

Reactive oxygen species, inflammation and calcium oxalate nephrolithiasis

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Abstract: Calcium oxalate (CaOx) kidney stones are formed attached to Randall's plaques (RPs) or Randall's plugs. Mechanisms involved in the formation and growth are poorly understood. It is our hypothesis that stone formation is a form of pathological biomineralization or ectopic calcification. Pathological calcification and plaque formation in the body is triggered by reactive oxygen species (ROS) and the development of oxidative stress (OS). This review explores clinical and experimental data in support of ROS involvement in the formation of CaOx kidney stones. Under normal conditions the production of ROS is tightly controlled, increasing when and where needed. Results of clinical and experimental studies show that renal epithelial exposure to high oxalate and crystals of CaOx/calcium phosphate (CaP) generates excess ROS, causing injury and inflammation. Major markers of OS and inflammation are detectable in urine of stone patients as well as rats with experimentally induced CaOx nephrolithiasis. Antioxidant treatments reduce crystal and oxalate induced injury in tissue culture and animal models. Significantly lower serum levels of antioxidants, alpha-carotene, beta-carotene and beta-cryptoxanthine have been found in individuals with a history of kidney stones. A diet rich in antioxidants has been shown to reduce stone episodes. ROS regulate crystal formation, growth and retention through the timely production of crystallization modulators. In the presence of abnormal calcium, citrate, oxalate, and/or phosphate, however, there is an overproduction of ROS and a decrease in the antioxidant capacity resulting in OS, renal injury and inflammation. Cellular degradation products in the urine promote crystallization in the tubular lumen at a faster rate thus blocking the tubule and plugging the tubular openings at the papillary tips forming Randall's plugs. Renal epithelial cells lining the loops of Henle/collecting ducts may become osteogenic, producing membrane vesicles at the basal side. In addition endothelial cells lining the blood vessels may also become osteogenic producing membrane vesicles. Calcification of the vesicles gives rise to RPs. The growth of the RPs is sustained by mineralization of collagen laid down as result of inflammation and fibrosis.

Keywords: Reactive oxygen species (ROS); nephrolithiasis; NADPH oxidase; Randall's plaque (RP); oxidative stress (OS)

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Kidney stones are comprised of mineral and organic components. Approximately 80% of the kidney stones contain calcium oxalate (CaOx) as the major mineral phase mixed mostly with calcium phosphate (CaP) and sometime uric acid (1). Stone formation involves crystal nucleation, growth, aggregation and their retention in the kidneys (2). These processes are modulated by a variety of urinary

macromolecules which become incorporated in the growing crystals and stones and eventually constitute stones' organic component or matrix. A better understanding of the pathogenesis of kidney stone formation has been developed through examination of clinical data and the use of animal models and tissue cultures. Based on clinical and experimental data, it is becoming obvious that stone

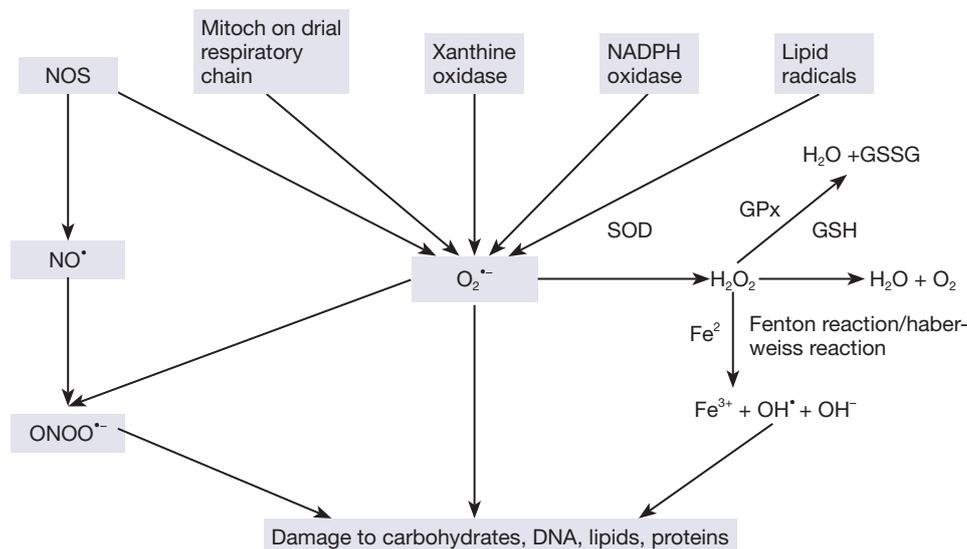


Figure 1 Sources and reactions involved in the production of superoxide ($O_2^{\bullet-}$), nitric oxide (NO^{\bullet}), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^{\bullet-}$), and hydroxyl radicals (OH^{\bullet}). GSH, glutathione; GPx, glutathione peroxidase; GSSG, oxidised glutathione; NOS, nitric oxide synthase; SOD, superoxide dismutase.

formation is not a simple physicochemical disorder. Renal epithelial cells as well as others respond to changing urinary environment; dysregulated mineral metabolism and in the case of CaOx nephrolithiasis, abnormal calcium, citrate, oxalate, phosphate, and CaOx/CaP crystals, by increased production of a variety of crystallization modulating macromolecules, epithelial to mesenchymal transition (EMT), epithelial to osteoblast transformation (EOT), and remodeling of extracellular matrix (ECM). It appears that reactive oxygen species (ROS) are intimately involved as signaling molecules as well as agents of injury and inflammation during stone formation (3-6).

Reactive oxygen species (ROS)

ROS comprising free radicals, atoms or molecules with unpaired electrons, and their metabolites, are highly reactive and play a critical role as signaling molecules. But they can also produce chemical modifications of, and damage to proteins, lipids, carbohydrates and nucleotides (7,8). Major cellular ROS include superoxide anion ($O_2^{\bullet-}$), nitric oxide radical (NO^{\bullet}), hydroxyl radical (OH^{\bullet}), and hydrogen peroxide (H_2O_2), which are generated by several pathways (Figure 1). $O_2^{\bullet-}$ anions are produced by NADPH oxidases, xanthine oxidase, lipooxygenase, cyclooxygenase, hemeoxygenase and as a byproduct of mitochondrial respiratory chain. Lipid radicals can also produce $O_2^{\bullet-}$. NO^{\bullet} radicals are

produced by the endothelial nitric oxide synthase (eNOS) mediated oxidation of L-arginine. In addition, eNOS can also produce $O_2^{\bullet-}$ rather than NO. The reaction between superoxide and nitric oxide can produce the highly reactive peroxynitrite $ONOO^{\bullet-}$.

Cells are equipped with a number of scavenging systems to control ROS availability (Figure 1). These include superoxide dismutase (SOD) to eliminate $O_2^{\bullet-}$, and glutathione (GSH) peroxidase (GPx) and catalase to detoxify H_2O_2 (Figure 1). Superoxide has a short half-life and spontaneously converts to H_2O_2 which is long-lasting and far more reactive than superoxide ions. The reaction is noticeably enhanced by SOD. Moreover, in a more complex transition metal catalyzed reaction called metal catalyzed Haber-Weiss reaction, H_2O_2 yields an even more reactive hydroxyl radical, which is however, short lived and works at short range. Initially, superoxide anions donate single electrons to ferric ions resulting in molecular oxygen and ferrous ions. The Fenton Reaction between ferrous ions and H_2O_2 leads to the formation of OH^{\bullet} . H_2O_2 is subsequently metabolized to water via catalase or by glutathione peroxidase in the presence of reduced glutathione.

Under normal conditions the superoxide anions ($O_2^{\bullet-}$), NO radicals (NO^{\bullet}) and their metabolites are generated by tightly controlled enzymes and serve as mediators in a variety of regulatory processes and signaling pathways including proliferation, activation or inactivation of

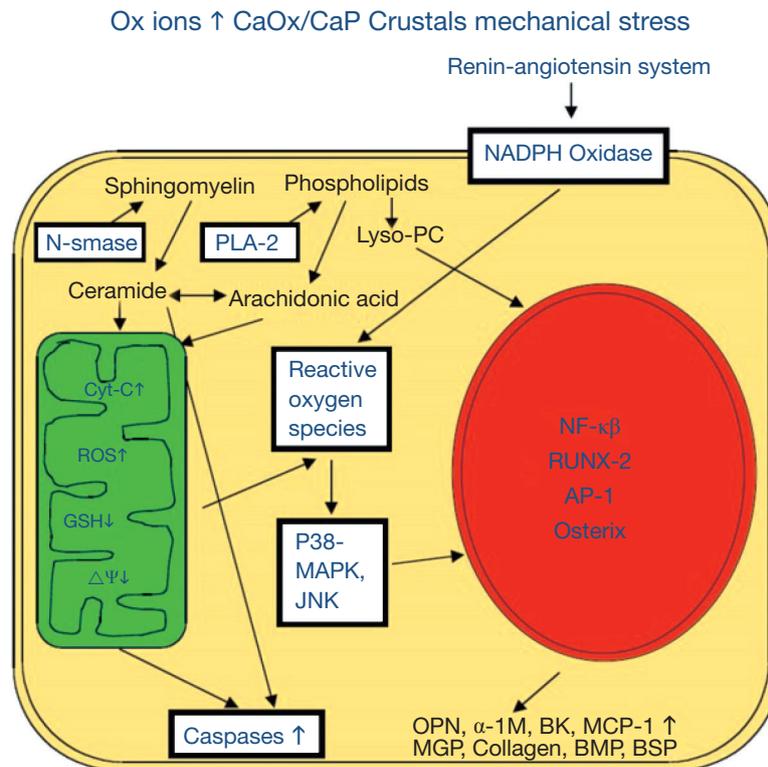


Figure 2 Proposed scheme for NADPH oxidase and mitochondrial involvement in the generation of reactive oxygen species (ROS) and their role in CaOx nephrolithiasis. Hyperoxaluria/CaOx/CaP crystal deposition causes renin upregulation and generation of angiotensin II which activates NADPH oxidase and production of ROS. Mitochondrial permeability transition pores are opened. The activation of phospholipase A2 (cPLA2) and neutral sphingomyelinase (N-Smase) affect mitochondria through lipid products archidonic acid, lysophosphatidylcholine (Lyso-PC) and ceramide. The production of cytochrome C (Cyt-C) goes up. There is a reduction in mitochondrial membrane potential ($\Delta\Psi$) and reduced glutathione (GSH). These actions lead to activation of caspases and apoptosis. ROS activate transcription factors such as nuclear factor κ B (NF κ B), activated protein-1 (AP-1) and growth factors including TGF β through P38 mitogen-activated protein kinase (-MAPK)/JNK. Runt-related transcription factor-2 (RUNX-2) and Osterix which are associated with osteoblast differentiation are also upregulated. Secondary mediators such as isoprostanes and prostaglandins are generated. Macromolecules involved in chemoattraction of monocyte-macrophages, crystallization modulation, bone morphogenesis, and fibrosis are produced. $O_2^{\cdot-}$, superoxide; OH^{\cdot} , hydroxyl radical; H_2O_2 , hydrogen peroxide; OPN, osteopontin; BK, bikunin; MCP-1, monocyte chemoattractant protein-1; MGP, matrix gla protein; BMP, bone morphogenetic protein; BSP, bone sialoprotein [Modified from Khan SR (4)].

regulatory biomolecules, and regulation of transcriptional activities. ROS regulate many calcium signals as well as such genes as *c-fos*, *c-myc*, and *c-jun* and transcription factor activation protein-1 (AP-1) and nuclear factor κ B (NF- κ B).

ROS and reactive nitrogen species (RNS) normally occur at steady state levels, generated when needed and then cleared by activities of various antioxidants and scavengers. But uncontrolled generation of the reactive oxygen or nitrogen species and/or a reduction in the endogenous antioxidant capacity creates oxidative stress (OS). Most cells respond to OS by boosting the levels of intracellular antioxidants such as glutathione. The oxidants can react

with all the basic constituents of cells: lipids, carbohydrates, proteins and nucleic acids severely affecting their structure and function. Pathological changes may result from the damaging effects of ROS and from ROS-mediated changes in gene expression and signal transduction.

Sources of ROS in CaOx nephrolithiasis

ROS are produced through the involvement of both mitochondria (4,9-12) and NADPH oxidase (Figure 2) (4,13,14). NADPH oxidase is a major source of ROS in the kidneys (15,16), particularly in the presence of Angiotensin

II (17). NADPH oxidase consists of six subunits, the two transmembrane units, p22^{phox} and gp91^{phox}; and four cytosolic units, p47^{phox}, p67^{phox}, p40^{phox} and the small GTPase rac1 or rac2 (18). The two transmembrane units, gp91^{phox} and p22^{phox} and a flavin make cytochrome b₅₅₈. The cytosolic units translocate to the membrane and assemble with the cytochrome to activate the enzyme.

ROS in response to oxalate and CaOx crystals are in part produced with the involvement of NADPH oxidase through the activation of the rennin angiotensin system (RAS). Reduction of angiotensin production, by inhibiting the angiotensin converting enzyme as well as blocking the angiotensin receptor, increased renin expression, reduced osteopontin (OPN) expression, crystal deposition and ameliorated the associated inflammatory response (Figure 2) (19-21). NADPH oxidase inhibition by apocynin treatment reduced the production of ROS, urinary excretion of kidney injury molecule (KIM) and renal deposition of CaOx crystals in hyperoxaluric rats (22). Atrovastatin, which has been shown to reduce the expression of gp91^{phox} and p22^{phox} subunits of NADPH oxidase (23), also inhibited crystal deposition in rats with experimentally induced hyperoxaluria (24).

Mitochondria are generally the most common source of superoxide and H₂O₂ in most cells and tissues. Hyperoxaluria and CaOx crystal deposition in rat kidneys causes mitochondrial damage. Treatment with taurine which has been shown to prevent oxidative injury of the mitochondria, reversed mitochondrial changes in the hyperoxaluric rat kidneys and decreased crystal deposition (25). Selective probes, substrates and inhibitors show mitochondria to be significant site of CaOx crystal induced superoxide production and glutathione depletion in both LLC-PK1 and MDCK cells (9). Exposure of LLC-PK1 cells to oxalate significantly increased cellular ceramides (26), however, pretreatment with glutathione precursor N-acetylcysteine (NAC) blocked this increase. Isolated mitochondria responded to oxalate exposure by the accumulation of ROS, lipid peroxides and oxidized thiol proteins (11). Citrate is also involved in maintaining endogenous antioxidant defenses. Administration of exogenous citrate to LLC-PK1 and MDCK cells bolstered these defenses and diminished the cellular injury inflicted by exposure to increased Ox and CaOx crystals (27). The presence of citrate in the culture medium was associated with a significant increase in GSH peroxidase and a drop in the production of H₂O₂ and 8-isoprostane (8-IP), which is an end product of lipid breakdown. There was a significant improvement in cell

viability as demonstrated by decreased LDH release and increased trypan blue exclusion.

Mitochondrial damage is suggested to be induced by the opening of mitochondrial permeability transition pore (mPTP). mPTP opening depends upon the activation of cyclophilin D in the mitochondrial matrix by ROS produced by NADPH oxidase and is inhibited by cyclosporine A (CSA) (28). CSA prevented the depolarization of mitochondrial membrane, decrease in SOD expression, increase in 4-hydroxy-2-nonenal (4HNE) and release of cytochrome-c into the cytosol in NR52E renal epithelial cells exposed to CaOx monohydrate crystals *in vitro*. CSA treatment of hyperoxaluric rats resulted in reduced mitochondrial damage, OS and CaOx crystal deposition in the kidneys.

Association of inflammation and injury with human stone formation

Most idiopathic stones are formed attached to Randall's plaques (RPs), the sub-epithelial deposits of CaP on renal papillary surfaces (29). RPs are postulated to start as deposits of poorly crystalline biological apatite in the basement membrane of the loops of Henle (30,31) or collecting ducts (32) or vasa recta (33,34). The deposits, consisting of aggregated CaP spherules, grow through the interstitium towards the renal papillary epithelium, where they eventually ulcerate to the surface (35). Interestingly, all RPs are not connected to stones and kidneys of non-stone formers also contain interstitial plaques (36).

Stones such as cystine, brushite, CaOx in primary hyperoxaluria and after bariatric surgery, some idiopathic stones and CaP in primary hyperparathyroidism are found attached to Randall's plugs (the tubular crystal deposits in the ducts of Bellini (31,37,38), which were most likely formed as a result of higher supersaturation with respect to the precipitating salt (39). Crystal deposition is associated with renal cell injury, cell loss, inflammation and fibrosis (4,40-45). The inflammation is generally localized to areas around crystal deposits in the renal papillae. In brushite stone formers, however, inflammation and fibrosis reach the cortex showing wide spread renal tubular atrophy and glomerular pathology (42).

It has been suggested that RPs are formed without causing renal injury and inflammation (31,46). But a close examination of published illustrations (30,32,36,47) clearly demonstrate the presence of necrotic tubules with thickened and layered basal lamina, along with perfectly normal

ones in association with the CaP spherules embedded in a matrix of collagen and other fibers. Similarly we have found injured tubules associated with the interstitial deposits of apatitic mineral (35,38). The molecules generally involved in inflammatory pathways, such as OPN (48,49), heavy chain of inter- α -inhibitor (50,51), collagen (30,36,47), and zinc (52) have been seen in the interstitial plaques strongly suggesting that inflammation may have been an early and local participant (4), which was resolved by the time stone was discovered. Biopsies are taken at the time of stone removal, many months after stone formation. Moreover, only a very small amount of tissue from limited number of patients has so far been investigated. "It is important to emphasize that urolithiasis is merely a final manifestation of diverse and systemic etiological and pathogenic events" (53). Inflammation is a complex biological response to various irritants. Osteopontin and inter- α -inhibitor are protective mediators, which are most likely produced to inhibit crystallization and protect the kidneys. In normal human kidneys, OPN is localized primarily to the distal nephron and is strongly expressed in the thick ascending limbs of the loops of Henle and papillary surface epithelium. OPN expression is increased during inflammation and interstitial fibrosis (54).

Renal CaOx crystal deposits have been seen in a variety of disorders with increased production and excretion of oxalate. In biopsies from a patient with primary hyperoxaluria, crystals were seen within tubular epithelial cells as well as in the interstitium of the transplanted kidney (55) and were associated with vascular and interstitial inflammation, cell proliferation and multinucleated giant cells. 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 (56,57).

Higher than normal levels of renal enzymes, gamma-glutamyl transpeptidase (GGTP), angiotensin 1 converting enzyme (ACE), β -galactosidase (GAL), and N-acetyl- β -glucosaminidase (NAG) were found in the urine of idiopathic CaOx stone formers (58), indicative of renal proximal tubular injury. The urine had significantly increased NAG, β -GAL, α -glutathione S-transferase (α -GST), malondialdehyde (MDA) and thiobarbituric acid-reactive substances (TBARS) (59), suggesting that stone-associated injury was most likely caused by the production of ROS. Urinary 8-hydroxydeoxyguanosine (8-OHdG), a marker of oxidative damage of DNA, was increased in stone patients and was positively correlated with tubular damage as assessed by urinary excretion of NAG (60). Recurrent

idiopathic calcium stone formers with and without stones, exhibited antioxidant deficiency. Investigators concluded that lithiasis started with oxidatively damaged cells (61). Anti-inflammatory proteins calgranulin, α -defensin, and myeloperoxidase (62), were increased in urine of stone patients and were also found in the inner core of the CaOx stones.

Members of I α I family of proteins, which are important participants in wound healing, were significantly increased in the urine of male stone formers (63), and found in the stone matrix as well as the matrix of CaOx and CaP crystals induced in the urine (64,65). Hyaluronan, which plays an important role in renal injury and repair, was a major constituent of the organic matrix of stones (66). Prothrombin fragment-1, a member of thrombin family of proteins which are extensively involved in tissue repair, was also excreted in urine, present in stone matrix and preferentially bound to CaOx crystals (67). Kidneys of stone formers expressed mRNA for MCP-1 as well as IL-6 (68).

Nephrolithiasis and chronic kidney diseases (CKDs)

Kidney stone formation has been linked with a number of chronic diseases (69), such as obesity (OB) (70), diabetes mellitus (DM) (71), hypertension (HTN) (70), metabolic syndrome (MS) (72), and CKD (*Figure 3*) (73). Nephrolithiasis is a risk factor for the development of hypertension (74), while similarly hypertensive patients are at greater risk to develop nephrolithiasis (75). There is an association between stone disease and DM (71,76-81), as diabetics persistently produce acidic pH and have a greater risk to form uric acid stones (82). Kidney stone formers are also at greater risk for coronary artery disease (83), myocardial infarction (84) and CKD (83,85). Not surprisingly, stone formation is common in adults with metabolic syndrome and the frequency of stone formation is directly correlated with weight and BMI (86-88).

Both clinical and experimental investigations indicate that OS and inflammation play a significant role in the development of cardiovascular diseases (89). OS is a common feature of HTN, DM, atherosclerosis, and myocardial infarct (*Figure 4*) (69). An increase in the production of ROS/RNS, and/or decrease in the extra and intracellular antioxidants has been demonstrated in both clinical and experimental HTNs (90) and leads to OS which may not only initiate HTN but also be developed by the hypertensive state (91). Experimental studies have shown the involvement of NADPH oxidase in the development of

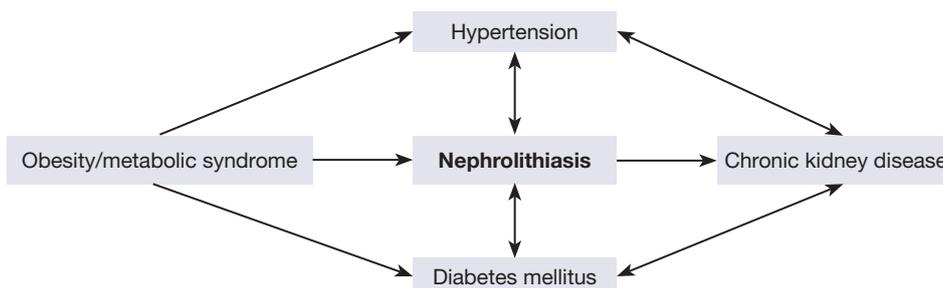


Figure 3 Diagram showing associations between nephrolithiasis and other diseases with renal involvement.

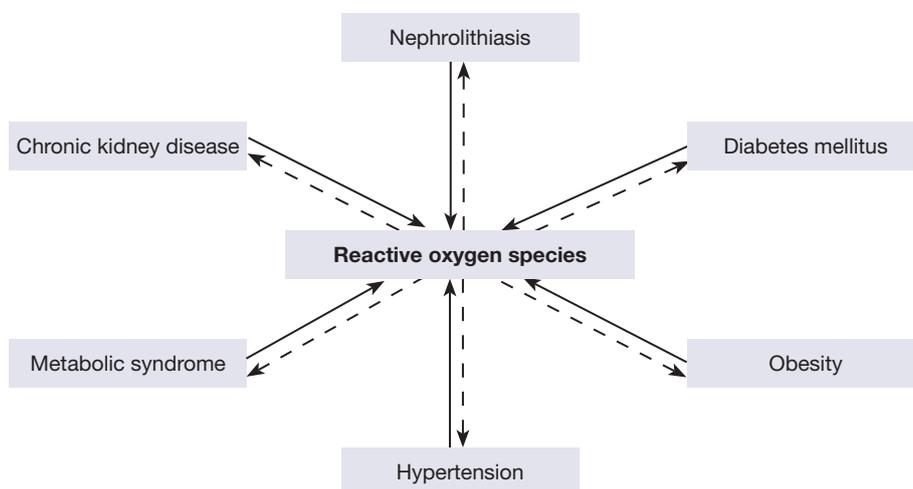


Figure 4 Role of ROS in nephrolithiasis and co-morbidities. ROS play a role in the progression of many disorders including nephrolithiasis. ROS produced during one disorder may instigate the other under conditions suitable for the specific disease. ROS, reactive oxygen species.

hypertension (91-93). OB associated OS eventually leads to systemic inflammation and endothelial cell dysfunction (94,95). Proper endothelial performance requires NO which acts on pericytes, and is depleted during OS because of its inactivation by $O_2^{\cdot-}$ (96,97). Oxidation of the NO also results in the formation of highly active $ONOO^-$ and enhancement of OS. NADPH oxidase is a major source of ROS in the kidneys and is activated by Ang II, mostly through the AT1 receptor. Both NO and $O_2^{\cdot-}$ are produced by the renal epithelial cells, in addition, NO is also produced by endothelial cells. There is a tubulovascular cross talk, whereby NO produced by the epithelial cells of the medullary thick ascending limb affect the interstitial pericytes and endothelial cells.

NADPH oxidase is the major source of ROS in the kidneys and cardiovascular system (93). The kidneys of spontaneously hypertensive rats (SHR) showed increased production of $O_2^{\cdot-}$ and upregulation of p47^{phox} subunit (98). Administration of SOD mimetic tempol produced a

reduction of blood pressure and renal vascular resistance (99). Significantly higher p22^{phox} mRNA levels and NADPH oxidase driven $O_2^{\cdot-}$ production were found in the aorta of SHR which were ameliorated by treatment with irbesartan, an angiotensin II receptor antagonist (100). The importance of p47^{phox} is also shown by moderate hypertensive response to angiotensin II in mice lacking the p47^{phox} (101,102). Inhibition by membrane permeable gp91ds-tat of p47^{phox} assembly with gp91^{phox} in Dahl salt sensitive (DS) rats fed a 4% salt diet, normalized ROS production and endothelium dependent relaxation as well as expression of LOX-1 and MCP-1 (103). Administration of apocynin, an antioxidant and an inhibitor of the p47^{phox} assembly with gp91^{phox}, to DS rats on high salt diet produced significant reductions in the mRNA expression of gp91^{phox}, p47^{phox}, p22^{phox}, and p67^{phox} subunits in addition to significantly reducing interstitial superoxide and mean arterial pressure (104). Apocynin also reduced NADPH oxidase activity, renal cortical $O_2^{\cdot-}$, monocyte/macrophage infiltration and glomerular and

interstitial damage (105). Experimental studies involving other animal models of hypertension have similarly shown the involvement of NADPH oxidase in the development of hypertension (91-93).

NaDPH oxidase also plays an important role in diabetic nephropathy (106-108), particularly in the presence of high glucose. Levels of Nox 4 as well as p22^{phox} mRNA were increased in kidneys of rats with STZ-induced diabetes along with an increase in immunostaining of 8-OHdG (109). Insulin treatment reduced them to control levels. STZ induced diabetes also increased excretion of H₂O₂, lipid peroxidation (LPO) products, and nitric oxide products (Nox) (110). Kidneys showed increased expression of gp91^{phox} and p47^{phox} and endothelial eNOS, increased mesangial matrix, fibronectin and type I collagen. The treatment with apocynin, which inhibits assembly of the cytosolic p47^{phox} with the membranous gp91^{phox}, inhibited the increases in membrane fraction of p47^{phox}, and excretion of H₂O₂, LPO and Nox.

Dietary Approaches to Stop Hypertension (DASH) diet, which reduces the risks for stroke and cardiovascular diseases, also reduced the risk for stone formation by up to 45% (111). The relationship between DASH type diet and the incident symptomatic kidney stones was examined in a prospective analysis of data from Health Professionals Follow up Study (n=45,821), Nurses' Health Study-1 (n=94,108) and 2 (n=101,837) and found that men and women with higher DASH scores were significantly less likely to develop kidney stones than those with lower DASH scores. Low sodium DASH diet reduces OS and improves vascular functions, lowers blood pressure (112,113). Analysis of data from National Health and Nutrition Examination Survey (NHANESIII) showed that serum levels of antioxidants alpha-carotene, beta-carotene and beta-cryptoxanthin were significantly lower in stone patients. Lower levels of these antioxidants were associated with decreasing incidents of stone disease (114).

The association between nephrolithiasis, CKD, DM, OB, HTN, and MS is most likely a result of common pathophysiological mechanisms (70). ROS and OS are common feature of CKD, nephrolithiasis, DM, OB, HTN, and MS. It is conceivable that ROS produced by one disease may lead to another under appropriate circumstances (*Figure 4*) (69,115,116). For example mild hypercalciuria, hyperoxaluria, hypocitraturia which under normal conditions may just be a curiosity or nuisance, can promote crystallization and stone formation when cells are injured by ROS produced through another disorder.

Epithelial exposure to oxalate or calcific crystals, inflammation and injury

Tissue culture studies in which renal epithelial cells are exposed to Ox and/or CaOx or CaP crystals have provided new insights into mechanisms involved in stone formation. Cell response is time and concentration dependent and cell specific. Exposure to high concentrations of Ox as well as CaOx and CaP crystals for longer duration is injurious to renal epithelial cells (117-121). Crystals bind rapidly to the surface of epithelial cells and are internalized (122-127). Cells of proximal tubular origin are more susceptible to injury than cells originating from distal sections of the renal tubules. Lower Ox levels induce expression of immediate early genes, stimulate DNA synthesis and promote cellular proliferation, while higher Ox levels induce cell damage and death.

The response of renal epithelial cells to COM crystals is characterized by increased expression of specific genes that encode transcriptional activators, regulators of the ECM, and growth factors (118,128), and production of pro and anti-inflammatory molecule, such as OPN, monocyte chemoattractant-1 (MCP-1), prostaglandin E2 (PGE2), bikunin and components of inter- α -inhibitor (I α I), α -1 microglobulin, CD-44, calgranulin, heparin sulfate, osteonectin, fibronectin and matrix-gla-protein (MGP) (14,21,129-134). Even though many of these molecules are integral to inflammation and fibrosis, they are also modulators of calcification (135,136). Gene expression of vimentin, a mesenchymal marker, is also increased (137).

Tissue culture studies have also provided the evidence for the involvement of free radicals in toxicity, production of various crystallization modulators, and inflammatory and anti-inflammatory macromolecules (14,138-141). Renal cells exposed to CaOx crystals secrete superoxide (142) and cellular injury could be ameliorated by antioxidants and free radical scavenger (5,14,138-140,143). Free radical scavengers, catalase and SOD provided protection from oxalate induced injury to LLC-PK1 and MDCK cells (138). Catalase treatment also reduced MCP-1 mRNA as well as protein in the oxalate, CaOx, CaP and uric acid treated NRK52E renal epithelial cells (132,133).

Inflammation and injury in animal models of CaOx nephrolithiasis

A number of rat and mice models (144,145), have been developed to investigate the pathogenesis of kidney

Table 1 Urinary macromolecules and their role in crystallization and inflammation

Name	Role in nephrolithiasis	Role in inflammation and repair
Tamm-Horsfall protein (THP)	Modulator of CaOx crystal nucleation, growth and aggregation as well as adherence to epithelium	Renoprotective, elicits immune response
Osteopontin (OPN)	Modulator of CaOx crystallization, aggregation and crystal attachment	Calcium binding, renoprotective, anti-inflammatory, chemoattractant for monocytes
Prothrombin fragment-1 (PTF-1)	Inhibitor of crystal growth and aggregation	Calcium binding, coagulation
Bikunin and inter- α -inhibitor family (I α I)	Inhibitor of CaOx crystallization and attachment	Metastasis, tissue repair and remodeling
α -1-microglobulin (α 1m)	Modulator of crystallization	Immunosuppressive, protective against oxidative stress
Hyaluronic acid (HA)	A major constituent of stone matrix, modulator of crystallization and adherence to renal epithelium	Major constituent of extracellular matrix
CD-44	Promoter of crystal attachment	Tissue repair and remodeling
Calgranulin (Calprotectin)	Inhibitor of crystal growth and aggregation	Calcium binding, tissue remodeling and inflammation
Heparan sulfate (HS)	Inhibitor of crystal aggregation and attachment	Tissue remodeling
Osteonectin		Calcium binding, tissue remodeling
Fibronectin	Inhibitor of crystal aggregation, attachment and endocytosis	Morphogenesis, wound healing and metastasis
Matrix gla protein (MGP)	Inhibitor of crystal deposition	Inhibitor of biomineralization
Fetuin	Increased urinary excretion by stone patients	Anti-inflammatory
Albumin	Modulator of crystal nucleation	
Interleukin-6	Increased urinary excretion by stone patients	Mediator of inflammation
Monocyte chemoattractant protein-1 (MCP-1)	No known role in crystallization	Attracts monocytes, memory T cells, dendritic cells to site of inflammation

CaOx, calcium oxalate.

stones. None of the models completely replicate the process of idiopathic stone formation and develop stones on papillary surface attached to the RPs. Instead the crystals are intraluminal, reminiscent of Randall's plugs. CaOx nephrolithiasis is produced by inducing hyperoxaluria through the administration of oxalate or its precursors such as glyoxylate, ethylene glycol (EG) and hydroxyl-L-proline (HLP). Both hyperoxaluria and CaOx crystal deposition trigger morphological and pathophysiological changes in the kidneys and alter urinary composition (22,146). Renal expression of OPN (21,147), Tamm-Horsfall Protein (THP) (148-150), bikunin (130,131), I α I (151), α -1microglobulin (152), prothrombin fragment-1 (PTF-1) (153), calgranulin, heparin sulfate (HS) (154), matrix gla protein (MGP) (155,156), are generally increased (*Table 1*) and often found at locations not normally seen. For example THP

is specifically produced by epithelial cells lining the thick ascending limbs of the loops of Henle. However, in the rat model of CaOx nephrolithiasis THP is seen associated with crystals throughout the kidneys including the cortex (150,157). In addition the expression of NF κ B, KIM, proliferating cell nuclear antigen (PCNA), and CD 44, e-cadherin, is also increased indicating both injury and proliferation (22,158). Urinary excretion of many of these molecules is increased as well (22,131,146,147,151,153,159). There is migration of monocyte and macrophages to the sites of crystal deposition. We examined kidneys at different times after induction of acute hyperoxaluria in male Sprague-Dawley rats, and found that CaOx crystals appeared first in the tubular lumen, then moved to inter- and intracellular locations, eventually relocating into the interstitium, where they became surrounded by

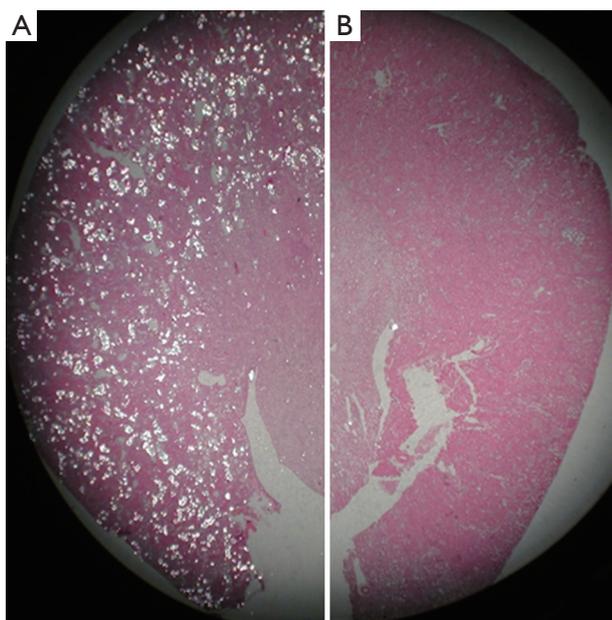


Figure 5 Hematoxylin and eosin stained paraffin section of a kidney from a rat made hyperoxaluric by feeding hydroxyl-L-proline for 28 days (146). Sections were examined by light microscope equipped with polarizing optics ($\times 4$). (A) Hyperoxaluric rat. Renal tubules are full with birefringent CaOx crystals; (B) hyperoxaluric rat receiving apocynin. Only a few birefringent CaOx crystals are present. CaOx, calcium oxalate.

macrophages. After a few weeks, the interstitial crystals disappeared, indicating the existence of a mechanism to remove the CaOx crystals (160). Multinucleated giant cells were also identified in the interstitium (161).

Lipid peroxides increased in both the renal tissue and urine in rats with hyperoxaluria and CaOx nephrolithiasis (162). Additionally, treatment with antioxidant vitamin E improved the tissue levels of antioxidant enzymes, reduced injury and totally eliminated CaOx crystal deposition in the kidneys (163). Deposition of CaOx crystals in the kidneys was associated with reduction of total renal cellular glutathione and an increase in lipid peroxides (19). Rats who received ACE inhibitor losartan, known to reduce OS, showed a significant increase in glutathione concentration and a decrease in the thiobarbituric acid reactive substances in the kidneys. The activities of catalase and MnSOD increased in kidneys while α - and μ -glutathione-S-transferase (GST) levels increased in the urine of hyperoxaluric rats (164). Microarray analysis of the kidneys of hyperoxaluric rats also revealed the development of OS during hyperoxaluria and CaOx crystals deposition. Expression of genes for SOD, GPx,

GST, aldehyde dehydrogenase, mitochondrial uncoupling protein and ceruloplasmin was increased in hyperoxaluric rats (165). Administration of apocynin, an antioxidant and inhibitor of NADPH oxidase, to rats with hydroxyproline induced hyperoxaluria nearly completely reversed the effects of hyperoxaluria (146), in addition, the deposition of CaOx crystals in the kidneys was also significantly reduced (Figure 5), and the urinary excretion of OPN, KIM, MCP-1 was significantly reduced without any effect on hyperoxaluria (Figure 6).

CaOx crystal deposition caused inflammation and attracted many inflammatory cells including leukocytes, monocytes, and macrophages (146,161,166,167), and multinucleated giant cells were identified around the crystals. The mechanism by which inflammatory cells enter the renal interstitium is not known, but chemotactic factors and adhesion molecules are involved. Leukocytes (neutrophils, monocytes, and lymphocytes) infiltrate the kidneys during a variety of inflammatory diseases. They mediate renal injury and subsequent sclerosis induced by such pathologies. Chemotactic factors are produced by renal cells and are found in the kidney and urine during inflammation. Results show that approximately 70-80% of monocyte chemotactic activity produced by cultured human mesangial cells (168), renal cortical epithelial cells (169), proximal tubular epithelial cells (170), and bovine glomerular endothelial cells (171), is accounted for by MCP-1. As discussed earlier, exposure to oxalate and both the CaP and CaOx crystals is associated with the production of MCP-1 by rat kidney cells culture (14,132,133).

Epithelial to osteoblast transformation (EOT)

Vascular calcification, which plays a major role in the development of CKD, was considered to occur by a passive, unregulated physicochemical mechanism as an irreversible degenerative process. Now however, it is considered to be an actively regulated process in which vascular smooth cells (VSMC) acquire osteogenic phenotype (172-174). Exposure of VSMC to elevated levels of calcium and phosphate triggers osteogenic transformation of VSMC (175-178), which involves an increased expression of osteoblast specific genes and a decrease in smooth muscle cell markers (179,180). Bone morphogenetic proteins, BMP 2 and BMP 4, and Wnt signaling pathways are activated through up-regulation of transcription factor, Runt-related transcription factor 2 (RUNX2)/msh homeobox 2 (MSX-2). Cells produce matrix proteins. Crystallization starts in

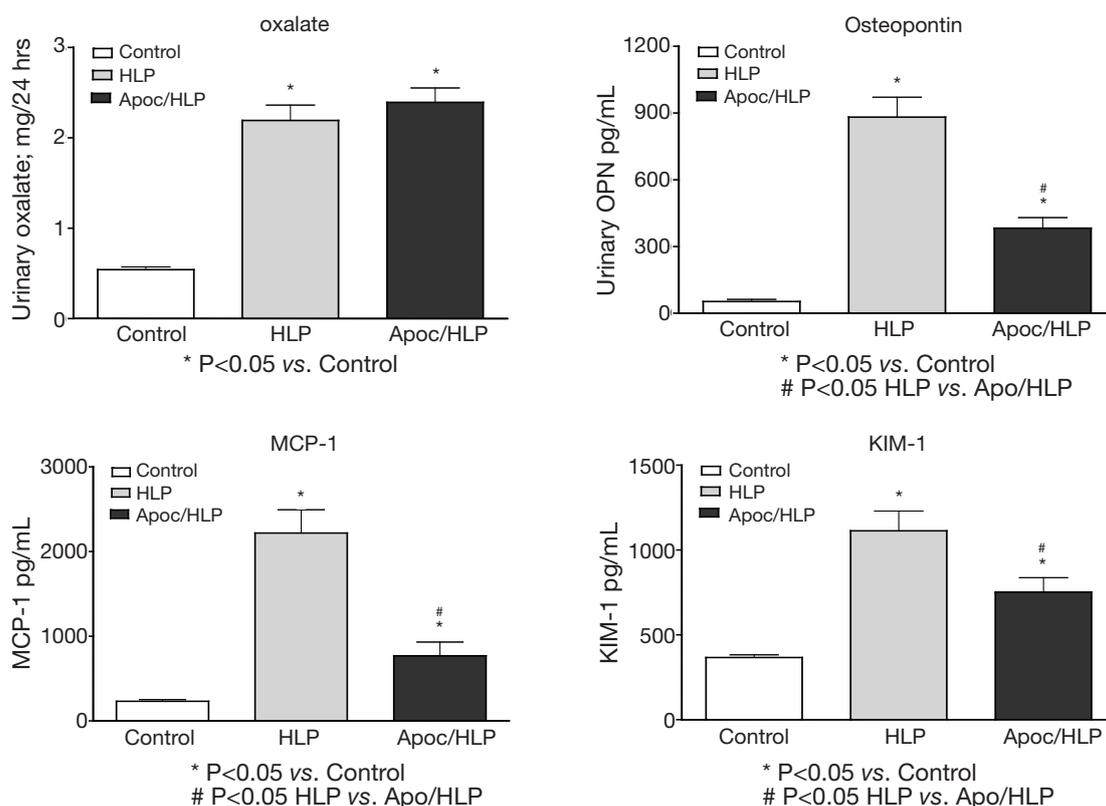


Figure 6 Urinary excretion of oxalate, osteopontin (OPN), monocyte chemoattractant protein-1 (MCP-1), and kidney injury molecule (KIM-1) by rats with hyperoxaluria induced by feeding hydroxyl-L-proline (HLP) (146) with or without apocyanin (HLP/Apo). There were significant reductions in the production of OPN, KIM-1, and MCP-1 by treatment with apocynin, an inhibitor of NADPH oxidase as well as an antioxidant.

membrane bound matrix vesicles produced by the viable transformed vascular smooth muscle cells or apoptotic bodies produced on their death (178,181,182). The vesicles are similar in composition to the matrix vesicles derived from chondrocytes and provide sites for the nucleation of CaP crystals (176). Once mineralized, the crystals poke through the limiting membrane of the vesicles and help mineralize the nearby ECM which sustains calcification. In addition to abnormal mineral metabolism, OS, inflammation and aberrant crystallization inhibition play significant role in vascular calcification. ROS are likely involved in the VSMC transformation to osteogenic phenotype by regulating RUNX-2 transcription factor (183,184). Advanced glycation end-products commonly seen in blood and arteries of diabetic patients and older individuals can promote vascular calcification mediated by NADPH oxidase induced ROS (185). Cytokines such as interleukin (IL)-1 β , IL-6, IL-8, tumor necrosis factor (TNF)- α , transforming growth factor

(TGF)- β produced by macrophages induce transformation of VSMCs (186). Inflammatory cells also produce proteolytic enzymes such as metalloproteinases (MMP)-2 and -9 which degrade matrix and promote calcification (187-190).

Calcification of vascular smooth cell is inhibited by MGP, pyrophosphate, OPN and Fetuin-A (179). MGP is a vitamin K-dependent protein functioning primarily as an inhibitor of vascular calcification (191). MGP also regulates BMP-2 activity (192). Mutations in the MGP gene lead to keutel syndrome, a disorder associated with extensive soft tissue and vascular calcification (193). MGP knockout mice die within two months as a result of arterial calcification and blood vessel rupture (194) while restoration of MGP in these mice prevents arterial calcification (195). Polymorphism of MGP may play a role in vascular calcification (196) and has shown an association with myocardial infarction (197).

Fetuin A, a member of cystatin family of protease

inhibitors, is a serum protein, produced by the liver and specifically enriched in mineralized tissues (198-200). Irrespective of its origin and posttranslational modifications fetuin-A prevents precipitation of hydroxyapatite *in vitro* (201). *In vivo*, serum fetuin A levels are lower in patients with CKD (202), and ectopic calcification is seen in fetuin A $-/-$ mice (200).

Cardiovascular complications are significantly increased in patients with CKD (203,204), and coronary artery calcification is considered an independent predictor of future cardiac event (205). Carotid atherosclerotic lesions of CKD patients frequently become calcified (206,207). The calcified plaques are more unstable and contain significantly lower amounts of collagen. Serum levels of MMPs are significantly increased (206-209). Enhanced calcification and reduced collagen, perhaps through the activities of MMPs, lead to plaque instability and rupture (206).

It is our hypothesis that stone formation is yet another case of pathological biomineralization. Renal epithelial cells under OS may become osteogenic (210) as happens to vascular smooth muscle cells during vascular calcification (177). The production of OPN (147,149), MGP (155,211), collagen, fibronectin, osteonectin and fetuin (unpublished results) by renal epithelial cells of rats with experimentally induced CaOx nephrolithiasis are indicative of such a transition. The presence of OPN, osteocalcin, fibronectin, and collagen (212) in stone matrices also suggests their increased production and excretion into the urine. Renal crystals in a CaOx stone patient were also associated with bone sialoprotein (BSP) (213). EMT (214) as well as endothelial to mesenchymal transition (215,216) are regularly seen in the diseased kidneys. Mesenchymal stromal cells have the ability to differentiate into osteoblast. Interestingly, perivascular cells or pericyte were heavily stained for MGP in kidneys of hyperoxaluric rats (211).

Stone patients excrete lower amounts of fetuin-A (217), and more BMP-2 (218). Single nucleotide polymorphism of MGP gene is associated with CaOx kidney stones disease in the Japanese (219) and Chinese populations (220). Brush border membrane vesicles of renal epithelium promote nucleation of CaOx and CaP crystals *in vivo* as well as *in vitro* (221-223).

Discussion and concluding remarks

Supersaturation is the driving force behind crystallization and in most idiopathic CaOx stone formers hypercalciuria, hyperoxaluria and hypocitraturia alone or in combination

are the main abnormalities. As a result, most stone therapies attempt to lower urinary supersaturation with respect to the crystallizing salt, yet 30% to 50% stone patients still continue to form stones (224). The risk of stone recurrence increases with each new episode (225), and nearly all stone formers are expected to form another provided they live long enough after the first episode (226). Even initial interventions do not stop stone recurrence in about fifty percent of the patients (227,228). Apparently stone formation does not occur by a passive unregulated physicochemical mechanism, but by a regulated process, similar to pathological biomineralization at other sites in the body including kidneys during vascular calcification (172, 229,230).

Renal epithelial cells are exposed to high oxalate and/or CaOx and/or CaP crystals during stone formation. Experimental studies suggest that renal cellular exposure to high oxalate and/or CaOx or CaP crystals results in increased gene expression and production of molecules involved in tissue remodeling, inflammation and biomineralization (Figure 2). Hyperoxaluria and CaOx crystal deposition induce rennin upregulation and generation of angiotensin II (21). Non phagocytic NADPH oxidase is activated (14,22,231,232) leading to the production of ROS (5,231,233) which is mediated by protein kinase C (PKC) (232). The activation involves phosphorylation of p47phox and translocation of Rac1 (234) and p47 phox to the membrane. P-38 MAPK/JNK transduction pathway is turned on (235,236), in addition to a variety of transcriptional and growth factors, including NF κ B, AP-1, TGF β , become involved (19,20,237). There is the generation of secondary mediators such as isoprostanes, cytoplasmic phospholipase A2 and prostaglandins (4,5,238), and an increased production of chemoattractants such as MCP-1 (132-134) and crystallization modulators OPN (134), bikunin (130,131), α 1-microglobulin (152), I α I (151) and prothrombin fragment-1 (153). Macrophages infiltrate the renal interstitium around the crystal deposits and phagocytic NADPH oxidase is also activated producing additional ROS resulting in inflammation, ECM production and fibrosis. Clinical data also provide the evidence of ROS generation and byproducts of their activities have been detected in both the kidneys and urine of the patients who form CaOx kidney stones.

The macromolecules, produced on exposure to oxalate and or various types of crystals through ROS dependent pathways, have dual functions (Table 1). They regulate crystallization and are also involved in inflammatory

processes. For example, HS, an inhibitor of crystal aggregation and attachment, regulates ECM production (3,239). Bikunin, a constituent of ITI, an inhibitor of CaOx crystal formation and attachment, is a proteinase inhibitor, and stabilizes the ECM (240-242). THP a modulator of CaOx crystal nucleation, growth and aggregation, is renoprotective and present in the renal interstitium during many tubulointerstitial diseases (243). OPN, an inhibitor of crystal nucleation, growth and aggregation, is also a chemoattractant involved in inflammation and fibrosis (244,245). Prothrombin is the precursor of thrombin and fragments 1 and 2 and plays a major role in the recruitment and activation of infiltrating immune cells. Fragment-1 is inhibitor of CaOx crystal growth and aggregation. Inflammation is primarily a protective response, therefore in the presence of impending mineralization, the body responds by producing macromolecules to inhibit crystallization and once crystal are formed to attract the inflammatory cells for their elimination (4,161,246). Crystals are phagocytosed and eliminated (246) or fenced in by a "wall" of macromolecules adsorbed to crystal surfaces rendering them harmless (247). This is likely the case with the interstitial plaques which are not attached to kidney stones and are common in the kidneys (32,36,47,248).

In summary, disturbance in the physiochemical milieu leads to the production of ROS and development of OS. ROS start a signaling cascade culminating in the production of macromolecules to inhibit crystal nucleation, growth and aggregation. In case of transitory disorder, either no crystal will form or crystals formed will stay small, well dispersed and passed out as crystalluria particles. In the face of persistent disorder, for example, hyperoxaluria, hypercalciuria and hypocitraturia, there is a loss of the balance between oxidative and antioxidative forces. ROS induced damage to the cells leads to cell death and the formation of membrane bound vesicles which support crystal nucleation (221,249). As a result crystallization inhibitors which are produced may be defective or damaged by exposure to the free radicals and thus not able to provide adequate protection resulting in crystal growth and aggregation. Cell death also leads to the formation of new cells to repopulate the epithelium. The surfaces of the new cells as well as the exposed basement membrane are conducive to crystal attachment and retention (250). Crystals retained in the terminal collecting ducts produce Randall' plugs (38,251) which will act as stone nidus when exposed to the pelvic urine. A recent study has shown that plugging is quite common in stones patients (37).

As far as interstitial RPs are concerned, CaP crystals may originate in the tubular lumen, endocytose on the luminal side and exocytose on the basolateral side (213,251,252), to initiate the formation of the plaque. Alternatively renal epithelial cells under OS may become osteogenic (210). In addition to epithelium to osteoblast transformation, vascular endothelial cells may also become osteogenic (216). The transformed epithelial or endothelial cells will produce a membrane bound vesicle on the basolateral side of the epithelium. Renal epithelial cells have been shown to produce *in vitro*, CaP crystal deposits in the basement membrane under a variety of growing conditions (213,253,254).

Calcification of the membrane vesicles and their aggregation produces calcified RP's in the basement membrane of the renal tubules. ROS induced inflammation results in the formation of collagen which is deposited during fibrosis (255), and is an excellent nucleator of CaP (182,256) playing a critical role in biomineralization processes in the body (182,257). Calcification which starts with membrane bound vesicles is propagated through the mineralization of collagen (35). Mineralization of collagen leads to the growth of the plaque, which eventually reaches the papillary epithelium, ulcerates to the surface and develops into a stone nidus. Once exposed to the pelvic urine, the plaque is overgrown by CaOx crystals, and promotes the formation of an idiopathic CaOx kidney stone attached to the sub-epithelial RP (258).

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Footnote

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