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ORIGINAL PAPER

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Oxalate binding proteins in calcium oxalate nephrolithiasis

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Abstract The existence of several oxalate specific binding proteins have been demonstrated in human and rat kidney. These occur in both cortical and medullary cells and are distributed mostly in the subcellular organelles. About 1/3 of the total cellular oxalate binding was localised in the inner mitochondrial membrane while the rest was in the nucleus. The purified mitochondrial oxalate binding protein (62 kDa) was composed, with a higher molar proportion, of basic amino acids, and could accumulate oxalate on incorporation into liposomes. In the nucleus, histone H_{1B} (27.5 kDa), nuclear membrane protein (68 kDa) and nuclear pore complex protein (205 kDa) were present with oxalate binding activities. In addition, a 45 kDa calcium oxalate binding protein was identified in most of the subcellular organelles. Both mitochondrial and nuclear oxalate binding proteins and calcium oxalate binding protein have shown the kinetic properties of specificity, saturability, pH and temperature dependency, energy of activation and inhibition by substrate analogues. All oxalate binding proteins were sensitive to the transport inhibitor 4′-4′ diisothiocyano stilbene-2–2 disulphonic acid (DIDS), which is known to interact with the lysine moiety of the proteins. Calcium oxalate monohydrate (COM) crystals adsorbed oxalate binding proteins from human and rat kidney, and oxalate binding proteins isolated from human kidney stone matrix also exhibited the above kinetic properties. In experimental hyperoxaluria, all of the renal oxalate binding proteins showed enhanced oxalate binding activity with increased protein concentration. This enhanced oxalate binding activity was also attributed to increased lipid peroxidation, which correlated positively, and to decreased thiol status, which correlated negatively. A positive correlation was observed between the lipid peroxidation and both the oxalate binding activity of the in vitro peroxidised subcellular organelles and the purified protein. Similarly, in an in vivo hyperoxaluric condition, a negative correlation was observed between thiol content and both the oxalate binding activity of the peroxidised subcellular organelles and the purified protein. In the calcium oxalate crystal growth system, the oxalate binding proteins behaved either as promoters or inhibitors of the nucleation and aggregation of crystals. Following the peroxidation of the proteins, the degree of effect of the promoter protein was further stimulated while the degree of inhibition caused by the inhibitor protein further declined. Similar observations were duplicated with the proteins derived from hyperoxaluric rat kidney or kidney homogenate subjected to in vitro lipid peroxidation. The oxalate binding proteins were thought to modulate the crystallisation process in an hyperoxaluric condition similar to calcium specific binding protein modulators.

Keywords Oxalate binding · Lipid peroxidation · Protein thiol depletion · Nucleation · Aggregation

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Urinary stone disease, which has plagued and intrigued human beings, existed at least 7,000 years ago, as it has been found in the pelvis, presumably in the bladder, of a mummified Egyptian [112]. Renal stone disease is estimated to occur in 2–5% of the population, and the rate of recurrence increases with age if untreated [14]. The formation of a stone within the urinary tract is not a specific disease but a culmination of the potential complications of many different metabolic disorders such as hypercalciuria, hyperoxaluria, hypocitraturia, abnormal crystallization inhibition, hyperabsorption in the intestine, excess secretion in tubular fluid, hormone imbalance

and inborn errors in oxalate metabolism [40, 72, 114, 133]. Despite the development of novel means of treatment, such as dietary modifications, supplementation of citrate, magnesium oxide, antioxidants like vitamin E, lipoic acid, indigenous medicines and by surgical methods [9, 90, 93], the basic mechanism of stone formation, the identity of predictors of recurrence, and the factors involved in retention are still largely shrouded in uncertainty.

Abnormality in oxalate metabolism has been suggested for the pathogenesis of stone disease. The urinary excretion of oxalate depends on various factors such as dietary intake, endogenous synthesis, intestinal absorption, and intestinal elimination by secretion, intestinal elimination by *Oxalobacter formigenes* and renal secretion [45, 81, 113]. Excessive excretion of oxalate leads to calcium oxalate (CaOx) crystalluria. Oxalate can be voided freely in the urine of healthy subjects who also pass crystals with normal oxalate excretion. The question of stone formation arises only when these crystals are retained in the tubules due to their size as well as their association with the epithelium, as proposed by Finlayson and Reid in the fixed particle hypothesis [28]. The factors which facilitate the retention and further growth of the crystals may play an important role in the development of nephrolithiasis. The specific molecular processes that mediate the attachment of crystal to kidney epithelium are yet to be identified. The attachment of crystals to cells may be dependent on the specific composition and arrangement of cell surface proteins, glycoproteins, glycolipids and lipids, as well as to cell damage mediated by lipid and protein peroxidation [15, 67, 96, 124]. Membranes are the prime sites involved in the transport, uptake and binding of oxalate during the absorption process in the intestine, as well as the secretion process in renal tubules. Many of the oxalate uptake studies have been performed using membrane vesicles [78], microperfused renal tubules [121], stripped intestinal tissue as a two compartment system [37], and cultured epithelial cells [84]. All of these systems have inherent defects, as membrane vesicles and culture do not provide the same information as the intact and functional epithelia under hormonal coordination. Most of the oxalate uptake studies present data on its ion-exchange system, such as chloride, bicarbonate, proton and formate [130]. But these studies do not throw light onto the mechanism of oxalate retention. Excellent reviews are available on calculi formation mechanisms, both with the involvement of altered papillary epithelium and also in the cavities without any attachment [35], as well as the interaction of macromolecules with stone forming calcific crystals [56, 123]. All of the macromolecules implicated in the crystallization processes so far have some form of calculi binding activity, either through specific domains or anionic sites [56]. This review presents evidence for the presence of oxalate/CaOx binding proteins and CaOx crystal adsorbing proteins with oxalate binding activity in the renal cells which are thought to be involved in the retention of crystals under altered physiological conditions.

Intestinal oxalate binding protein

The hyperabsorption of dietary oxalate has been attributed to idiopathic CaOx nephrolithiasis [46]. In the rabbit, oxalate is transported through the intestine by a diffusion mechanism that is not energy but concentration dependent [27]. Oxalate absorption from the gut takes place by simple passive diffusion in humans and experimental animals [38]. In rat colon mucosa, oxalate and chloride share a common transport pathway in which chloride exchanges with bicarbonate [49]. The energy dependent net absorption of oxalate is found to be 4′,4′-diisothio-cyano stilbene 2-2 disulfonic acid (DIDS) sensitive and carrier mediated. Studies by Pinto and Paternain showed, for the first time, the presence of an oxalate transport system mediated by a carrier protein having the characteristics of a transport protein with a molecular weight of 73 kDa [83]. They speculated that two different oxalate transport systems exist, one operating at low oxalate concentration mediated by the transport protein and the other at high oxalate concentration by passive diffusion.

Pyridoxine deficiency causes hyperoxaluria in humans and animals [31]. In pyridoxine deficiency, oxalate absorption takes place with biphasic carrier mediated characteristics in which a carrier-mediated saturable component facilitates oxalate uptake from the lumen into enterocytes at low mucosal oxalate concentrations [27]. Koul et al. identified an oxalate binding protein in the brush border membrane having the kinetic properties of reversibility, saturability, temperature sensitivity and inhibition by substrate analogues [63]. The protein was induced under pyridoxine deficiency with two distinct classes of receptor sites for oxalate, one with high affinity and the other with low affinity (Table 1). The protein had a molecular weight of 79 kDa and may be involved in oxalate binding and transport in the rat intestinal brush border membrane during pyridoxine deficiency.

Red blood cell band 3 protein

The anion transporter band 3 protein is ubiquitous, not only being present in cell membranes, but also in nuclei, Golgi's complex and mitochondrial membranes [55]. It is involved in respiration, acid-base balance and is the major structural protein linking the plasma membrane to the cytoskeleton. The transport of chloride and bicarbonate, physiologically important anions, is rapid in human red blood cells (RBC) from adults and late fetuses [22], and also from adult chickens [21]. Band 3 protein has been cloned in many species [70], and the gene is located on chromosome 17. It is a 911 amino acid protein having a hydrophilic cytoplasmic domain, a hydrophobic transmembrane domain and an acidic

Table 1 Cellular localization of oxalate transport/binding proteins. BBM: brush border membrane, Bmax: maximal binding capacity; Kd: dissociation constant, IC 50: concentration of inhibitior at 50% of maximal binding. Equilibrium dialysis technique; ↑ = increase, ↓ = decrease

	Small intestine		Kidney											Liver	Red blood cells	
	BBM	B_6 deficient BBM	Mitochondria				Nucleus					Ca.Ox binding protein (Homogenate)		Histone (H_1)	Chicken	Human
			Inner membrane		Purified protein		Histone (H_1)		Nuclear membrane	Nuclear pore complex						
			Rat	Human	Rat	Human	Rat	Human	Rat	Rat	Human	Rat	Human			
Sp. activity (pmol/mg protein)	6.3		23.5	7.7	63	51	940	1,570	14.6	144	220	4.98	4.3	660	1,354 Flux rate 1.24	36 Flux rate 0.51
Optimum pH	7.5	7.0			7.0	7.0	4.5	4.5	7.4	7.4	7.4	5.5	5.5	4.2	7.4, 4.5	7.4
Optimum Temperature (°C)		34	23				30	30				30	30	28	37	37
Energy of activation		19	12.15				10.07	16.47				8.07	8.87	27.54		
Equilibrium time (min)	480 at 4°C	6	2		90	90*	2	2		3	3				10	10
Bmax (pmol/mg ptn)		30,156	49	13	21	14	42	75			197	6.5	6.2	40		320
							145	143						210		
Kd		24.3	43	149	33	41	42.4	76			2.98	39.7	39.5	133, 262		3.79
Binding site		2			1	1	2	2				1	1	2		
Inhibitors (% inhibition) EGTA			10	8			33	38	12	15	15	7	0	0		
DIDS			100	100			100	100		50	50	90	90	50	100	100
IC 50 (μM) oxalate	↓	4	0.25	0.20	0.22	0.25	0.35	0.8	0.5			0.3	0.3	0.5		
Glyoxalate		10	70	10	220	22	14	22				700	700	700		
Malate		300	660	75			105	80				0.75	0.75	1200	60% (inhibition)	45% (inhibition)
Thiol modification (% activity) oxidation			170				135	120				100	100			
Reduction			20				35	37				35	56			
Alkykation			100				100	100				50	51	15		
Molwt (kDa)	73	79			58	62	27.5	29	68		205		45	27		91, 97
Hyperoxaluria (% increase)	↑	Induction ↑	↑				150		150		130	150		↑		↑
References	[83]	[64]	[65, 73]	[65, 73]	[98]	[98]	[103]	[103]	[110]	[130]	[130]	[2]	[2]	[104]	[21]	[106]

C-terminal domain. The amino acids involved in anion exchange include lysine, arginine, histidine and glutamic acid [85]. Band 3 protein is involved in the transport of oxalate across the human RBC membrane [11]. It behaves as an oxalate exchanger in a phosphorylation dependent manner [12]. Increased arachidonic acid content of the RBC membrane of stone formers was attributed to enhanced oxalate exchange through the phosphorylation reaction. Diet induced reduction of arachidonic acid content of RBC membranes resulted in the modification of RBC oxalate transport and urinary oxalate excretion by human subjects and animals [30], suggesting that membrane phospholipid arachidonic acid content can control cellular oxalate transport by modulating protein kinases involved in the band 3 phosphorylation process [13].

Band 3 like proteins are present along the gastrointestinal tract as well as the renal tubules [4]. Immunological studies using highly purified antibodies raised against the intra-membranous domain of band 3 protein have shown specific fluorescence along the basolateral membrane's alpha intercalated cells of the collecting duct [126]. A link between RBC abnormality and renal stone formation has been suggested [19]. Patients with primary CaOx nephrolithiasis have a significantly elevated RBC oxalate exchange [10, 74].

Renal oxalate uptake and oxalate binding proteins

Renal oxalate uptake

The transcellular movement of substrates from the peritubular capillaries to the tubular fluid requires the crossing of two barriers: the basolateral plasma membrane, which is in contact with the internal milieu facing the blood supply, and the apical plasma membrane, which is in contact with the external milieu and faces the tubular fluid. The transport of oxalate across these membranes is mediated by anion exchange mechanisms.

Renal oxalate secretion is mediated by the cellular entry of oxalate in exchange for sulfate and/or bicarbonate at the basolateral membrane, followed by the cellular exit of oxalate in exchange for sulfate, bicarbonate or hydroxyl ions at the apical membrane [122]. To create a driving force for the exit of sulfate via the central luminal membrane, sulfate must first accumulate in the cell. This happens by virtue of sodium coupled sulfate transport in the luminal membrane. Using LLC-PK1 cell lines, as well as rabbit renal microvillus membrane, transport studies have suggested that chloride/oxalate exchange plays a major role in the cellular exit of oxalate at the apical membrane [54, 131]. The presence of distinct oxalate transporters in the mammalian proximal tubule has been demonstrated [33]. In addition, a sulfate/bicarbonate anion exchanger having affinity for oxalate in the rat renal cortex basolateral membrane vesicles [61], oxalate exchange for bicarbonate and sulfate in rabbit renal cortex apical membranes [53], and an oxalate/OH^- exchanger [134] in rat renal proximal brush border vesicles have been demonstrated. All of these anion exchangers, including subtypes of organic anion transporters are thought to be involved in the transport of organic anions [121]. Several oxalate specific binding proteins had been identified in renal tissues in our laboratory and their kinetic properties and influence on crystal growth have been studied.

Oxalate binding proteins

Mitochondria

The presence of an oxalate binding protein had been identified in tissues such as the human kidney, where it is found in mitochondria, and rat kidney and liver [65]. Other rat tissues like heart, lung, skeletal muscle, spleen, stomach, and small and large intestinal homogenate showed no binding with oxalate. About a third of the total oxalate binding was found to be localized in the inner mitochondrial membrane. The binding of oxalate was specific and the other substrate analogues compete with it less efficiently. The binding of oxalate was rapid, reversible, dependent on oxalate concentration, and temperature sensitive. Scatchard plot analysis has shown the maximum binding capacity (Bmax) to be 49 pmol/mg protein with a dissociation (Kd) of 43 nM. Calcium had no effect on oxalate binding [65], suggesting that the binding was oxalate specific. The purified proteins from human as well as rat mitochondria showed molecular weights of 62 and 58 kDa, respectively. Both proteins had higher percentages of both basic and acidic amino acids [98]. Antibody raised to the rat protein inhibited oxalate binding and also cross-reacted with the human protein. The characteristic properties are listed in Table 1. Proteoliposomes prepared with the proteins showed an accumulation of oxalate, confirming a transport function for the protein. The uptake of oxalate in mitochondria was mediated through the dicarboxylate transport carrier [115].

Nucleus

About 2/3 of the total cellular oxalate binding activity was distributed in the kidney and liver nuclei [73]. Most of the radioactive oxalate binding was present in the histone-H_1 fraction, and in particular the H_{1B} fraction [103]. Purified protein showed oxalate binding with the characteristics of rapidity, reversibility, saturability and pH dependency. Trypsin treatment abolished oxalate binding. Scatchard plot analysis showed the characteristics of protein ligand binding (Table 1). Two distinct binding sites were found, one with high affinity and the other with low affinity for oxalate. The oxalate binding activity was inhibited by the transport inhibitors DIDS and phenyl succinate. Similarly, liver H_{1B} was found to have a maximal oxalate binding activity with similar characteristics to renal protein [104]. This binding was

not associated with calcium binding. Different tissues histone H_1 fractions also exhibited oxalate binding, showing its ubiquitous nature.

About 40% of the total nuclear oxalate binding was localized in nuclear membrane. Two oxalate binding proteins were identified on the nuclear membrane, one having a molecular weight of 68 kDa [110] and the other in the nuclear pore complex having a molecular weight of 205 kDa [128]. Both oxalate binding proteins had maximal binding at pH 7.4 for both rats and humans.

All nucleated cells had oxalate uptake mechanisms. Oxalate binding and uptake were studied using, for example, intact, nucleated chicken RBC, nuclear membranes and nuclear histones. The rate of oxalate anion exchange by nucleated erythrocytes was found to be higher (K = 1.24/min) than that of human RBC (K = 0.5/min), but less than that of nuclei (K = 1.55/min) [106]. The flux rate was pH and temperature dependent. Oxalate exchange was reduced by 35% for chicken RBCs and nuclei in the presence of sulfate, bicarbonate, phosphate and succinate, suggesting their common transport nature. Both plasma membrane and nuclear membrane showed maximal oxalate binding at pH 7.4, while for the nuclear basic protein fraction this occurred at pH 4.5, similar to renal and hepatic histones. DIDS inhibited both exchange as well as oxalate binding, suggesting that oxalate specific binding proteins are involved in the transport systems.

Calcium oxalate binding proteins

Renal calcium binding proteins have been identified in several biological systems [41]. Resnik et al. [90] reported excess excretion of calcium binding proteins in kidney stone formers. Calcium binding proteins as well as molecules like sialic acid, γ carboxy glutamic acid and phosphatidic acid allow high local concentrations of calcium inside the cell in hyperoxaluric conditions [5, 87, 88].

Using calcium-^{14}C oxalate, the existence of CaOx binding protein has been demonstrated in several rat tissues [2]. Kidney showed the maximum binding activity among the various tissues studied. Renal medulla exhibited higher CaOx binding activity than papilla or cortex. Subcellular studies revealed the enrichment of this protein in nuclei. The purified protein has a molecular weight of 45 kDa and the kinetic properties of concentration and time dependency, optimum temperature, substrate saturability with a single affinity site with a Kd of 41 nM and Bmax of 6.5 nmol/mg protein (Table 1). This binding was inhibited by DIDS, while EGTA and ruthenium red had no effect on the binding, suggesting that the protein-oxalate binding was oxalate specific and not calcium site specific. Further lysine group might be involved in oxalate binding since DIDS is known to react with lysine-amine residues.

All of the above listed oxalate specific binding proteins, located in the different membranes of renal cells, may serve the function of carrier proteins for the transport systems since all of the transport systems exhibit energy of activation as well as transition point during temperature effect studies and inhibition by transport inhibitors. Carrier proteins of the transport system are generally constituted with lipids and they undergo phase transition at a temperature which is a function of the fatty acid composition of the lipid components [20, 91].

Calcium oxalate monohydrate adsorbing proteins

All urinary stones contain an organic matrix which comprises approximately 2–5% of the total stone weight. Organic matrix has been considered to be essential for the genesis and mineralization in the growth of urinary calculi during stone formation [76]. As the matrix proteins are thought to be present due to inclusion by tissue trauma or by coprecipitation along with crystals, the actual proteins in the active involvement of the crystallization process are still unknown. Thus, studies have been attempted with the proteins which were adsorbed with calcium oxalate monohydrate (COM) crystals in urine. Several proteins have been identified by this method which resemble those identified in stone matrix. [8, 26, 44, 48]. All of these proteins, such as nephrocalcin, Tomm-Horsfall glycoprotein, crystal matrix protein or prothrombin fragment F1 and bikunin, have the characteristic properties of calcium binding and inhibition of crystal growth. None have been tested for oxalate binding activity. Several oxalate-binding proteins have been identified in COM crystal adsorbed proteins as well as stone matrix proteins in our laboratory.

Human kidney

The proteins were adsorbed on CaOx crystals by allowing them to interact with triton-extracted human kidney homogenate. They were then subjected to DEAE-cellulose column chromatography and three protein peak fractions were eluted (designated as fraction I–III) according to their order of elution with increasing concentration of NaCl [100]. Among the three eluted protein peaks, the protein eluted with 0.3 M NaCl (fraction III) had the maximum oxalate binding activity of 270 pmol/mg protein at pH 4.5. The protein had no oxalate binding at pH 7.4. The purified protein had a molecular weight of 23 kDa. Amino acid analysis showed that 18% of the total molar proportion was made up of basic amino acids (lysine and arginine) while acidic amino acids accounted for only 11%. Modification of the lysine group abolished oxalate-binding activity.

In contrast to fraction III, fraction I had oxalate binding activity at pH 7.4.The purified protein had a molecular weight of 45 kDa with an activity of 280 pmol oxalate/mg protein. The protein had the kinetic properties of saturability, with a Kd of 1.96 nmol and Bmax of 200 pmol/mg protein [99].

Rat kidney

Among the different tissues, the COM adsorption of proteins derived from kidney was found to be maximum. DEAE-cellulose chromatographic fractionation yielded three proteins with oxalate binding activities, fractions I–III according to their order of elution. The molecular weight of these fractions were determined to be 74, 20 and 23 kDa, respectively. Subcellular distribution studies of these proteins revealed that the 20 kDa one was largely distributed in microsomes, that of 74 kDa in mitochondria and the 23 kDa protein in nuclei [101]. The 74 kDa protein exhibited maximum oxalate binding activity at pH 7.4, while for the 23 kDa protein this was at pH 4.5 with no activity at pH 7.4.

Human kidney stone matrix

When EDTA-extractable proteins from human kidney stone matrix were subjected to DEAE-cellulose chromatography, three protein peaks were identified with oxalate binding activity in the order of elution, fractions 1–3. Fraction 1 on further passage through a Sephadex G-200 column, separated into 48 kDa and 29 kDa proteins. The 48 kDa protein had maximum oxalate binding activity compared to the other. Both proteins exhibited the binding characteristics of renal proteins [34] (Table 2).

Effect of lipid and protein peroxidation on oxalate/CaOx binding proteins in hyperoxaluria

Mitochondrial oxalate binding

Oxalate binding was increased by 150–180% of control values in kidney and liver after subjecting them to lipid peroxidation (LPO) [107]. Renal mitochondrial oxalate binding was stimulated by promoters in the order of $Fe^{2+} > tBH > ascorbic\ acid > Fe^{3+} > H_2O_2$. The iron induced oxalate binding was inhibited by reduced glutathione, ß-mercaptoethanol, alpha tocopherol, and the hydroxyl radical scavengers histidine and mannitol. Catalase also inhibited both Fe^{2+} and H_2O_2 induced enhanced oxalate binding and LPO reaction suggesting that the oxalate binding was mediated through a hydroxyl radical reaction. Increased oxalate binding had a positive correlation ($r = +0.98$) with LPO and a negative correlation with reduced GSH content ($r = -0.80$), observed during lipid peroxidation in rat kidney mitochondria [108] (Table 2). In the presence of oxidized glutathione (GSSG), peroxidized mitochondria lost 48% of their protein-SH with a concomitant threefold increase in oxalate binding activity, while control mitochondria lost only 20% of their protein-SH content with only a 0.8%-fold increase in oxalate binding activity [108]. The affinity of oxalate (Km) to GSSG treated mitochondria increased from 1×10^{-8} M to 0.6×10^{-8} M. Reduced GSH inhibited oxalate binding activity competitively ($Ki = 1.4\times10^{-31}$ M). Urolithic kidney mitochondria showed a 30–50% increase in oxalate binding activity along with a depletion in total GSH and protein-SH as observed in vitro [79]. Similarly, feeding dehydroascorbic acid to rats also increased renal oxalate binding activity [102]. This increased activity was associated with increased LPO and decreased activities of the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase, glutathione-S-transferase (GST) and the scavengers, reduced glutathione (GSH) ascorbic acid and vitamin E. These studies suggest that oxalate binding is regulated by the thiol status of the mitochondria. This is further supported by the following studies: (1) feeding a vitamin B_6 deficient diet or calculi producing diet such as sodium glycolate or ethylene glycol in drinking water increased oxalate binding activity in mitochondria with the depletion of thiol content [79, 108]; (2) the depletion of GSH by buthionine sulfoximine in the presence of ethylene glycol feeding [80] increased oxalate binding activity. Further, oxalate binding activity was correlated negatively with the thiol status of mitochondria and calcium accumulation was also negatively correlated with thiol content; (3) ischemia reperfused kidney also showed stimulated mitochondrial oxalate binding with the depletion of thiol content [109]. A well pronounced effect was seen when ischemia reperfusion was combined with hyperoxaluria. (4) In addition, oxalate administration to rats also increased mitochondrial oxalate binding activity, and peak activity was correlated with the deposition of crystals [119].

Nuclear oxalate binding proteins

An increase in oxalate binding activity was observed when nuclear fractions were subjected to lipid peroxidation. The increased oxalate binding was associated with the depletion of -SH content. The binding activity was correlated positively with LPO and negatively with thiol content in the nuclear as well as residual membrane fractions [52]. In contrast, histone oxalate binding activity was correlated negatively with LPO (Table 2). Increased nuclear oxalate binding was also associated with experimental urolithiasis. The hyperoxaluric rat kidney nucleus exhibited a 50% increase in oxalate binding in both the residual fractions containing nuclear envelope and the histone fraction, with a concomitant increase in basal lipid peroxidation and a decrease in thiol content [53]. Hyperoxaluric rat kidney showed an increased H_1 content and oxalate binding activity. The distribution of H_{1B} was higher than in the control, showing that hyperoxaluria increased the expression of H_{1B}. Similarly, the expression of nuclear pore complex protein was found to be dependent on the cell cycle and oxalate concentration. Maximum expression was associated with telophase [129].

Table 2 Effect of in vitro lipid/protein peroxidation on oxalate binding and calcium oxalate crystal growth

	Oxalate binding				Crystal growth, crystal nucleation and aggregation				References
	Control (pmol/mg protein)	Peroxidation (%increase)	Correlation		Control	Peroxidation	Correlation		
			Vs peroxidation	Vs-SH content			Vs LPO	Vs thiol	
Mitochondrial membrane	18.9	180	+0.98	−0.80	Promoted crystallisation by 75%; promoted nucleation and aggregation	Increased nucleation and aggregation	+	−	[34, 98]
Mitochondria	9.7	148	+	−0.54					
Histone	16.7	24	−0.62	+0.62	Stimulates crystal growth by 33–50%	Stimulates crystal growth by only 25%	+	+	[104, 103]
Nuclear membrane	4.1	256	+0.98	−0.74	Inhibitor of crystal nucleation and aggregation by 65%	Less inhibitory effect	+	−	[52, 110]
Nucleus	7.8	130	+	−0.54	Promoter of crystal nucleation and aggregation	Peroxidized nucleus, promoted nucleation	+	−	[34]
Microsomes	1.1	120	+		Inhibited nucleation and aggregation	Decreased effect of nucleation and aggregation			[6]
COM fraction I (73 kDa)	149	112	+	−	Promoted nucleation and aggregation, COD crystal turned into COM crystal	Further promoted nucleation and aggregation. More COM crystal aggregation	+	−	[101]
Fraction II	165	120	+	−	Inhibitor of nucleation and aggregation	Decreased inhibition of nucleation and aggregation	− (Increased COD size)	−	[101]
Fraction III (23 kDa)	211	138	+	−	Inhibitor of nucleation and aggregation. COD crystals with altered morphology	Induced aggregation of crystals	+	−	[51, 101]
Stone matrix fraction 1	110	122	+	−	Promoted nucleation and aggregation	Enhanced promoting effect on nucleation and aggregation	+	+	[34]
Fraction 2	88	111	+	−	Inhibited nucleation and aggregation	Reduction in inhibitory effect on nucleation and aggregation	+	+	[34]
Fraction 3	35	114	+	−	Promoted nucleation and aggregation	Enhanced promoting effect on nucleation and aggregation	+	+	[34]

Calcium oxalate binding protein

Renal CaOx binding activity was increased in rats treated only with cyclosporin A, a condition which increases lipid peroxidation leading to nephropathy [3]. A further increase was observed when combined with hyperoxaluria. This increased CaOx binding was associated with LPO as well as with a concomitant decrease in total thiol in both rat and human kidney [3]. The increase in CaOx binding activity observed in hyperoxaluria was associated with increased availability of the protein, suggesting an enhanced expression of this protein.

COM binding proteins

Proteins derived from different rat tissues show that all organs have the capacity for COM adsorption. Maximum adsorption occurred in rat kidneys and pancreas. Microsomes showed maximum adsorption in the kidney. Hyperoxaluric rat tissues showed a greater percentage of adsorption to crystals suggesting an enhanced affinity. Among COM binding proteins derived from hyperoxaluric rat kidney, both the 74 kDa and 23 kDa proteins showed an increased concentration with increased oxalate binding activity [51].

Stone matrix proteins

Similar to mitochondrial, nuclear and COM adsorbed proteins, stone matrix fractions obtained from DEAE-cellulose chromatography showed increased oxalate binding by 22%,11% and 14%, respectively, for fractions 1–3. During peroxidation, there was no loss of protein content but thiol content was drastically decreased [33] (Table 2).

Effect of oxalate binding proteins on cCaOx crystal growth: the role of peroxidation

Differences in the effect of oxalate binding proteins on CaOx crystal growth have been noted (Table 2). Purified mitochondrial oxalate binding proteins derived from rat and human kidney showed a promoter effect on CaOx crystallization [98]. Among them, rat protein showed higher promoter activity and the antibody of this protein inhibited both oxalate binding activity and crystal growth in vitro, suggesting that the oxalate binding site plays an important role in crystal growth.

Histone oxalate binding protein also exhibited CaOx crystal growth promoter activity [103]. Histone may be involved in the initiation of crystal formation in nuclei, since calcium and calcium binding protein concentrations are also higher in the nucleus [32]. In contrast to histone oxalate binding protein, nuclear pore complex oxalate binding protein (205 kDa) showed an 87% inhibition of CaOx crystal growth in vitro [128].

Among the COM adsorbed human kidney proteins, the basic protein (23 kDa) was found to inhibit crystal growth by 82% at 0.8 μmol/l. It inhibited the nucleation and aggregation of the crystals by 6% and 28%, respectively, at 49 nmol/l. The inhibition of both nucleation and aggregation was higher at pH 5.7 than at pH 7.4. Significantly, the protein induced the formation of intertwined CaOx dihydrate crystals in a medium known to induce the formation of individual dihydrate crystals [78]. In contrast, the 45 kDa protein was found to promote crystal growth with −38.61 inhibitory units at 125 μg/l protein concentration. The protein promoted crystallization to a greater extent when modified with oxidized glutathione by promoting both nucleation and aggregation [100]. It transformed the structure of crystals from COD to COM even in the presence of citrate. The COD crystals were of 5–7 μm in size while the COM crystals were of 3–5 μm [51]. These studies suggest that the 45 kDa protein seems to be a strong candidate for promoting crystal formation, since the antibody to this protein cross reacted with the matrix stone proteins as well as urinary proteins (unpublished data).

Among the COM adsorbed rat renal proteins, the 74 kDa protein was found to be a promoter while the other protein fractions inhibited crystallization. In hyperoxaluria, the crystal growth promoting activity of the 74 kDa protein was further increased while the degree of inhibition by the 20 and 23 kDa proteins was decreased. The 74 kDa protein derived from control rats formed single COM crystals in crystal growth while the hyperoxaluric rat fraction induced the aggregation of COM crystals [51].

In order to determine whether LPO of membranes alters the binding properties of COM crystals, the kidney homogenate was subjected to lipid peroxidation and COM adsorbing proteins were isolated and studied in relation to their effect on CaOx crystal growth. A positive correlation between LPO and COM adsorption and a negative correlation between reduced glutathione and COM adsorption were observed [51]. However the peroxidized proteins did not show any alterations in the elution profile on the DEAE-cellulose column. The -SH content of the peroxidized fractions was lower than that of the control fractions, but their oxalate binding activities were increased. Peroxidized fraction I (74 kDa) promoted crystal growth to a greater extent than control fraction I (74 kDa). Peroxidized fractions II and III were found to be less inhibitory in nature compared to their control fractions. Light microscopic examination of the crystals formed in the presence of peroxidized fractions showed the formation of large aggregates of COM (Table 2). These results suggest that peroxidation may be one of the mechanisms altering the crystal growth modulatory activity of the proteins in hyperoxaluria [101].

Kidney stone matrix protein fractions eluted from the DEAE-cellulose column showed increased oxalate binding activity and correlated negatively with reduced thiol content. Fraction 1 (eluted in Tris-HCl, pH 7.4)

and fraction 3 (0.3 M NaCl in buffer) showed an inhibitory effect in an in vitro crystallization system [33]. On peroxidation, fractions 1 and 3 showed a further increase in the promoting effect whereas fraction 2 showed a reduction in the inhibitory effect of nucleation and aggregation of CaOx crystals. Protein peroxidation was negatively correlated with the inhibitory activity of the protein in CaOx nucleation and aggregation. A similar promoting effect of nucleation and aggregation was seen with mitochondria and nuclei after peroxidation. These studies suggest that the peroxidation of proteins or tissue has a substantial influence on nucleation and aggregation in CaOx crystal growth.

In all of the above studies, it is very interesting to note that in the oxalate binding protein fraction eluted in buffer from DEAE-cellulose columns from human or rat kidney, COM adsorbed proteins and human stone matrix proteins behaved as strong promoters of CaOx crystal growth. The other fractions eluted in salt buffer eluate behaved as strong inhibitors. Peroxidation of the protein fractions either increased the promoting effect of the promoter proteins or decreased the inhibitory effect of the inhibitor proteins. These functional alterations are attributed directly to the thiol status of the cell. This is also reflected by urinary protein in crystal growth behaviour.

Similar to tissue proteins, hyperoxaluric rat urinary proteins also showed a different behaviour in crystal growth studies. The protein eluted in 0.3 M NaCl on DEAE-cellulose columns having a molecular weight of 45 kDa had maximaum nucleation as well as aggregation inhibitory effect. In hyperoxaluria, the excretion of this protein was increased significantly. In the crystal growth assay, the control 45 kDa protein inhibited nucleation by 75% and aggregation by 100%[111]. In contrast, it is very interesting to note that the protein derived from 28th day hyperoxaluric urine, behaved as a promoter of nucleation (−113%) and a weak inhibitor of aggregation (28%). A highly significantly negative correlation ($r = -0.97$) between oxalate excretion and the inhibitory effect of the 45 kDa protein was observed suggesting a modification of the protein by oxalate.

Apart from the peroxidation of proteins, the carboxylation of proteins was also found to facilitate COM crystal adsorption. Microsomal proteins were adsorbed maximally with COM crystals. On carboxylation of the renal microsomal proteins, a significant increase in the COM crystal adsorption by 2.5-fold was observed in the hyperoxaluric condition [6]. Further, carboxylated microsomes of ethylene glycol fed rats showed significant binding with CaOx. The enhanced carboxylation reaction was associated with enhanced carboxylase activity due to stimulation of the enzyme oxalate/CaOx, and lipid peroxidation [5]. The microsomal γ-glutamyl carboxylation system has been shown to be an integral membrane protein in the renal tubule [29], and enhanced γ-carboxy glutamic acid and carboxylated protein excretion has been observed in calcium nephrolithiasis [25, 77]. Both γ-carboxy glutamic acid and carboxylated proteins exert a marked enhancing effect on the nucleation rate of CaOx.

Physiological significance of oxalate binding proteins in relation to other proteins in CaOx stone formation

The interaction between CaOx crystals and tubular epithelial membrane is considered to be an essential event for the development of renal stones. Several mechanisms have been proposed for the aetiology of stone formation and crystal retention [62, 120]. The principal causative factors for the formation of calcium salt stones are attributed to the supersaturation of precipitating salts and decreased excretion of inhibitory substances such as citrate, pyrophosphate or glycosaminoglycans and inhibitory proteins which form complexes with calcium or elements such as magnesium and sodium which bind oxalate [24, 72]. The events leading to the attachment of crystals followed by retention of crystals by renal cells are still not well understood. In order for the renal cell to retain CaOx crystal, the crystal should bind to the cell surface or, primarily, to the subcellular organelles. The retention of crystals in the tubular luminal side may occur due to the size of the crystals exceeding the diameter of the nephron or increased passage time due to an abnormal nephron morphology [23, 35].

The adherence of crystals is considered to be the first event in the final phase of retention. Khan et al. identified renal cell injury as a high risk factor for CaOx nephrolithiasis [58]. Cell injury can be induced during mild hyperoxaluria, as evidenced by enzymuria and membranuria [59], in the absence of crystalluria and crystal deposition, implying that hyperoxaluria plays a significant role in membrane physiology. Our studies have established a positive correlation between free radical induced lipid peroxidation of the membrane and oxalate binding. This increased oxalate binding is negatively correlated with decreased thiol content of the membrane/protein [2, 93,79, 98, 103, 106, 108] in stone forming condition. That the involvement of free radical mediated lipid peroxidation is the major cause of tissue injury is further confirmed by our studies with the supplementation of antioxidants to urolithic rats, in which not only the LPO reaction but also CaOx deposition was prevented in the renal cells [1, 79, 93, 94, 97, 105, 116]. The role of lipid peroxidation and free radical mediated changes in renal stone formation have been further confirmed by Schied et al. [92] and Thamilselvan et al. [117, 119]. Oxalate and not CaOx is found to be more effective in inducing lipid peroxidation [95], and this effect is also substantiated by studies on the exposure of cells to a metastable concentration of oxalate along with CaOx [118]. Similarly, Koul et al. [64] have also shown a specific interaction of oxalate and not CaOx crystals in altering the cell reaction for the promotion of retention of COM crystals. In addition, oxalate is known to interact with the cellular machinery to induce the

expression of several gene products such as early growth response-1, Nur 77, C-Jun, C-myc, zinc finger transcription factor, fast acting plasminogen activator inhibitor, osteopontin and platelet derived growth factor [117].

Several crystal-adhering molecules have been proposed (Table 3). The urinary epithelial cell can bind a crystal only after injury to the cell that results in the exposure of membrane phosphatidyl serine on its luminal surface. This is correlated with a corresponding increase in COM crystal attachment. The apical membrane lipid asymmetry due to the loss of cell polarity occurs by the exposure of crystals [132]. Specific sialic acid containing glycoproteins and glycolipids have been suggested as the critical determinants of the face-specific nucleation of COD crystals on the apical renal cell surface [69]. Crystal retention is attributed to alterations in the number or composition of these cell surface molecules on genetic modification by genetic alterations, cell injury or drugs in the tubular fluid [125]. Evidence has been provided for the interaction of CaOx crystals with sialic acid [47, 135], hyalouranan, chondroiton sulphate and heparan sulfate molecules, which are thought to play a major role in the attachment of crystals [127]. It has been suggested that in the absence of structural injury, sub-lethal injury mediated by ischmeia or oxalate induced lipid peroxidation reaction can expose previously concealed sialic acid residues so that they protrude into the tubular lumen in a different orientation which enable them to interact with the crystals, resulting in crystal adhesion. Moreover, the increased expression of sialic acids in the distal tubule glycoprotein as well as glycolipids with altered sialic acid linkages has been suggested for the increased nucleation and attachment of crystals [49].

Several crystal adhesion inhibitor molecules have been identified and their expression is found to increase during crystal formation [50, 60]. These molecules such as osteopontin, nephrocalcin, Tomm-Horsfall glycoprotein and bikunin inhibit crystal nucleation as well as aggregation [56, 60] (Table 3). Mild hyperoxaluria promotes the increased production of crystallisation modulators/inhibitors [49]. It has been suggested that these molecules control not only crystal nucleation, growth and aggregation but also crystal interaction with the tubular epithelium and their retention in the cell [56].

All the crystal binding molecules or adherence molecules facilitating crystal adherence to the epithelium or molecules which inhibit or modulate crystal growth behaviour are specific calcium binding molecules. They form a molecular contact with the calcium atoms on the surface of CaOx crystals [56]. All of the crystal binding molecules possess anionic sites such as carboxylate contributed by either sialic acid (glycoprotein, glycolipids) or serine, glutamate/aspartate (Tamm-Horsfall glycoprotein, bikunin, prothrombin F1); carboxylate and phosphate contributed by the membrane phospholipids of phosphatidyl serine and phosphatidyl inositol, and sulfate contributed by surface carbohydrates (glycosaminoglycans, heparan sulphate and chondroiton sulphate).

Apart from calcium binding sites, we now have evidence of oxalate specific binding molecules with similar crystal growth modulating activity. Both crystal promoting and crystal growth inhibitor proteins are known [51, 98, 100, 101, 103, 111]. They occur in the renal medulla and cortex and are differentially abundant in the subcellular organelles. All of the proteins possess lysine in the active binding site with oxalate. Lysine modification abolishes oxalate-binding activity. Oxalate binding activity is influenced by the thiol status of the cell. Oxalate itself is known to deplete the thiol status of the protein by means of inducing lipid peroxidation [1, 94, 97, 105], and peroxidised proteins modulate the crystal growth behaviour differently. Following peroxidation, the promoter activity is increased for the promoter protein while the degree of crystal growth inhibition is decreased for the inhibitor proteins, with a net result of promoting crystal growth. This situation is also reproducible in CaOx nephrolithiasis (Table 3) and these proteins are at higher concentration under this condition.

All of the oxalate specific binding proteins are distributed in the subcellular organelles. Their functions, although not defined at present, may be implicated in the movement of oxalate/CaOx from the basolateral side to the luminal side for secretion and/or from the luminal side to the lateral side for possible detoxification through macrophage mediated destruction [56]. Adhesion followed by internalisation of CaOx crystal by endocytosis has been suggested to promote crystal retention in the nephron by a positive feedback loop that favours the adhesion of additional crystals [66]. In addition to the endocytic process, the cellular depletion of thiol could contribute further for the accumulation of both calcium and oxalate [80, 93]. The increased concentration of calcium and oxalate in the cells may turn on the expression and cell proliferation by the presence of calcium and oxalate binding proteins in the nucleus and mitochondria. The oxalate binding nature of histone H_{1B}, and the involvement of nuclear membrane pore complex oxalate binding proteins in the cell cycle reaction suggest that oxalate is not like a simple dicarboxylate molecule alone but has a specific genomic interaction to modulate its activity.

In conclusion, oxalate specific binding proteins could be involved in the initiation of renal stone formation under altered physiological conditions. In hyperoxaluria, these membrane proteins undergo lipid/protein peroxidation with the loss of sulfhydryl groups, resulting in enhanced binding with the oxalate. The peroxidised membranes/proteins have a net promoting effect of CaOx crystallisation by way of enhancing the promoter effect and decreasing the efficiency of the inhibitory effect (Fig. 1). The crystal promoting activities could be increased further by increased availability of these proteins in hyperoxaluria. The lipid peroxidation reaction could lead to fragmentation of the

Table 3 Molecules that are involved in the interaction of calcium oxalate crystals in renal cells. COM: calcium oxalate monohydrate

Serial no.	Binding molecules	Tubular localization	Chemistry of the molecule	Crystal attachment site	Effect on crystal growth attachment	Expression in nephrolithiasis	References
1	Cell surface carbohydrates	Epithelium	Sialic acid-glycoprotein	Calcium	Facilitation of crystal attachment	Increased	[47, 66, 68, 69]
		Medullary interstitium, epithelium of distal and proximal tubules	Hyaluronan chondroitin sulphate	Calcium	Crystal attachment, crystal growth inhibition	Increased	[88, 127]
2	Cell surface lipids	Epithelium	Sialic acid-glycolipids	Calcium	Facilitates crystal attachment, growth inhibition	Increased	[47]
		Epithelium	Phospholipids-Phosphatdyl serine	Calcium	Attachment of crystals	Depolarization and asymmetry	[15, 16, 133]
3	Proteins prothrombin fragment I/crystal matrix protein	Epithelial cells of thick ascending limb of Henle and distal collecting tubule.	Glu, Asp rich	Calcium	Crystal growth inhibitor	Increased	[26, 36]
	Inter α trypsin inhibitor (220 kDa)	Proximal tubule	Glycoprotein-Gla rich	Calcium	Crystal growth inhibitor	Increased	[56, 75]
	Bikunin (35 kDa)	Tubular epithelial cells	Chondroitin sulphate	Calcium	Crystal growth inhibitor	Increased	[8, 49, 56, 75]
	Osteopontin/uropontin	Loop of Henle	Glu, Asp rich; RGD sequence	Calcium	Crystal growth inhibitor	Increased	[48, 50, 58]
	Nephrocalcin (14–64 kDa)	Proximal cells	Glu, Asp rich	Calcium	Crystal growth inhibitor	Increased	[42, 44, 56]
	Tamm-Horsfall Protein (96 kDa)	Thick ascending limb of loop of Henle	Glu, Asp rich	Calcium	Crystal growth inhibitor	Structural modification	[42, 43, 49, 56]
	Uronic acid rich protein (35 kDa)	Tubular epithelial cells	Uronic acid rich	Calcium	Crystal growth inhibitor	Increased	[8, 56]
	γ Carboxy glutamic acid rich proteins	Microsomes	Gla rich	Calcium	Promoter of crystal growth	Increased	[6, 77]
4	Oxalate binding proteins 58/62 kD	Cortex, medulla mitochondria	Rich in Lys and Arg	Oxalate	Promoter of crystal growth	Increased	[98]
	68 kDa	Cortex/medulla/nuclear membrane	DIDS-sensitive-Lys	Oxalate	Crystal growth inhibitor	Increased	[110]
	205 kDa	Nuclear pore complex	DIDS-sensitive-Lys	Oxalate	Crystal growth inhibitor	Increased	[130]
	27.5 kDa (histone-1)	Nuclear chromatin	Basic protein rich in Lys, Arg	Oxalate	Promoter of crystal growth	Increased	[104, 103]
	45 kDa (CaOx binding protein)	Subcellular organelles; enriched in nucleus	DIDS-sensitive-Lys	Oxalate	Promoter of crystal growth	Increased	[3]
5	COM adsorbed proteins 23 kDa (human and rat kidney)	Nucleus	Basic protein rich in Lys and Arg	Oxalate	Crystal growth inhibitor	Increased	[100]
	45 kDa (human kidney)	Mitochondria, nucleus	Rich in basic amino acids	Oxalate	Promoter of crystal growth	Increased	
	73 kDa (rat kidney)	Mitochondria, nucleus	Rich in basic amino acids	Oxalate	Promoter of crystal growth	Increased	[51, 107]
6	48 kDa -stone matrix protein	Mitochondria, nucleus	DIDS-sensitive-Lys	Oxalate	Promoter of crystal growth	Increased	[34]
7	96 kDa, band 3 protein	RBC membrane	Basic amino acids in active site/Ser-Thr	Oxalate	Increases oxalate flux	Increased phosphorylation	[12, 18, 71, 85]

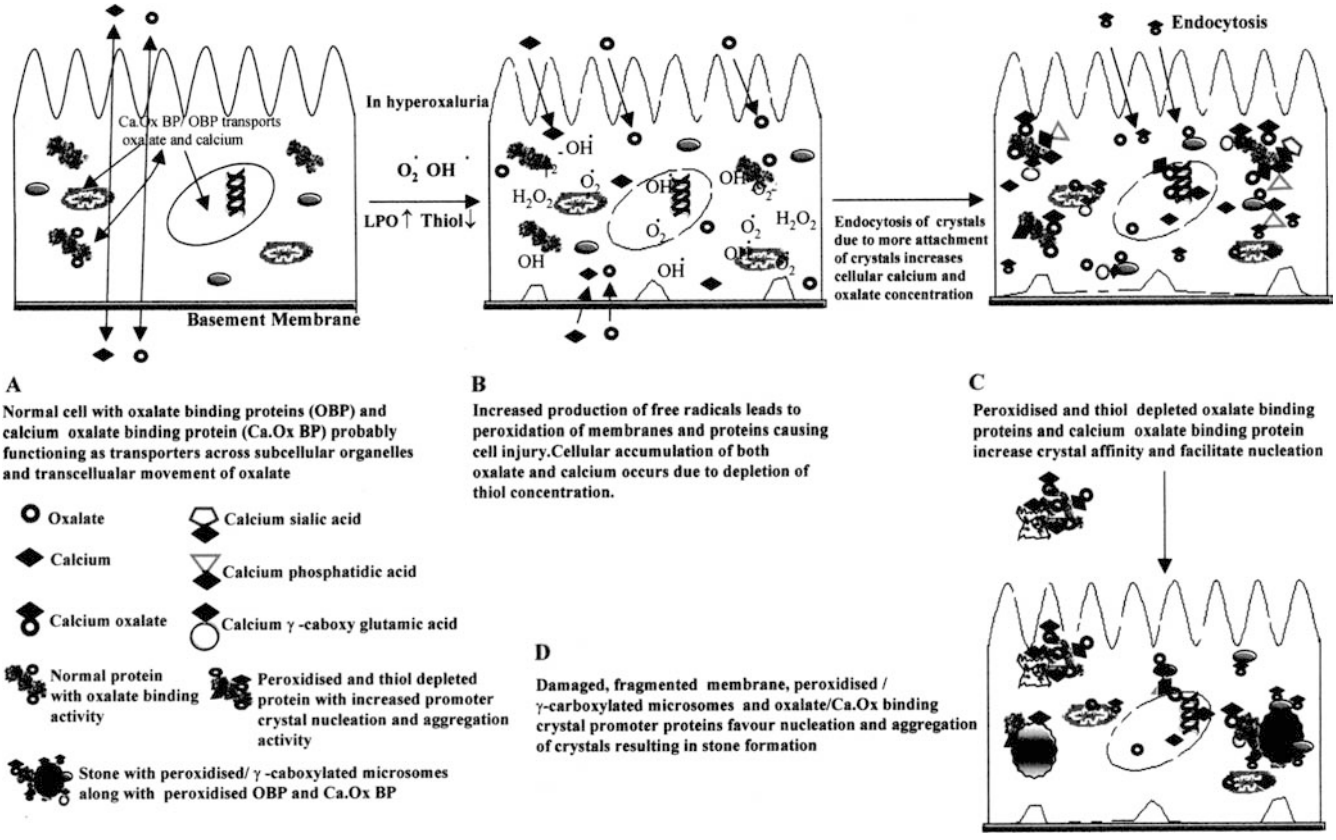

Fig. 1 Involvement of peroxidised oxalate/calcium oxalate binding proteins in the pathogenesis of calcium oxalate stone disease

membrane and peroxidised membrane/protein could act as a nidus by facilitating increased binding with oxalate on the crystal, culminating in more and more deposition of crystals. This reaction might further lead to crystal incorporation into stones or/and pass along with the flow of urine.

Editorial comment (C.F. Verkoelen)

Since the mid-1980s investigators at the Department of Medical Biochemistry of the University of Madras have regularly reported on renal oxalate binding proteins in subcellular organelles such as mitochondria and nuclei. Although the proteins were purified and partially characterized, they were not identified. Oxalate has been reported to recycle across the apical membrane of renal tubular cells via anion-exchange proteins to promote the transcellular transport of chloride, But it is doubtful whether dicarboxylic acids, like oxalate, are capable of accumulating inside renal tubular cells. Renal cell oxalate handling has been engaging nephrolithiasis research for many years. The possibility cannot be excluded that oxalate binding proteins are somehow involved in the etiology of this disease. Progress can only be expected, however, after the identity and function of these proteins has been revealed.

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