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Increased calcium oxalate crystal nucleation and aggregation by peroxidized protein of human kidney stone matrix and renal cells

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Abstract Kidney stone matrix protein fractions eluted from DEAE cellulose column showed increased oxalate binding activity and had negative correlation with reduced thiol content. Fraction 1 (eluted in Tris-HCl, pH 7.4) and fraction 3 (0.3 M NaCl in buffer) showed nucleation and aggregation-promoting properties while fraction 2 (0.05 M NaCl in buffer) showed an inhibitory effect in an in vitro crystallization system. 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 calcium oxalate crystals. Protein peroxidation was negatively correlated with the inhibitory activity of the protein on calcium oxalate nucleation and aggregation. A similar promoting effect of nucleation and aggregation was seen with mitochondria and nucleus after peroxidation. These studies suggested that peroxidation of protein or tissue had greater influence on the nucleation and aggregation property of calcium oxalate crystal growth.

Key words Urolithiasis · Kidney stone matrix · Crystal nucleation · Peroxidation · CaOx crystal lization · Peroxidation of renal cells

Introduction

Kidney stones do not form if crystals of calcium oxalate do not nucleate within the kidney [7]. Nucleation of the stone-forming minerals takes place within the urinary tract, possibly under the influence of a wide range of supersaturation. These nuclei or nidi are subsequently able to grow, aggregate and dissolve [14].

The urinary macromolecules are suggested to play a major role in the crystallization events such as nucleation, growth and aggregation. Protein peroxidation is shown to modify proteins and enhance oxalate binding activity [17], and the peroxidation process increases the oxalate binding activity of renal nuclear [6] and mitochondrial subfractions [17].

We have shown that renal tissue damaged due to peroxidative stress is more prone to calcium oxalate crystal retention. In conditions which promote lipid peroxidation in renal cells by feeding pyridoxine deficient diet [13], ethylene glycol [6], glycolate or buthionine sulfoximine [11], crystal deposition is severe. Arresting lipid peroxidation reaction, by way of supplementation of antioxidants such as vitamin E [21] or glutathione monoester [11] to urolithic rats, abolished the retention of calcium oxalate stone formation.

Very little study has been done on the effect of peroxidation of proteins on crystal nucleation and aggregation. This work presents the enhanced crystal nucleation and aggregation following peroxidation of stone matrix proteins as well as tissue proteins.

Materials and methods

Human cadaver kidneys were obtained from the Forensic Sciences Department (Madras Medical College, Chennai, India) within 12 h of expiration. Kidney stones were obtained from the Urology Department (Madras Medical College Hospital, Chennai, India).

Chemicals and their sources

DEAE cellulose (1.0 meq/g) and sephadex G 200 were purchased from Sigma Chemical (St. Louis, Mo., USA). [^{14}C]Oxalate with specific activity of 4 mCi/mmole was obtained from Bhabha Atomic Research Centre (Trombay, Mumbai, India).

Malondialdehyde, Tris [(hydroxymethyl) aminomethane] and all other fine chemicals used were of analytical grade and highest quality available.

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Methods

Human cadaver kidneys were placed in ice-cold saline, trimmed free of connective tissues, blotted with filter paper and weighed. The kidneys were minced and a 10% homogenate was prepared in Tris-HCl buffer (0.01 M containing 0.25 M sucrose, pH 7.4) using a power driven Teflon homogenizer. Subcellular fractionation was carried out by the method of Sottocossa et al. [20] by differential centrifugation. Nuclei were pelleted at 800*g* for 15 min, and mitochondria at 8000*g* for 20 min. The post mitochondrial supernatant was treated with 80 mM $CaCl_2$ in the ratio of 1:10 and subjected to 17,500*g* for 30 min to pellet the microsomes, and the cytosol remained as the supernatant. Nuclei or mitochondria were resuspended in Tris-HCl buffer (0.01 M containing 0.25 M sucrose) and washed thrice to obtain a purified fraction. The microsomal pellet was washed, resuspended in a buffer containing 0.25 M sucrose, 0.25 M imidazole, 0.5 M potassium chloride and 2 mM dithiothreitol, pH 7.2 (SIK buffer), and repelleted. All the procedures were carried out at 2 °C in a high speed refrigerated centrifuge (Kubota).

Extraction of stone matrix

Stones with high calcium oxalate content were used for matrix isolation. Matrix was isolated using the procedure of Khan and Hackett [8]. Ten millilitres of 0.25 M EDTA (pH 7.4) was added per gram of powder and the suspension was stirred for 4 days at 4 °C. The suspension was centrifuged for 30 min at 6000*g* at 4 °C and the supernatant filtered; the residue extraction was repeated on the pellet five times. The supernatants were pooled and extensively dialysed against deionized water at 4 °C. The retentate was lyophilized.

About 2 mg of the isolated stone matrix protein was loaded onto a DEAE cellulose column (15 × 1.5 cm) which was previously equilibrated with Tris-HCl buffer (0.05 M, pH 7.4). The elution was carried out first with 0.05 M Tris-HCl buffer (pH 7.4) followed by 0.05 M NaCl in the above buffer and finally in 0.3 M NaCl in the same buffer. Twenty "2-ml" fractions were collected in each step of elution and the elution of protein was monitored in a UVIKON 930 spectrophotometer at 220 nm.

Three major peaks were obtained. The protein contents of the fractions were estimated by the method of Lowry et al. [10].

[^{14}C]Oxalate binding assay

Oxalate binding studies were carried out in stone matrix protein fractions as well as in the subcellular fractions of human and rat kidney using the method of Seethalakshmi et al. [16] and expressed as picomoles of oxalate bound per mg protein.

Peroxidation system

Stone matrix fractions (50 μg) or human kidney subcellular organelles were incubated with ascorbate system (50 mM $FeCl_3$, 1 mM ADP, 1 mM KH_2PO_4, 0.4 mM ascorbate) for 30 min. After the incubation period, aliquots were taken and the following estimations were carried out.

Estimation of total, protein and non-protein thiols

Protein and non-protein thiols were estimated using the method of Sedlack and Lindsay [15]. The amount was expressed as microgram TSH per milligram protein.

Estimation of lipid peroxidation

Lipid peroxidation was assayed using the method of Devasagayam and Tarachand [3]. The thiobarbituric acid reactive substances (TBARS) of the sample were expressed as nanomoles MDA formed per milligram protein.

Spectrophotometric crystallization assay

The spectrophotometric crystallization assay was carried out according to the method of Hess et al. [5].

To 1 ml of potassium oxalate solution taken in the quartz cuvette, 1 ml of $CaCl_2$ solution was added, so that the final concentration of the incubation mixture was 4.25 mM for calcium and 0.75 mM for oxalate. All the solutions were prepared in deionized water containing 200 mM NaCl and 10 mM sodium acetate (pH 5.7). Automated time course measurements of OD at 620 nm were performed with a UVIKON 930 spectrophotometer (Kontron Instruments, Italy). The experiments were carried out in the presence of 10 μg of purified stone matrix protein fractions prior to and after modification with ascorbate system.

OD_{620} increases initially during nucleation phase and decreases during the aggregation phase. Slopes of the nucleation (till the maximum) and aggregation (after the peak) phases were calculated using linear regression analysis and the percentage inhibition of the proteins was calculated using the formula:

$$\text{Percentage inhibition} = [1 - Sm/Sc] \times 100 \quad (1)$$

where *Sm* is the slope in the presence of the modifier and *Sc* the slope of the control.

Statistical analysis was carried out using Student's *t*-test.

Results

The effect of peroxidation on oxalate binding activity of stone matrix fractions 1–3 is shown in Table 1. Stone matrix protein fractions 1–3 showed increased oxalate binding by 23%, 11% and 14%, respectively, after peroxidation. During peroxidation of protein there was no loss of its content.

In order to determine which of the subcellular fractions resemble the behaviour of the stone matrix fractions, lipid peroxidation studies were also undertaken in the subcellular fractions of both human and rat kidney. Among the subcellular organelles, mitochondria and nuclei of both human and rat renal cells showed increased oxalate binding activity by 30–40% on peroxidation when compared to that of unperoxidized organelles (Table 2). Figure 1a, b shows the effect of peroxidized protein on nucleation and aggregation, respectively. While stone matrix protein fractions 1 and 3 promoted calcium oxalate crystal nucleation, fraction 2 showed an inhibitory effect on nucleation. The promoting effect of fraction 1 was significantly enhanced following peroxidation. In contrast, on peroxidation, the inhibitory effect of fraction 2 was reduced. Both

Table 1 Oxalate binding activity of CaOx stone matrix fractions before and after peroxidation. Values are expressed as means ± SD for six experiments and as pmoles oxalate bound per mg protein

Stone matrix fractions	Before peroxidation	After peroxidation
Fraction 1	110 ± 10	135 ± 9.8*
Fraction 2	88.0 ± 12	98.0 ± 11.6*
Fraction 3	35.4 ± 1.95	40.2 ± 5.2*

* $P < 0.05$ when compared to unperoxidized condition

Table 2 Oxalate binding activity in human and rat kidney subcellular fractions before peroxidation and after lipid peroxidation. Values are expressed as means ± SD for six experiments and as pmoles oxalate bound per mg protein

	Human		Rat	
	Before peroxidation	After peroxidation	Before peroxidation	After peroxidation
Homogenate	2.6 ± 0.45	3.7 ± 0.42*	2.90 ± 0.1	4.2 ± 0.49*
Nucleus	7.8 ± 0.98	9.8 ± 0.65*	5.7 ± 0.8	7.3 ± 0.70*
Mitochondria	9.7 ± 1.27	13.5 ± 1.02*	8.9 ± 0.93	12.7 ± 0.86*
Microsomes	0.96 ± 0.02	0.925 ± 0.09**	1.2 ± 0.09	1.4 ± 1.9**
Cytosol	0.13 ± 0.08	0.19 ± 0.103**	0.18 ± 0.02	0.22 ± 0.03**

* $P < 0.05$ when compared to unperoxidized condition
** Not significant when compared to unperoxidized condition

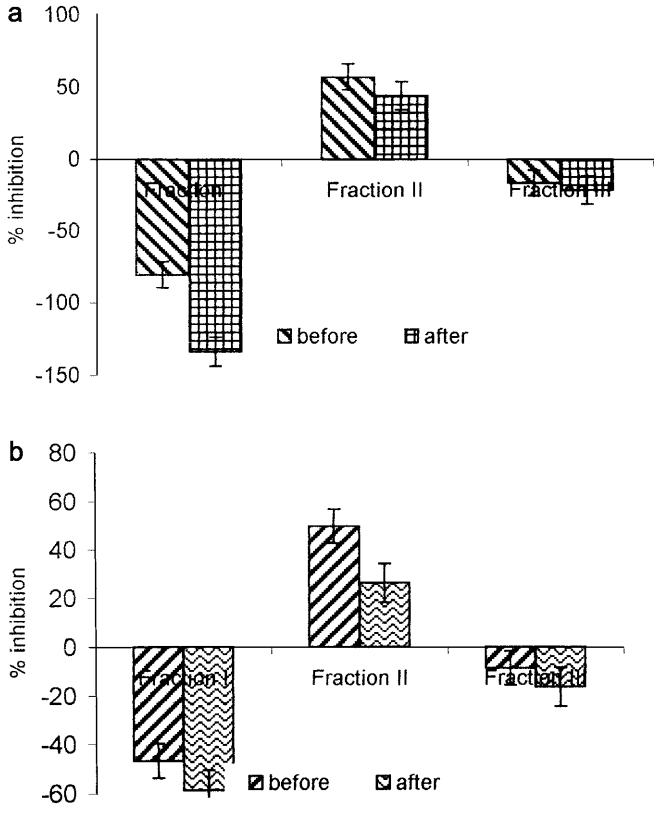

Fig. 1 **a** Effect of peroxidized calcium oxalate stone matrix fractions on nucleation. **b** Effect of peroxidized calcium oxalate stone matrix fractions on aggregation

fractions 1 and 3 promoted crystal aggregation, while fraction 2 showed an inhibitory effect on aggregation. The aggregation-promoting effect was enhanced for fractions 1 and 3 following peroxidation. In contrast, the inhibitory effect of fraction 2 was reduced following peroxidation.

Figure 2 shows the correlation between TSH, nucleation and aggregation of stone matrix fractions. Nucleation and aggregation were negatively correlated with the total thiol content of the protein. Peroxidation of stone matrix proteins led to significant loss of thiol content and showed a negative correlation ($v = -0.864$).

The effect of peroxidation of human kidney subcellular organelles on nucleation and aggregation is shown in Table 3. Homogenate, microsomes and cytosol inhibited nucleation by 127%, 98% and 132%, respectively, whereas mitochondria and nuclei promoted crystal nucleation by 24% and 32%, respectively. After peroxidation, the promoting activities of nucleation and aggregation by mitochondria and nuclei were further enhanced while the inhibitory activities of other subcellular organelles were decreased.

Lipid peroxidation of mitochondria and nuclei depleted TSH content and a negative correlation was observed ($v = -0.54$) for mitochondria. A positive correlation between LPO and nucleation or aggregation and a negative correlation between TSH and nucleation or aggregation were observed (Fig. 3).

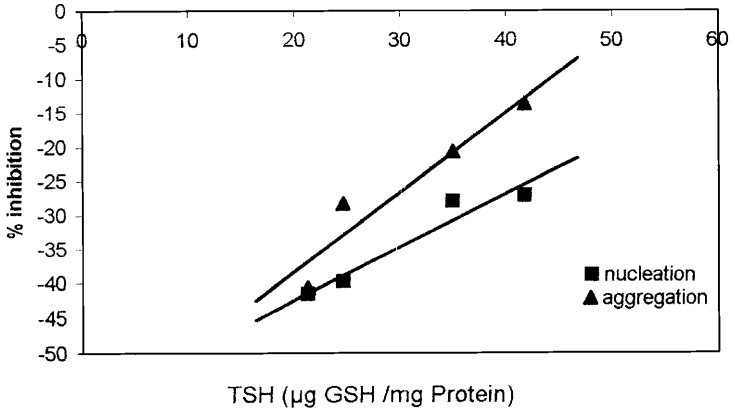

Fig. 2 Correlation between TSH and % inhibition of nucleation or aggregation of stone matrix fractions

Table 3 Effect of peroxidized human kidney subcellular organelles on calcium oxalate crystal nucleation and aggregation. Values for LPO, TSH and nucleation and aggregation are expressed as means ± SD for six determinations. LPO values are expressed as nmoles MDA released per mg protein. TSH values are expressed as microgram TSH per milligram protein. Nucleation and aggregation are expressed as % inhibition

Contents	LPO		TSH		Nucleation		Aggregation	
	Before peroxidation	After peroxidation	Before peroxidation	After peroxidation	Before peroxidation	After peroxidation	Before peroxidation	After peroxidation
Homogenate	9.936 ± 1.1	18.5 ± 1.8	52.11 ± 4.7	21.98 ± 2.4	126.92 ± 11.5	98.35 ± 10.5	28.72 ± 3.5	18.26 ± 2.2
Nucleus	26.17 ± 2.9	49.7 ± 4.2	35.07 ± 2.8	21.3 ± 1.8	−24.19 ± 2.5	−41.62 ± 5.8*	−29.75 ± 3.6	−40.75 ± 5.8*
Mitochondria	11.56 ± 2.1	38.25 ± 4.2	41.78 ± 5.1	24.6 ± 2.1	−32.5 ± 2.6	−39.84 ± 3.1*	−13.76 ± 1.2	−28.36 ± 2.5*
Microsomes	26.71 ± 2.7	51.62 ± 6.5	34.09 ± 3.8	16.86 ± 1.8	97.52 ± 10.5	86.12 ± 9.0	84.89 ± 9.5	No inhibition*
Cytosol	3.62 ± 0.6	6.78 ± 0.9	29.62 ± 3.2	22.73 ± 2.5	132.32 ± 12.5	95.5 ± 8.5	131.38 ± 12	No inhibition*

* $P < 0.05$ when compared to unperoxidized condition

Discussion

The process of crystallization comprises several steps that are not independent and occur simultaneously such as nucleation, aggregation and phase transformation of crystals [5]. Many biochemical changes are observed under stone-forming conditions, notably accumulation of oxalate in the kidney membrane fractions and enhanced lipid peroxidation. The enhanced LPO-mediated cell injury has been suggested as a possible predisposing factor for the retention of oxalate [12].

Stone matrix protein fractions show a negative correlation between lipid peroxidation and −SH content. Further peroxidized proteins show enhanced oxalate binding activities. Thus, the increased oxalate binding capacity under peroxidation must have been caused by depletion of thiol content of the protein. Depletion of protein thiol, anti-oxidants, GSH and ascorbic acid has been shown to facilitate retention of crystals [12] due to membrane damage. Khan et al. [9] have shown that damaged urothelium readily binds calcium oxalate crystals. Crystals appear to be retained either by attachment to the tubular epithelium or by aggregating with other crystals, thus becoming large enough to be retained by their collective size.

The nucleation of microcrystals occurs as the initial event of stone salt precipitation. Nucleation of CaOx crystals in presence of stone matrix fraction shows a negative correlation between percent inhibition of nucleation and LPO. Stone matrix fraction strongly promotes nucleation and aggregation of CaOx crystals following peroxidation. Further, the inhibitory fraction 2 loses its inhibitory potential following peroxidation, suggesting that under conditions in which the proteins are peroxidized, it could favour crystal growth promotion. As cellular fragments can serve as heterogeneous foci for the nucleation of calcium oxalate crystals [2], membrane damage produced by free radical mediated lipid peroxidation reaction or by administration of chemicals such as gentamycin [19] or ethylene glycol [4] can induce enhanced retention of calcium oxalate crystals. This is further supported by the present study where nucleation and aggregation are promoted following lipid peroxidation of proteins or subcellular organelles.

Peroxidized human or rat kidney subcellular organelles show increased oxalate binding activity, due to the loss of thiol content. A similar observation of enhanced oxalate binding activity has been reported in mitochondria of urolithic rat kidney with reduced TSH as well as protein −SH [18]. The oxalate binding activity exhibited by peroxidized nuclei may be associated with structural alterations of the membrane by oxidation of thiol groups during peroxidation [6]. Peroxidized mitochondria have also been shown to bind more oxalate than the unperoxidized mitochondria [17].

Mitochondria are found to be enriched with fraction 1 protein (unpublished observation) and so the increased oxalate binding activity may be due to more content of

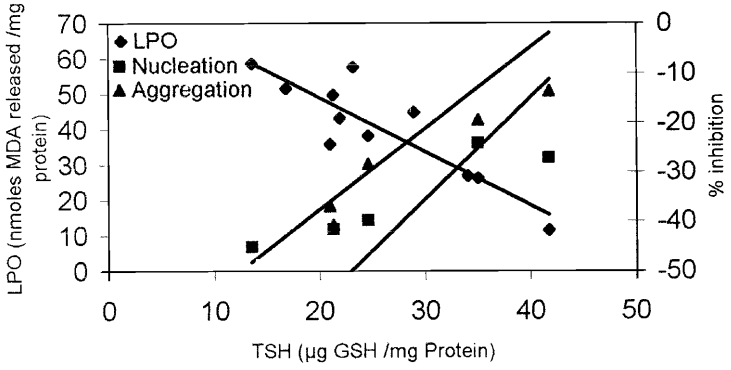

Fig. 3 Correlation between LPO, TSH and % inhibition of nucleation or aggregation of kidney mitochondria

fraction 1 protein. Under physiological conditions the crystal binding site of the protein may be minimally exposed, but could be unmasked when cells are injured by way of lipid peroxidation. This might favour the protein to bind the crystal better than the unperoxidized condition.

Conclusion

It is concluded that conditions which enhance peroxidation and depletion of thiol content increase the oxalate binding activity, which in turn promotes nucleation and aggregation property of stone matrix protein fractions. This behaviour is also associated with peroxidized mitochondria and nuclei, suggesting that peroxidation can be a causative factor for the initial stage of stone formation.

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References

1. Adhirai M, Selvam R (1997) Protection of cyclosporin and induced biochemical changes by vitamin E pretreatment in hyperoxaluric rat kidney. Nutr Biochem 8: 32
2. Balaji KC, Mani M (1997) Mechanism of stone formation. Urol Clin North Am 24: 1
3. Devasagayam TPA, Tarachand U (1987) Decreased LPO in rat kidney during gestation. Biochem Biophys Res Commun 145: 134
4. Hackett RL, Shevock PN, Khan SR (1990) Cell injury associated calcium oxalate crystalluria. J Urol 144: 1535
5. Hess B, Meinhardt U, Zipperle L, Giovanoli R, Jaegar P (1995) Simultaneous measurements of calcium oxalate crystals nucleation and aggregation: impact of various modifiers. Urol Res 23: 231
6. Kannabiran K, Selvam R (1997) Induction of renal nuclear oxalate binding activity in experimental hyperoxaluric rats. Nephron 75: 125
7. Khan SR (1997) Animal models of kidney stone formation: an analysis. World J Urol 15: 236
8. Khan SR, Hackett RL (1993) Role of organic matrix in urinary stone formation: an ultrastructural study of crystal matrix surface of calcium oxalate monohydrate stone. J Urol 150: 339
9. Khan SR, Cockrell CA, Finlayson B, Hackett RL (1984) Crystal retention with renal tubular epithelium, mechanism of crystal adhesion and its impact on stone development. Urol Res 132: 153
10. Lowry OH, Rosebrough NJ, Farr AL, Randall RT (1957) Protein measurement with Folins phenol reagent. J Biol Chem 193: 265
11. Muthukumar A, Selvam R (1998) Effect of depletion of reduced glutathione and its supplementation by glutathione monoester on renal oxalate retention in hyperoxaluria. Mol Cell Biochem 185: 77
12. Ravichandran V, Selvam R (1990) Increased lipid peroxidation in kidney of vitamin B_6 deficient rats. Biochem Int 21: 599
13. Ravichandran V, Selvam R (1991) Lipid peroxidation in subcellular fractions of liver and kidney of vitamin B6 deficiency. Med Sci Res 18: 369
14. Ryall RL (1993) The scientific basis of calcium oxalate urolithiasis. World J Urol 11: 59
15. Sedlack J, Lindsay RH (1968) Estimation of total protein-bound and non-protein-bound sulphydryl groups in tissues with Ellman's reagent. Anal Biochem 25: 192
16. Seethalakshmi L, Selvam R, Mahle CJ, Menon M (1986) Binding of oxalate to mitochondrial inner membranes of rat and human kidney. J Urol 135: 862
17. Selvam R, Sridevi D (1991) Induction of oxalate binding by lipid peroxidation. Biochem Int 24: 857
18. Selvam R, Sridevi D (1996) Oxalate binding to rat kidney mitochondria: induction by oxidized glutathione. Ind J Biochem Biophys 33: 62
19. Sigmon D, Kumar S, Carpenter S, Miller T, Menon H, Scheid C (1991) Oxalate transport in renal tubular cells from normal and stone forming animals. Am J Kidney Dis 17: 376
20. Sottocossa GL, Kuylenstierna B, Ernster L, Bergstrand (1967) Separation and some enzymatic properties of the inner and outer membranes of rat liver mitochondria. In: Colowik SP, Kaplan NO (eds) Methods in enzymology, vol 10. Academic Press, New York, p 49
21. Thamilselvan S, Selvam R (1997) Effect of vitamin E and mannitol on renal calcium oxalate retention in experimental urolithiasis. Ind J Biochem Biophys 34: 319