Journal of Cellular Biochemistry 93:1099–1106 (2004)

Nuclear Pore Complex Oxalate Binding Protein p62: Its Expression on Oxalate Exposure to VERO Cells

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Abstract Oxalate rich stones are the most common among the various stones. Oxalate binding protein plays a vital role in the transport of oxalate. Nuclear pore complex (NPC) contains a protein of molecular weight 62 kDa and it has maximum oxalate binding activity. The physiological significance of the presence of oxalate binding protein in the NPC is not well understood. In order to study its function, the expression of this protein during oxalate stress condition and the morphological changes on oxalate exposure to synchronized VERO cells have been determined. VERO cells were synchronized at different stages of cell cycle using cell cycle blockers and expression of the NPC p62 was assessed using enzyme linked immunosorbent assay (ELISA) technique with p62 antibody (MAb 414). Expression of NPC p62 was more pronounced in 1.0 mM oxalate concentration in mitotic phase than in S phase, suggesting cell cycle dependency. During oxalate exposure there is cell aggregation and complete degeneration of cell morphology occurs, which in turn lead to the expression of certain genes, including the NPC oxalate binding protein p62. Thus, oxalate induces degeneration of cells (may be due to the lipid peroxidation) and leads to the expression of NPC oxalate binding protein and the expression is of cell cycle dependent manner. J. Cell. Biochem. 93: 1099–1106, 2004.

Key words: VERO cells; p62; synchronized VERO cells; oxalate binding protein

Kidney stone disease is multifarious in origin and the precise mechanism by which the kidney stones originate is still not well understood. Oxalate, a metabolic end product forms calcium oxalate deposits in the kidney aided by the macromolecular components of the cell [Khan et al., 1990]. Two thirds of the total oxalate binding of the homogenate is localized in the nucleus while rest one third resides in the mitochondria [Seethalakshmi et al., 1986]. The presence of nuclear membrane oxalate binding with a molecular weight of 68 kDa from rat liver [Selvam et al., 1996] have been reported. Selvam et al. [2003] reports that nuclear pore complex (NPC) contains an oxalate binding protein with the molecular weight of 205 kDa. Oxalate has been shown to induce mitosis [Li et al., 1993], DNA synthesis [Koul et al., 1994], and expression of certain genes [Koul et al., 1996]. The physiological significance of the presence of oxalate binding protein in the NPC is not well understood. Our earlier report showed that there was an increased expression of the NPC oxalate binding protein 205 kDa on VERO cells which has the inhibitory effect in crystal promoting activity [Vijaya et al., 1999]. The present study aims to find out the physiological significance of the p62 and its expression under oxalate stress condition. In order to study its function, the expression of this protein during different stages of cell cycle was studied under oxalate stress condition.

MATERIALS AND METHODS

Minimum essential medium, penicillin, streptomycin, sodium bicarbonate, fetal calf serum, methotrexate, colchicine, cytochalasin, trypsin–ETDA solution, and molecular weight markers were obtained from Sigma Chemical Company (St. Louis, MO). Triton X-100 was purchased

Grant sponsor: Council of Scientific and Industrial Research (CSIR); Grant number: 9/115 (577) 2002 EMR-I.

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Received 4 April 2004; Accepted 8 July 2004
DOI 10.1002/jcb.20269

from Sisco Research Laboratories (Bombay, India).

Monoclonal antibody against NPC p62 (MAb 414) was obtained from Covance Research Products, Inc. (Berkley, CA). Goat anti-rabbit-IgG horseradish peroxidase was a gift from NII, New Delhi. All the other chemicals and solvents used were of the highest grade and purity.

VERO monkey kidney cells were serially passaged in minimal essential medium (MEM) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were maintained in an atmosphere of 5% CO_2/95% air in a humidified 37°C incubator.

Oxalate concentration was fixed according to Scheid et al. [1996a] to be 0.1 mM (low), 0.3 mM (medium), and 1 mM (high/toxic) total oxalate (which is equivalent to 30, 100, and 350 μM free oxalate concentrations, respectively). Cells (1×10^5) were incubated with MEM containing specified concentrations of oxalate.

Cells (1×10^5) were incubated with MEM containing corresponding cell cycle blockers in 25 ml culture flasks. Cells were synchronized at "S" phase using methotrexate; at metaphase using colchicine and telophase using cytochalasin B at the concentration of 10 μg/ml each and the cells were allowed to synchronize for 24 h. The morphological changes during each stage of the cell cycle were viewed under Phase Contrast NIKON DIAPHOT-P20 at 200× magnification.

Cells were trypsinized by incubating with trypsin–EDTA solution for 20 min and washed two times by centrifuging at 2,500 rpm/10 min with MEM and resuspended with the same. From the resuspended pellet, an aliquot of the cell was mixed with 0.05% trypan blue. Cells were then examined immediately under a light microscope and the relative abundance of the dead (stained) cells was assessed.

Whole cell extract was prepared according to the method of Brizuela et al. [1987]. The cells suspended in MEM was centrifuged at 2,500 rpm/5 min and resuspended in the lysis buffer (100 mM Tris-HCl, pH 7.4, containing 1% Triton X-100, 0.4% SDS, and 2 mM EDTA) and incubated for 30 min at 4°C. The Triton X-100 extract containing the membrane protein, p62 was obtained by centrifuging the whole cell extract at 15,000 rpm/30 min and the residual pellet was resuspended in Tris-HCl buffer (100 mM; pH 7.4). Protein concentration was estimated by the method of Lowry et al. [1951].

Enzyme linked immunosorbent assay (ELISA) was done according to the method of Sharma and Singh [1989]. Ten microliters of Triton extract and residual pellet were coated onto 96-well micro titre plates, probed with 1:500 diluted monoclonal antibody (MAb 414) against NPC p62 and then incubated with 1:20,000 diluted secondary antibody (goat anti-human IgG horseradish peroxidase). Diamino benzidine tetra hydrochloride DAB/H_2O_2 in 10 ml of 0.05 M Tris-HCl buffer (pH 7.6), add 10 μl of 30% H_2O_2 to 10 ml of substrate prior to use. One hundred microliters of substrate was added and incubated in dark for 30 min. The color developed on adding 1 N H_2SO_4 was read at 490 nm in MR600 DYNATECH ELISA reader.

All biochemical values were expressed as mean ± SD for three experiments with two replicates in each experiment. The "*P*" significance was arrived at using Student's *t*-test ($P < 0.05$**, $P < 0.001$***).

RESULTS

Exposure of VERO cells to oxalate in the culture medium produced changes in cell density. Twenty four hours exposure to varying concentrations of oxalate (0.1, 0.3, and 1.0 mM) produced biphasic effects on cell numbers. Cells exposed to 0.1 mM oxalate showed 30% increase in cell density, while cells exposed to 0.3 mM oxalate showed 20% increase in cell density and that exposed to 1.0 mM oxalate showed a slight decline of 10% in cell density when compared to that of the control cells (unexposed to oxalate) (Fig. 1).

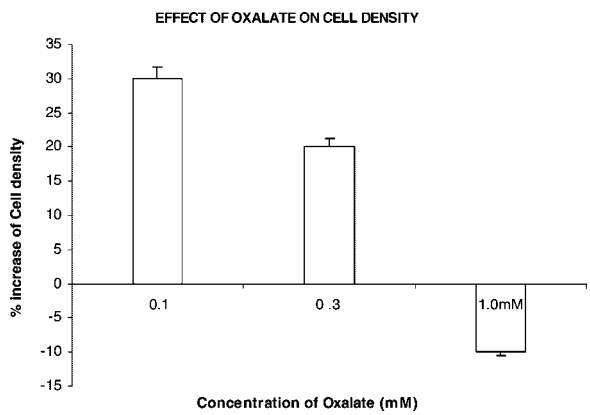

Fig. 1. Values are average of three experiments of two duplicates. Cells (1×10^5) were incubated with growth medium containing varying concentrations of oxalate (0.1, 0.3, and 1.0 mM). After 24 h, 10 μl of cells from each category were mixed with 0.05% Trypan blue and the cell density was assayed as described in "Materials and Methods."

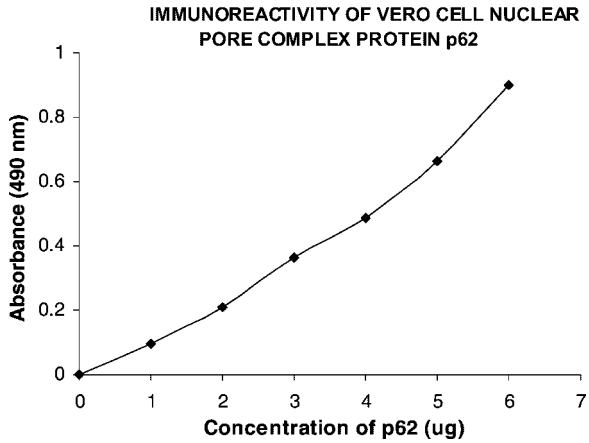

Fig. 2. Values are mean ± SD for six determinations. Different concentrations of whole cell extract was coated onto enzyme linked immunosorbent assay (ELISA) plates, the immunoreactivity was assessed using a constant antibody concentration as described in ''Materials and Methods.''

When different concentrations of whole cell extract from VERO cells were incubated with the monoclonal MAb 414 antibody (Covance Research Products), a concentration dependent increase in the antigen–antibody complex formation was observed (Fig. 2). The presence of NPC p62 was confirmed by Western blot (Fig. 3).

Effect of Oxalate on VERO Cells

The cell morphology on exposure of oxalate (0.1, 0.3, and 1.0 mM) to VERO cells in culture medium were assessed. Figure 4a depicts the control cells (unexposed cells) where the cells are normal, spindle shaped with elongated epithelioid morphology. Figure 4b–d shows the toxic cells due to 0.1, 0.3, and 1.0 mM oxalate concentration. In 0.1 mM oxalate toxicity, cells are normal and increased cell density was observed, in 0.3 mM oxalate toxicity, cells morphology gets degenerated and dark patches of cells were observed due to the aggregation of cells and the adherent property maintains whereas in 1.0 mM oxalate toxicity, cell morphology gets completely degenerated, aggregation of cells were seen along with refractive CaOx crystals.

Effect of Oxalate on the Expression of NPC p62

When the Triton extract of cells grown in different concentrations of oxalate were quantitated for p62 by ELISA, there was a significant increase in its expression. When cells were exposed to 0.1 mM ($P < 0.05$), 0.3 mM ($P < 0.001$), and 1.0 mM ($P < 0.001$) oxalate, p62 concentration were found to be 72.25, 124.33, and 265.4 μg of p62/1 × 10^5 cells, respectively, while in the control cells, it was about 58.16 μg of p62/1 × 10^5 cells (Table I).

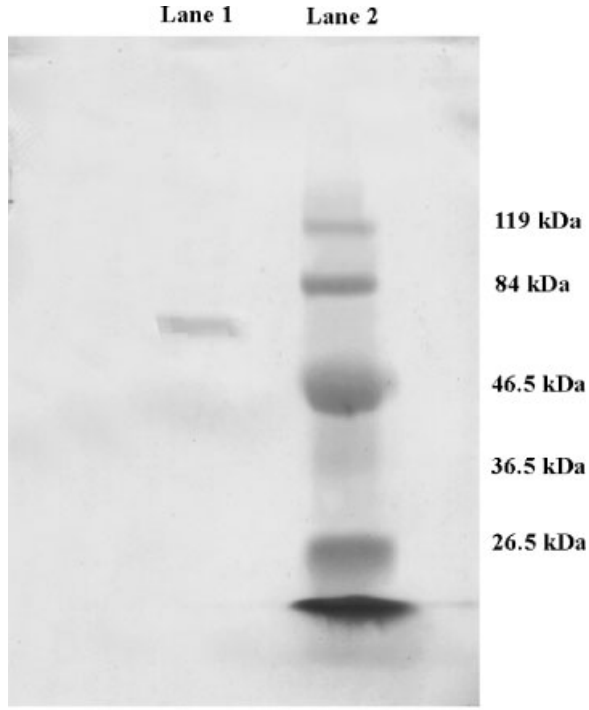

Fig. 3. Western blot of nuclear pore complex (NPC) oxalate binding protein from control VERO cells. The SDS-gel was transblotted onto PVDF membrane and processed as described in ''Materials and Methods.''

Oxalate Induced and Cell Cycle Dependent Expression of p62

When the cells were grown in different concentrations of oxalate (0.1, 0.3, and 1.0 mM) and synchronized at different phases, "S" phase using methotrexate, metaphase using colchicine and telophase using cytochalasin B, morphological changes in the cells were observed. Figure 5(i–iii) represents the cells at "S," meta and telophases. Figure 5(i. a) represents the cells at "S" phase of control cells, where cells show normal architecture with elongated epithelioid cells with nucleus whereas Figure 5(i. b) represents the synchronized "S" phase cells exposed to 1.0 mM oxalate toxicity where the morphological changes were observed, fusion of cells were seen as big multi nucleated cells but

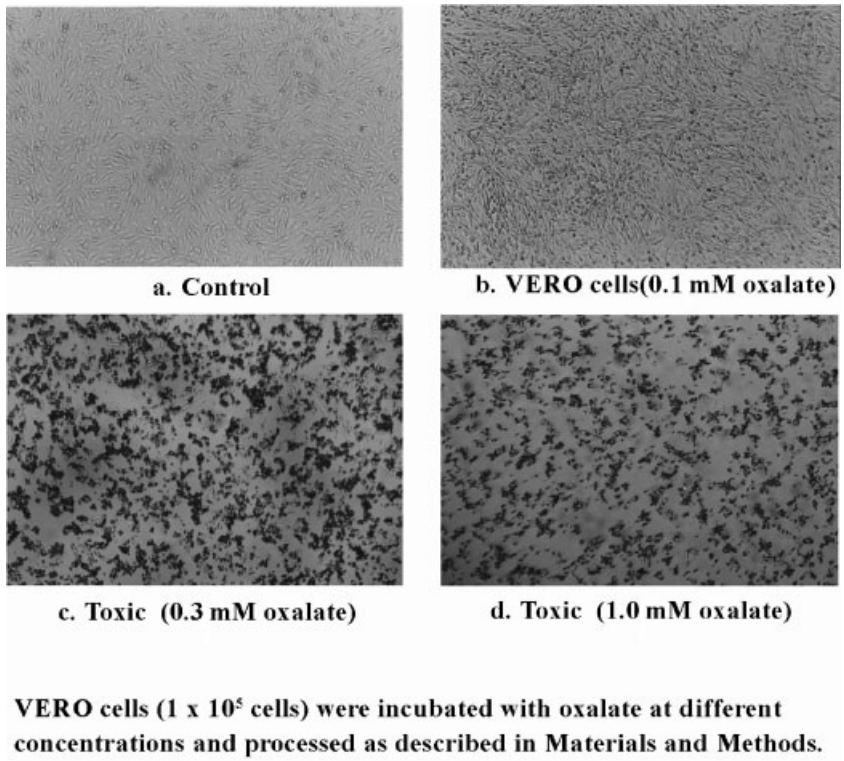

Fig. 4. Morphological changes on oxalate exposure to VERO cells. **a**: Control VERO cells, normal, spindle shaped, elongated epithelioid cell morphology were observed. **b**: VERO cells (0.1 mM oxalate), cell morphology maintained as control and increased cell density. **c**: Toxic VERO cells (0.3 mM oxalate), cell morphology gets degenerated, aggregation of cells observed as dark patches with maintenance of adherence property. **d**: Toxic VERO cells (1.0 mM oxalate), cell morphology gets completely degenerated, aggregation of cells seen along with refractive CaOx crystals. The cells were viewed under phase contrast NIKON DIAPHOT microscope (Magnification at 40×).

with marked membrane separation characterizing the toxicity due to oxalate.

Figure 5(ii. a) represents the synchronized cells at metaphase of control cells where the morphological changes were observed as giant round cells with elongated nuclei region, whereas the Figure 5(ii. b) represents the cells exposed to 1.0 mM oxalate at metaphase, cell morphology degenerated and aggregation of cells were observed as dark patches.

Figure 5(iii. a) represents the synchronized cells at telophase of unexposed cells, multinucleated cell morphology was observed. Cell morphology gets changed from elongated to round and aggregation of cells were seen. The Figure 5(iii. b) represents the cells exposed to 1.0 mM oxalate at telophase, where the cells with aggregated nuclear region seen as dark spots and fragments of nuclear region were seen.

The concentration of p62 in synchronized cells grown in the medium containing different concentrations of oxalate showed an increased expression when compared to that of the control cells (unexposed to oxalate).

In the control cells, p62 concentration was increased to a maximum at telophase of the mitotic phase than that of "S" phase. When the cells were exposed to 0.1 mM total oxalate, the concentration of p62 was significantly

TABLE I. Effect of Oxalate on Expression of Nuclear Pore Complex (NPC) p62

Particulars	Micrograms of p62 protein/ 1×10^5 cells
Control	58.16 ± 5.99
0.1 mM	72.25 ± 7.89*
0.3 mM	124.33 ± 10.15**
1.0 mM	265.4 ± 25.69**

Values are mean ± SD for three experiments with two replicates of each experiment. Thirty micrograms of Triton extract of each category was coated onto enzyme linked immunosorbent assay (ELISA) plates and probed with monoclonal p62 antibody (MAb 414) antibody and the concentration was assessed based on the data on Figure 2. Values are statistically significant when compared to control.
*$P < 0.01$.
**$P < 0.001$.

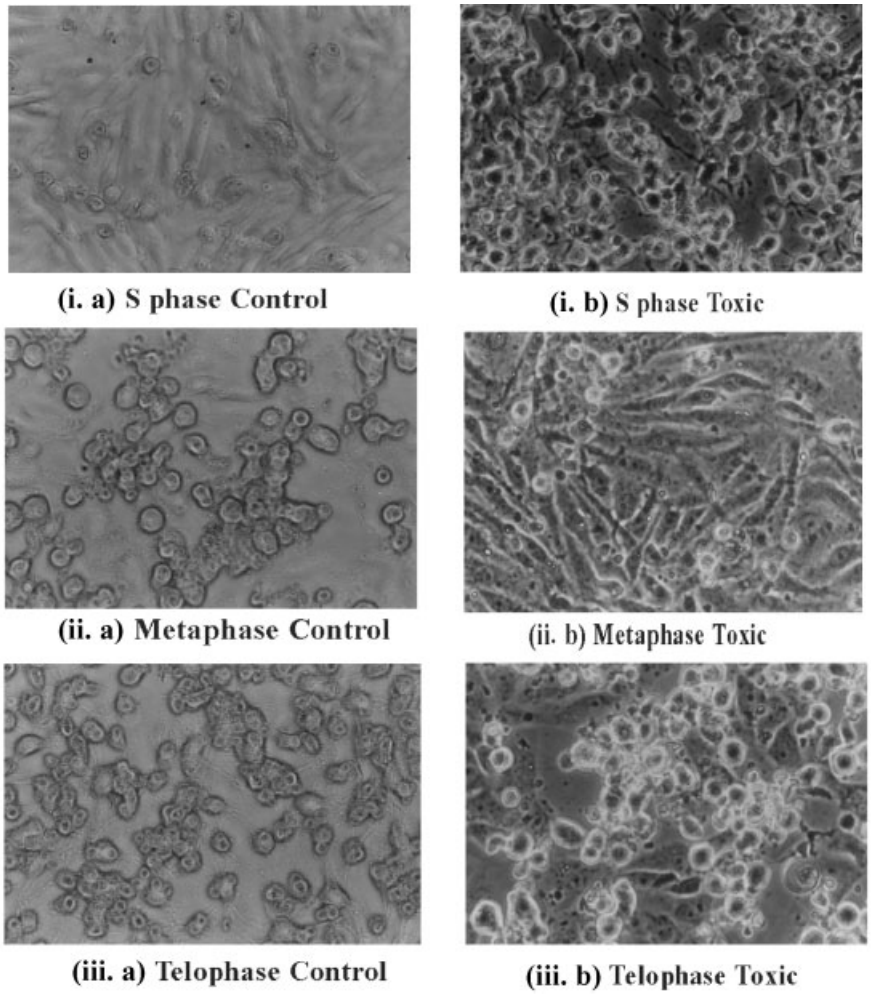

Fig. 5. Morphological changes on oxalate exposure to synchronized VERO cells. **i**. (**a**): S phase control, elongated epithelioid cells with nucleus were observed. (**b**): S phase toxic, morphological changes were observed, fusion of cells were seen as big multi nucleated cells but with marked membrane separation characterizing the toxicity due to oxalate. **ii**. (**a**): Metaphase control, giant round cells with elongated nuclei were observed. (**b**): Metaphase toxic, cell morphology gets degenerated and aggregation of cells were observed as dark patches. **iii**. (**a**): Telophase control, multinucleated cell morphology was observed. (**b**): Telophase toxic, toxic cells with aggregated nuclear region seen as dark spots and fragments of nuclear region were seen. The cells were viewed under phase contrast NIKON DIAPHOT-P20 at 200× magnification.

increased ($P < 0.01$), in "S," meta and telophases when compared to that of their respective controls. On exposure of the cells to 0.3 mM total oxalate, a significant ($P < 0.001$) increase in p62 concentration was observed in all the three phases when compared to that of the control. In the mitotic phase, the telophase showed a maximum concentration of p62 when compared to metaphase and "S" phase. When the cells were exposed to higher concentration of oxalate (1.0 mM total oxalate), further ($P < 0.001$) increase in p62 concentration in all the three phases was observed. Similar to the above, the maximum increase was observed in the telophase. Among the different concentrations of oxalate, the cells exposed to 1.0 mM oxalate showed a maximum concentration of p62 in the telophase (Table II).

The increase in p62 expression positively correlates with that of the oxalate-induced

TABLE II. Effect of Oxalate on Expression of NPC p62 Protein on Different Cell Cycles

	Micrograms of p62 protein/1 × 10^5 cells		
Particulars	S phase	M phase	T phase
Control	27.4 ± 2.61	29.5 ± 2.82	55.7 ± 5.61
0.1 mM	34.7 ± 3.23*	38.7 ± 4.65*	72.2 ± 7.54*
0.3 mM	68.8 ± 6.75**	88.6 ± 7.94**	122.4 ± 12.1**
1.0 mM	97.8 ± 8.74**	143.0 ± 14.12**	260.3 ± 24.1**

Values are mean ± SD for three experiments with two replicates for each experiment. Thirty micrograms Triton extracted protein of each category was coated onto ELISA plates and probed with the monoclonal p62 antibody (MAb 414) and the concentration was assessed based on the data on Figure 2. Values are statistically significant when compared to their respective controls.
*$P < 0.01$.
**$P < 0.001$.

apoptosis in VERO cells (data not shown). Table III represents the percentage of apoptotic cells after the exposure of oxalate at varying concentrations.

DISCUSSION

Urolithiasis, a multifactorial disease has been a subject of research since ancient times. Earlier studies have shown that oxalate induce mitosis, DNA synthesis, and expression of certain genes [Koul et al., 1996]. Even though oxalate induces mitosis, studies with LLC-PK_1 cells (a line of renal epithelial cells with characteristics of proximal tubular cells) reveal that, when exposed to high concentrations of oxalate, these cells show significant apoptotic changes, including condensation and margination of nuclear chromatin and DNA fragmentation [Gupta, 1996].

In order to study the expression of this protein during oxalate stress condition in vitro studies were carried out. Effect of varying concentration of oxalate on cell density has been studied. Similar to the above mentioned reports, lower concentrations of oxalate increase the cell density, while at higher concentration, a slight decline in cell density is observed.

TABLE III. Percentage of Apoptotic Cells After Exposure of Oxalate at Varying Concentrations

Particulars	Percentage of apoptotic cells
Control	8 ± 1
0.1 mM	10 ± 1
0.3 mM	17 ± 3
1.0 mM	51 ± 6

Values are expressed as mean ± SD for 100 cells per well and 6-wells per condition.

The adherent properties of the VERO cells are lost, when the cells are incubated with 0.3 mM oxalate. Madin–Darby canine kidney cells are injured by exposure to 0.25 mM oxalate and calcium oxalate crystals [Hackett et al., 1994]. Our report depicts the formation of CaOx crystals at 1 mM oxalate concentration. Earlier reports have demonstrated that monkey kidney epithelial cells (BSC-1) can internalize calcium oxalate crystals [Lieske et al., 1992]. Internalized crystals are found to be distributed to daughter cells during cell division. Available evidence suggests that oxalate levels are about 0.005–0.01 mM in the glomerular filtrate [Wolthers and Hayer, 1982] and would increase to 0.05–0.1 mM in the proximal tubules. Cortical oxalate levels might reach approximately 0.3 mM free oxalate concentration [Knight et al., 1981]. Thus, oxalate might well approach toxic levels in the proximal tubules, particularly in individuals with hyperoxaluria.

When VERO cells were incubated with 0.1 mM oxalate, the proliferation rate overwhelms the death rate and p62 expression at this oxalate concentration was only slightly increased, whereas, as the apoptotic events of the cell predominate at high concentration of oxalate, a tremendous increase in p62 expression is observed. These results suggest that a link between cell cycle events and oxalate toxicity might be mediated via p62. A similar involvement of p62 in apoptosis has already been established and it is expressed as one of the late genes [Carmo-Foncesca et al., 1991; Mc Morrow et al., 1994].

Worcester et al. [1992] have reported that the mouse kidney cortical cells when incubated with oxalate for 6–72 h, expressed a calcium oxalate crystal growth inhibitor protein, osteopontin in high concentrations. Though the expression of p62 oxalate binding protein is increased at each stage of cell cycle on exposing to different concentrations of oxalate, the maximal expression of p62 protein is found to be in telophase (threefold) than in interphase. This may be due to the nuclear membrane degeneration induced by oxalate.

During cell division, the nuclear envelope of higher eukaryotic cells undergoes dramatic structural changes [Mc Keon, 1991; Wiese and Wilson, 1993]. When the chromosomes condense during mitosis, the nuclear membranes

breakdown into vesicles, and the lamina and pore complexes disassemble into component polypeptides or smaller protein complexes. As cells enter anaphase, new nuclear envelopes begin to reassemble. Nuclear envelope reassembly is a stepwise process that proceeds through telophase and initiates with the targeting of membrane vesicles to decondensing chromosomes [Chaudhary and Courvalin, 1993]. New protein components are synthesized and targeted to the nuclear envelope during interphase so that the nucleus can enlarge during the G_1, G_2, and S phases of cell cycle and subsequent mitotic divisions can occur [Courvalin and Worman, 1997].

In the present study p62, an oxalate binding protein is expressed more in mitosis under oxalate stress condition. Oxalate has been shown to be mitogenic [Li et al., 1993] and induces the expression of *C-myc* gene [Koul et al., 1996]. Further, calcium oxalate crystals are shown to induce the expression of immediate early genes *C-myc*, *C-jun*, *EGR-1*, and *NUR-77* and genes encoding plasminogen activator inhibitor (PAI-1) [Hammes et al., 1994] and platelet derived growth factor (PDGF)-A in BAC-1 kidney epithelial cells in primary cultures of rat proximal tubular epithelium exposed to oxidative stress [Menon et al., 1993].

During experimental hyperoxaluria, the intracellular oxalate concentration is very high [Bhaskar and Selvam, 1985] and nuclei contain 2/3 of total oxalate [Selvam and Menon, 1983]. The presence of an oxalate binding protein in the pore complex is highly essential for the accumulation of oxalate in nuclei. The physiological significance of oxalate induced expression of p62, an oxalate binding protein may be considered as a pre-requisite step for entry of oxalate and hence the nuclear events that explain increased transcription of certain genes.

Oxalate is also reported to induce lipid peroxidation [Anuradha and Selvam, 1989; Khan et al., 1989; Selvam and Ravichandran, 1990; Selvam and Sridevi, 1991]. Khand et al. [2002] reported that exposure to high concentrations of oxalate can induce oxidative stress, as shown by increased lipid peroxidation, increase in free radical generation, increases in arachidonic acid release via phospholipase-A_2 and decreased glutathione concentrations [Kohjmoto et al., 1999]. Vaca and Harms-Ringdahl [1989] have reported interaction of nuclear membrane lipid peroxidation products with nuclear macromolecules such as histone and chromatin. Hockenberry et al. [1993] and Kane et al. [1993] have demonstrated that a resistance to oxidant stress can be conferred by over expression of BCl-2 gene product. Scheid et al. [1996b] have reported that over expression of BCl-2 protects against oxalate toxicity in LLC-PK_1 cells. Similarly, p62 might also be expressed as a result of oxidative stress induced by oxalate.

CONCLUSION

This study suggests that there is a link between cell cycle events and oxalate toxicity and it might be mediated via p62. Thus, oxalate induces the expression of the NPC oxalate binding protein p62.

ACKNOWLEDGMENTS

P. Sivakamasundari was a recipient of Senior Research Fellowship from the Council of Scientific and Industrial Research (CSIR), New Delhi. This work was partly supported by CSIR, New Delhi. The help rendered by Dr. P. Kalaiselvi, Department of Medical Biochemistry, Dr. ALM PGIBMS, Taramani is thanked.

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