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ORIGINAL PAPER

Oxalate exposure provokes HSP 70 response in LLC-PK1 cells, a line of renal epithelial cells: protective role of HSP 70 against oxalate toxicity

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Abstract We investigated the effects of oxalate on immediate early genes (IEGs) and stress protein HSP 70, commonly induced genes in response to a variety of stresses. LLC-PK1 cells were exposed to oxalate. Gene transcription and translation were monitored by Northern and Western blot analysis. RNA and DNA synthesis were assessed by [^{3}H]-uridine and [^{3}H]-thymidine incorporation, respectively. Oxalate exposure selectively increased the levels of mRNA encoding IEGs c-*myc* and c-*jun* as well as stress protein HSP 70. While expression of c-*myc* and c-*jun* was rapid (within 15 min to 2 h) and transient, HSP 70 expression was delayed (∼8 h) and stable. Furthermore, oxalate exposure resulted in delayed induction of generalized transcription by 18 h and reinitiation of the DNA synthesis by 24 h of oxalate exposure. Moreover, we show that prior induction of HSP 70 by mild hypertonic exposure protected the cells from oxalate toxicity. To the best of our knowledge this is the first study to demonstrate rapid IEG response and delayed heat-shock response to oxalate toxicity and protective role of HSP 70 against oxalate toxicity to renal epithelial cells. Oxalate, a metabolic end product, induces IEGs c-*myc* and c-jun and a delayed HSP 70 expression; While IEG expression may regulate additional genetic responses to oxalate, increased HSP 70 expression would serve an early protective role during oxalate stress.

Keywords LLC-PK1 cells · Immediate early genes · Heat shock protein · Nephrolithiasis · Oxalate toxicity · Gene expression

Sweaty Koul and Meiyi Huang contributed equally to this work.

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Introduction

Oxalate is a metabolic end product and is excreted primarily by the kidney. This organic dicarboxylate is freely filtered at the glomerulus and undergoes bidirectional transport in the renal tubules [23, 27, 31]. The commonest pathological condition involving oxalate is formation of calcium oxalate stones in the kidney [39]. Besides renal stone formation, oxalate deposits are also associated with hyperplasic thyroid glands [11], benign neoplasm of the breast [37, 44], and renal cysts in acquired renal cystic disease, and proliferating cells in the kidney [12, 36]. Many of these conditions are associated with aberrant cell proliferation and cell death. Previous studies from our laboratory and those of others [7, 20, 21, 25, 26, 28, 29, 41] have demonstrated that oxalate interaction with renal epithelial cells results in a program of events consistent with cellular stress. Studies from our laboratory demonstrated that oxalate-induced reinitiation of the DNA synthesis is inhibited by inhibitors of transcription and translation, indicating that the cellular adaptations to oxalate toxicity are dependent on new gene expression and protein synthesis [30]. Moreover, cells of the renal tubular epithelium are exposed to an environment with variable and elevated concentrations of the oxalate and must be able to adapt to oxalate stress. However, the genetic response of renal epithelial cells to oxalate exposure remains poorly understood.

Gene expression is regulated in response to numerous environmental stimuli including hormones, neurotransmitters, enzyme substrate availability, UV radiation, hypoxia, temperature, gravitation, and other environmental stresses [2, 5, 13, 43]. The immediate early gene (IEG) expression in response to cellular stress in general is rapid and transient [9]. The delayed gene expression or secondarily gene expression in response to cellular stress varies with each stress condition. Nonetheless, cells respond to elevated temperature by increasing expression of genes coding for heat shock proteins (HSPs), which range in size from 10 to 170 kDa. These include HSP 70, which is strongly induced by high temperature in most of the cells and exhibits considerable conservation across species. Induction of HSP confers resistance to subsequent elevation of temperature [1, 46]. The same proteins are also induced in response to various other stresses including heavy metals, hypoxia, arsenate, ethanol, and hypertonicity and hence the more general name "stress proteins." HSPs are thought to have important role in preventing protein denaturation during metabolic stress and enhancing the ability of the cells to recover after stress [3, 5, 33, 45].

LLC-PK1 cells, a line of porcine kidney epithelial with characteristics of S1–S3 segment of proximal tubular epithelium, has been used widely as an in vitro model of renal epithelial cells and these cells express transport systems for oxalate and other ions [27, 35]. It has been shown that LLC-PK1 cells are sensitive to oxalate and provide a useful system to study the effects of oxalate [7, 20, 25–31]. In the present study, we set out to determine IEG and heat shock response to oxalate toxicity in LLC-PK1 cells. Preliminary studies [14], presented in full in this report, show that oxalate exposure elicits a specific genetic response: oxalate exposure selectively increased the levels of mRNA encoding IEGs c-*myc* and c-*jun* mRNA. We also demonstrate for the first time that oxalate exposure results in the induction of HSP 70. While expression of c-*myc* and c-*jun* were rapid and transient, HSP 70 expression was delayed and stable. We demonstrate that prior induction of HSP 70 protected these cells against oxalate toxicity. IEG expression may regulate additional genetic response to oxalate, while HSP 70 would represent a protective response of renal epithelial cells to the nephro-toxic challenges of oxalate.

Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Life Technologies, Inc. (Rockville, MD). Goat anti-rabbit IgGs and goat anti-mouse IgGs were obtained from Kodak Biolabs Scientific Imaging Systems (Rochester, NY). Probes (40mer oligo) specific for c-*myc* and c-jun mRNA, and antibodies to c-Myc were obtained from Oncogene, Cambridge, MA. HSP 70 plasmid (PAT 153) was obtained from American Type Culture Collection, Rockville, MD, USA. Antibodies to HSP 70 and c-Jun were obtained from BD Transduction Laboratories (Los Angles, CA, USA). [Methyl-^{3}H]-thymidine and [^{3}H]-uridine were obtained from ICN Radiochemicals, Inc. (Costa Mesa, CA). All other chemicals were of analytical grade and were obtained mostly from Sigma Chemical Co. (St Louis, MD).

Cell culture

LLC-PK1 cells, a renal epithelial cell line of porcine origin (American Type Culture Collection, Rockville, MD, USA), were used between passages 216 and 240. The cells were serially passaged in Dulbeco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 mg/ml), and glucose (4.5 g/l). All cultures were maintained in an atmosphere of 5% CO_2/95% air in humidified 37°C incubator. For these studies, high-density confluent cultures were used at least 2–3 days postconfluence. Where indicated, the cells were serum-starved over night (∼18 h). The concentration of serum was 0.25% during serum starvation period. For Western blotting, DNA and RNA synthesis experiments, the cells were plated in six-well plates while for Northern blot analysis cells were plated in T75 flasks.

Stock solution of oxalate (20 mM) was prepared in PBS, pH adjusted to 7.4, and used within 2–3 days. Where indicated, stock solution of oxalate was added to the cells to a final concentration of 1.0 mM, which results in free oxalate concentration of 300 μM as calculated by EQUIL.

Northern blot analysis

The serum-starved and growth-arrested cells were exposed to oxalate (1 mM) for various time periods. After the end of the experimental periods, the medium was aspirated from the cells grown in T75 flasks and the cells were lysed by addition of 4 ml of guanidium isothiocyanate; cells were scraped off the dishes and RNA was extracted as described [8]. The RNA was precipitated and dissolved in denaturing solution. The RNA was again precipitated and dissolved in DEPC-treated water. Samples of total RNA (30 μg each) were fractionated on 0.8 agarose/6% formaldehyde gels, and transferred to a nylon membrane Immobilon NY+ (Millipore, Bedford, MA).

The blots were incubated with hybridization buffer [0.5 M sodium phosphate, pH 7.1; 2 mM EDTA, 7% (w/v) SDS; 0.1% (w/v) sodium pyrophosphate] for 2 h at 65°C. These membranes were then hybridized overnight at 65°C

with the labeled probe (2.5×10^6 cpm) in hybridization buffer. Unbound radioactivity was removed by washing the blots at 65°C in 1× SSC buffer (0.3 M NaCl, 0.03 M Na citrate) containing 0.1% SDS The autoradiograms were prepared at −80°C for 24–72 h by exposing the blots to X-ray film in X-ray cassette containing two intensifying screens.

The probes for c-myc and c-jun were prepared by end-labeling 40mer antisense oligos (oncogene) with γ ^{32}P-ATP as described previously [28]. The probe for HSP 70 was generated by releasing the insert from the HSP 70 plasmid (ATCC, 57494) and labeled by random hexamer priming with αp32 ATP using Prime-a-Gene labeling system (Promega, WI) as per manufacturer's recommended protocol.

Gene expression was corrected for RNA loading and transfer by making comparisons relative to 18s RNA on the blots. Each experiment was repeated at least three times.

Western blot analysis

LLC-PK1 cells were serum-starved, growth-arrested, and exposed to oxalate for the time points described. At the end of experimental period, cells were washed with ice-cold PBS and solubilized with lysis buffer (20 mM Tris, pH 7.4, 1% Triton X-100, 1 mM sodium orthovandate, 10 mM NaF, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin). Lysates were sonicated with a micro-ultrasonic cell disrupter and centrifuged at 14,000×g at 4°C for 15 min to remove insoluble material. Protein concentrations were determined by using the Bradford (Bio-Rad) method and the samples were prepared by adding 2× sample buffer (50 mM Tris, pH 6.7, 2% SDS, 2% β-mercaptoethanol, and bromophenol blue) and boiled for 3–5 min. Twenty-five micrograms of protein from each sample was fractionated on a 10% SDS polyacrylamide gel. Proteins were transferred to Immobilon membrane (Millipore) at 24 V for 40 min in a semi-dry transfer apparatus (Biorad). Western blot analysis was carried out as described previously [30]. Briefly, the membranes were incubated with blocking buffer {5% nonfat milk in Tris-buffered saline–Tween buffer (TBST, 10 mM Tris–HCl, pH 7.5, 135 mM NaCl, 0.05% Tween)} for 1 h at room temperature and then incubated with antibodies to HSP 70, c-myc, or c-jun for overnight at 4°C. Immunoblots were washed with several changes of TBST at room temperature and then incubated with anti-mouse IgG linked to horseradish peroxidase or anti-rabbit IgG linked to horseradish peroxidase. Immunoreactivity was detected with enhanced chemiluminescence detection system (Amersham Biosciences) according to manufacturer's recommended protocol and quantified using densitometric analysis (Strategene's Eagle Eye gel documentation system).

RNA synthesis

RNA synthesis was monitored to check the specificity of gene expression. [^{3}H]-uridine was incorporated into the TCA precipitable material and was used as an index of RNA synthesis. For these studies, serum-starved and growth-arrested LLC-PK1 cells were exposed to oxalate (0.2 mM or 1 mM), and incubated for various time periods. During the last 1 h of exposure, these cells received [^{3}H]-uridine, 1 μCi/ml. The cells were washed with cold PBS and precipitated with 10% trichloroacetic acid overnight. The TCA precipitable material was solubilized in a solution of 1% SDS and 0.1 N NaOH. [^{3}H]-uridine incorporation was determined by liquid scintillation counting.

DNA synthesis

For these studies, LLCPK1 cells were plated at a high density in six-well plates and grown to confluence. Forty-eight hours after the last media change, cells were incubated in DMEM (0.25% serum) for 12–18 h as described previously [28]. These quiescent cells were then exposed to oxalate (1 mM) or 10% FCS for 24 h. During last 6 h of exposure, 2–5 μCi of [^{3}H]-thymidine was added per well. At the end of experimental period, medium was removed and the cells were washed with two changes of ice cold phosphate buffered saline (PBS). The cells were washed with two changes of 10% trichloroacetic acid (TCA) for 2–4 h. The TCA insoluble material was dissolved in lysis solution (1% SDS, 0.1 N NaOH), 0.5 ml/well, and radioactivity was measured by liquid scintillation counting, and used as an index of DNA synthesis.

Cell viability assay using vital dyes

Cells were sub-cultured in 8 chamber slides and grown to 50–75% confluence. The cells were exposed to (DMEM + 0.25% FBS) or (DMEM + 0.25% FBS supplemented with 25 mM NaCl for overnight) prior to the addition of fresh medium alone (DMEM + 0.25% FBS) or in combination with varying concentrations of oxalate ranging from 0.2 to 1.0 mM. Cells were exposed to oxalate for 1 h at the end of which the media was aspirated and 300 μL of 0.4% Trypan blue added to each of the wells as described previously [28]. Cells were examined under light microscope and the number of cells that failed to exclude the dye per high power field (200×) were counted from five randomly selected fields in each chamber and used as an index of altered membrane permeability. The number of cells that could not exclude the dye was expressed as a percentage of the total number of cells seen per high power field.

Estimation of free radical production

Oxalate-induced changes in the reduction of nitro blue tetrazolium (NBT), a dye that reacts with superoxide, were used to evaluate oxidant stress as described previously [25]. For these experiments confluent LLC-PK1 cells grown in 12-well plates were exposed for varying periods (0–180 min) to DMEM containing 25 µg/ml NBT. Where indicated, cells were pretreated with SOD (super oxide dismutase) (50 µg/ml). Cells were exposed to oxalate for the predetermined intervals (120 or 180 min) the medium was removed and the reaction was halted by the addition of 70% methanol. The monolayers were washed with four changes of 100% methanol to remove the unreduced NBT, air-dried, and solubilized with a mixture of 2 M KOH and dimethyl sulfoxide (DMSO) in a ratio of 1:1.167 (v/v). Samples were then centrifuged for four min at 15,000 rpm and supernatants were read at 700 nm (density OD_{700}) using a Beckman DU-650 spectrophotometer against a blank containing KOH and DMSO.

Statistical analysis

Statistical analysis was performed using Student's *t* test.

Results

Effect of oxalate on the immediate early gene expression

The kinetics of the expression of IEGs (c-myc and c-jun) was investigated in cells following exposure to 1 mM oxalate, a concentration previously shown to maximally stimulate DNA synthesis. Total cellular RNA was extracted from the cells and Northern blots were probed with [γ32P]-ATP-labeled c-myc- or c-jun-specific probes.

Northern blot analysis showed that exposure of cells to oxalate (1 mM) resulted in transient overexpression of the c-*myc* message. Maximum expression reached in around 2 h of oxalate addition. At about 8 h, the expression subsided to about 30% above the control level (Fig. 1a). c-*myc* gene expression was corrected for 18s RNA and the relative expression was calculated and is presented in Fig. 1b. Maximum expression of c-*myc* mRNA (211 ± 26% stimulation over control) were observed at 2 h following oxalate exposure. These data demonstrate transient transcriptional activation of c-*myc* gene in renal epithelial cells by oxalate.

Oxalate exposure to growth arrested renal *epithelial* cells also resulted in overexpression of c-*jun* gene similar to that of the c-*myc* gene. However, c-*jun* overexpression was observed at earlier time points. The overexpression of c-*jun* gene was detected as early as 15 min of oxalate exposure with maximum expression observed at about 1 h of oxalate

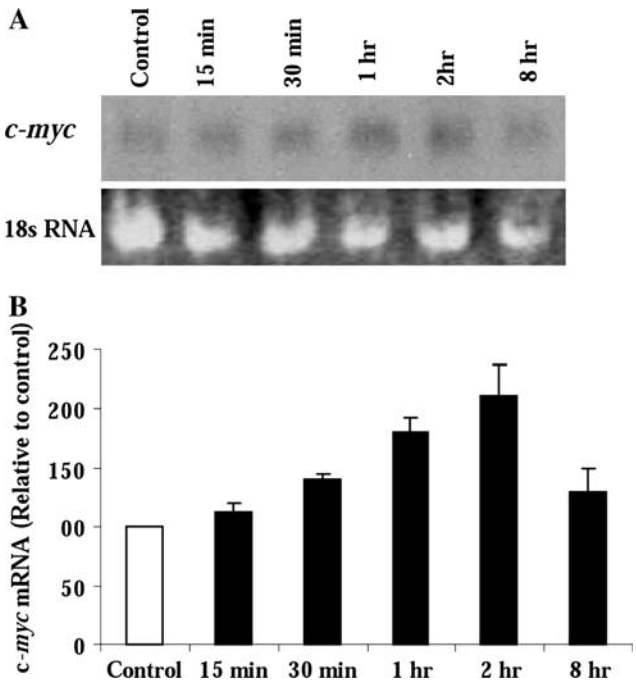

Fig. 1 Northern blot analysis showing effects of oxalate on the c-*myc* gene expression: for these studies, confluent, quiescent, growth-arrested, serum-starved LLC-PK1 cells were exposed to oxalate (1 mM) for various times (15 min to 8 h). Total RNA was isolated, separated on 0.8% agarose/6% formaldehyde gel, and blotted on nylon membrane. Blots were probed with ^{32}P-labeled c-*myc* probe. **a** Representative Northern blot showing expression of c-*myc* mRNA following oxalate exposure. **b** c-*myc* expression relative to 18s RNA as quantitated by densitometric analysis. Data are expressed as the average percentage of control ± SD. Please note that similar results were observed in three separate experiments

exposure. The c-*jun* message returned to the control level at around 8 h (Fig. 2a). The expression of c-*jun* was also corrected for 18s RNA and relative expression of c-*jun* gene is presented in Fig. 2b. These results demonstrate that maximum levels of c-*jun* mRNA (242 ± 30% compared to control) are seen at 30 min following oxalate exposure. These data illustrate that c-*jun* expression is also transiently upregulated following oxalate exposure. Taken together, these data demonstrate that oxalate exposure to renal epithelial cells results in transient over expression of IEGs c-*jun* and c-*myc*.

Effect of oxalate on HSP 70 gene expression

Results presented in Fig. 3 show that HSP 70 gene was constitutively expressed in LLC-PK1 cells. There was no effect of oxalate exposure on HSP 70 gene expression at early time points tested (up to 4 h of oxalate exposure). However, at later timepoints oxalate exposure significantly increased the expression of HSP 70 gene (Fig. 3a). HSP gene expression was corrected for 18s RNA and the relative expression of HSP 70 gene following oxalate exposure is presented in

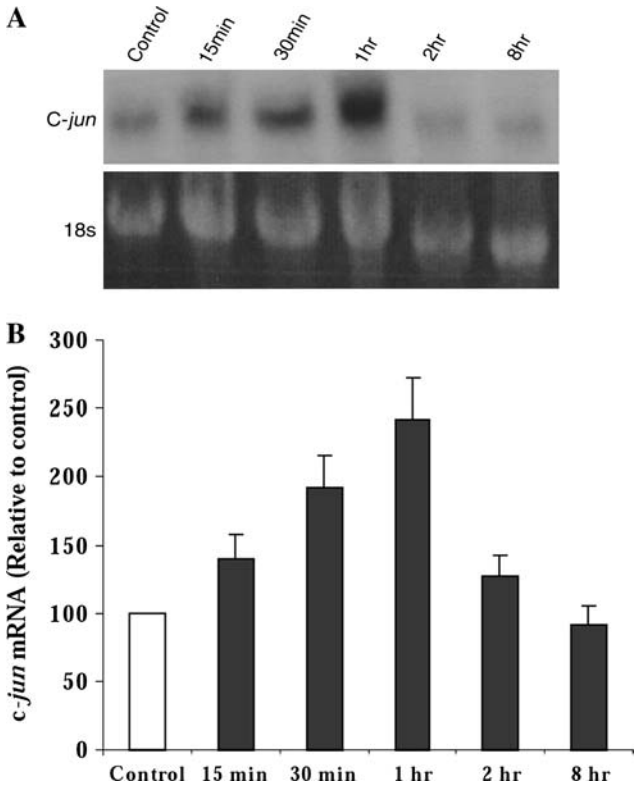

Fig. 2 Northern blot analysis showing effects of oxalate on the c-*jun* gene expression: for these studies, confluent, quiescent, growth-arrested, serum-starved LLC-PK1 cells were exposed to oxalate (1 mM) for various times (15 min to 8 h). Total RNA was isolated, separated on 0.8% agarose/6% formaldehyde gel, and blotted on nylon membrane. Blots were probed with ^{32}P-labeled c-*jun* probe. **a** Representative Northern blot showing expression of c-jun mRNA following oxalate exposure. **b** c-*jun* expression relative to 18s RNA as quantitated by densitometric analysis. Data are expressed as the average percentage of control ± SD. Similar results were observed in three separate experiments

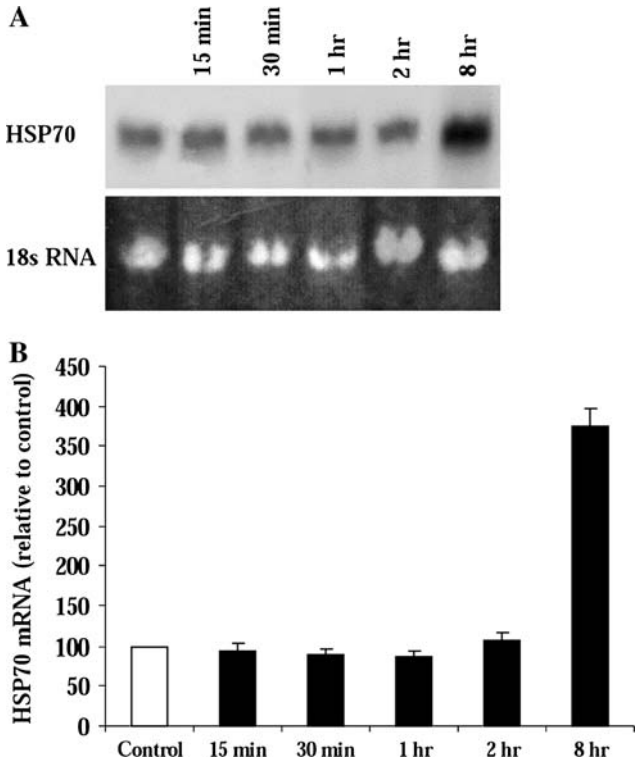

Fig. 3 Northern blot analysis showing effects of oxalate on the *HSP 70* gene expression: for these studies, confluent, quiescent, growth-arrested, serum-starved LLC-PK1 cells were exposed to oxalate (1 mM) for various times (15 min to 8 h). Total RNA was isolated, separated on 0.8% agarose/6% formaldehyde gel, and blotted on nylon membrane. Blots were probed with ^{32}P-labeled *HSP 70* probe. **a** Representative Northern blot showing expression of HSP 70 mRNA following oxalate exposure. **b** *HSP 70* expression relative to 18s RNA as quantitated by densitometric analysis. Data are expressed as the average percentage of control ± SD. Please note that similar results were observed in three separate experiments

Fig. 3b. These data reveal that maximum expression of HSP 70 (376 ± 20% compared to control) was observed at 8 h following oxalate exposure exposure to 1 mM oxalate. Thereafter, HSP 70 gene expression (mRNA levels) remained elevated for at least up to 24 h.

Next we evaluated the effect of oxalate exposure on the protein levels of c-Jun, c-Myc, and HSP 70 to assess the effects of overexpression of c-*myc*, c-*jun*, and *HSP 70* genes on cellular levels of these proteins.

Effect of oxalate on c-Myc, c-Jun, and HSP 70 protein expression

For these studies, serum starved growth arrested, monolayers of LLC-PK1 cells were exposed to oxalate (1 mM) for various timepoints. At the end of experimental periods, media were aspirated, cells were washed with two changes of ice cold PBS. The cells were lysed and proteins were separated by SDS-PAGE and levels of c-Myc, c-Jun, and HSP 70 proteins were evaluated by Western blot analysis as described under "Methods."

These Western blots data are presented in Fig. 4. Results demonstrate that oxalate exposure resulted in rapid and transient increase in c-Jun protein level (Fig. 4a). The data reveal that c-Jun protein levels started to increase as early as 5 min with maximum increase at 30 min. At 60 min, the protein level started following down. The levels of c-Myc protein (Fig. 4c) followed a pattern similar to the c-Jun protein: a rapid and transient increase in c-Myc protein levels. These data reveal that the c-Myc protein levels rose transiently with maximum increase at 2 h following oxalate exposure. However, c-Myc levels remained elevated upto 4 h of oxalate exposure. Results also show that the cells expressed more HSP 70 protein after oxalate treatment (Fig. 4e). There was no change in HSP 70 protein expression immediately following oxalate exposure. The overexpression of HSP 70 was first seen after 8 h exposure to oxalate, increased gradually, and reached maximum after 24 h treatment.

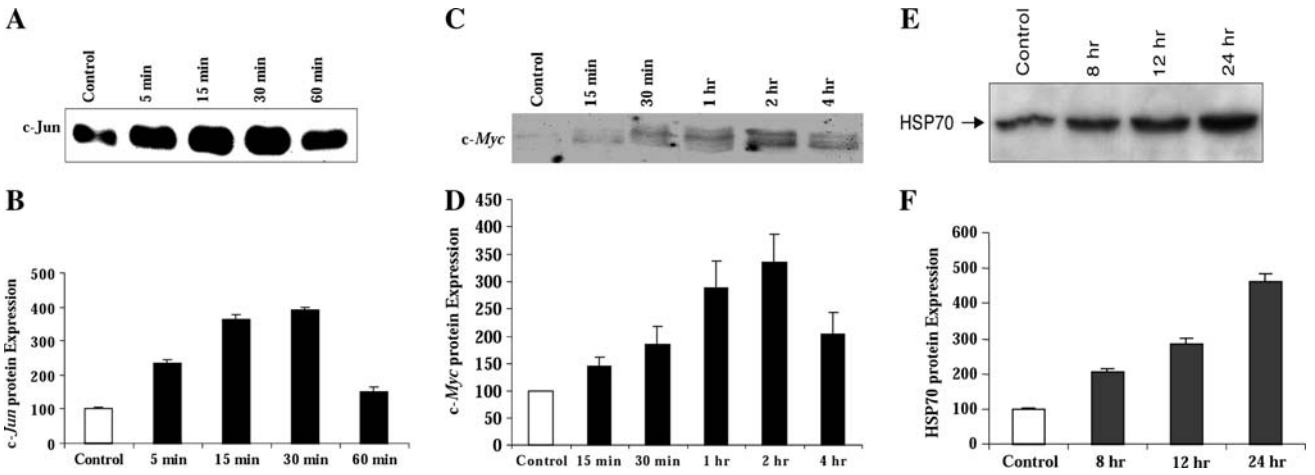

Fig. 4 Oxalate exposure increased the protein levels of c-Myc, c-Jun, and HSP 70: for these studies, confluent, quiescent, growth-arrested, serum-starved LLC-PK1 cells were exposed to oxalate (1 mM) for various times (15 min to 24 h). Protein expression was monitored by Western blot analysis, using specific antibodies that recognized c-Myc protein, c-Jun protein, or HSP 70 protein product. **a** Representative blot showing c-Jun protein levels following oxalate exposure. **c** Representative blot showing c-Myc protein levels following oxalate exposure. **e** Representative blot showing HSP 70 protein levels following oxalate exposure. **b**, **d**, **f** Relative levels of c-Jun, c-Myc, and HSP 70 proteins at various time points following oxalate exposure, as measured by densitometric analysis of Western blots in **a**, **c**, **e**, respectively. Please note that oxalate exposure resulted in early but transient elevation of c-Myc and c-Jun protein levels and a delayed and stable increase in HSP 70 protein levels

These western Blot data (Fig. 4a, c, e) were analyzed densitometricly and relative expression of c-Jun, c-Myc and HSP 70 expression is presented in Fig. 4b, d, f, respectively. As is shown in the Fig. 4b, oxalate exposure resulted in 390 ± 9% increase in c-Jun protein levels with maximum increase achieved at 30 min following oxalate exposure. Similarly, oxalate exposure resulted in increase in c-Myc protein expression with maximum protein levels 336 ± 50% achieved at 2 h following exposure to oxalate (Fig. 4d). In contrast to this early and transient over-expression of c-Myc and c-Jun proteins, no changes were observed in HSP 70 protein expression until 8 h. of oxalate treatment. Exposure of LLC-PK1 cells to oxalate for 8 h resulted in twofold increase in the amount of HSP 70 protein as compared to the control cells. At 12 h following the exposure to oxalate, the HSP 70 levels were threefold as compared to the control cells and at 24 h following oxalate exposure, the amount of HSP 70 protein was more than threefold higher than control cells. These data demonstrate that exposure of renal epithelial cells to oxalate results in increased HSP 70 protein synthesis as well as accumulation (Fig. 4f).

RNA synthesis after oxalate exposure

[^{3}H]-uridine incorporation into cultured LLC-PK1 cells was used as an index of total mRNA synthesis and to assess the specificity of the overexpression of c-*jun*, c-*myc*, and *HSP 70* mRNA after oxalate exposure (Fig. 5). Exposure of

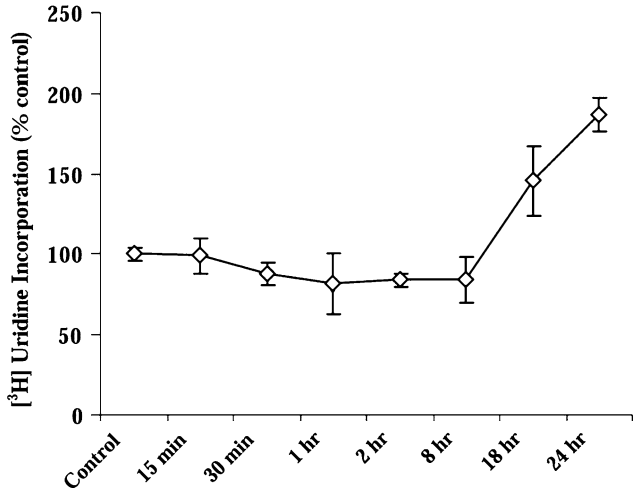

Fig. 5 Effect of oxalate on total cellular RNA synthesis: for these studies, confluent, quiescent, growth-arrested, serum-starved LLC-PK1 cells were exposed to oxalate (1 mM) for various times (15 min to 24 h). At the end of the experimental period, the RNA synthesis was indexed by [^{3}H]-uridine incorporation. Each point represents mean ± SD of four observations. *$P < 0.05$ versus control

LLC-PK1 cells to oxalate (1 mM) resulted in slight decreases (84 ± 5% vs. 100 ± 7%; oxalate vs. control) in [^{3}H]-uridine incorporation at early time points (15 min to 8 h). However, there was higher incorporation rate (186 ± 11% above control) at later time points (18 and 24 h) indicating generalized transcription. These data confirm that over expression of c-*myc,* c-*jun* and *HSP 70* reflect specific expression of those messages.

DNA synthesis after oxalate exposure

Kinetics of the re-initiation of DNA synthesis was evaluated by [^{3}H]-thymidine incorporation into the TCA precipitable material as described under "Methods." Results presented in Fig. 6 show that oxalate exposure to renal cells resulted in reinitiation of the DNA synthesis in a time-dependent fashion. There was a delay of about 18–24 h following oxalate exposure in the reinitiation of the DNA synthesis. After 24 h of oxalate exposure, the thymidine incorporation was 250 ± 17% over that of control. [^{3}H]-thymidine incorporation continued to rise with time; at 48 h following oxalate exposure thymidine incorporation reached over 260% of the control valve.

Role of free radical scavenge

Results presented in Fig. 7 show that oxalate exposure was associated with increased superoxide production, and pretreatment with superoxide dismutase was able to diminish free radicals generated upon oxalate exposure (Fig. 7a). We also observed that pretreatment of cells with superoxide dismutase resulted in partial ablation of oxalate-induced HSP 70 response (Fig. 7b, c). These data indicate that oxalate-induced HSP 70 response may in part be in response to free radical generation.

Role of HSP 70 in oxalate toxicity

Results presented in Fig. 8 demonstrate that exposure of cells to hypertonic media (supplementing media with 25 mM NaCl) resulted in induction of HSP 70 in a time-dependent fashion. Increased HSP 70 levels were observed

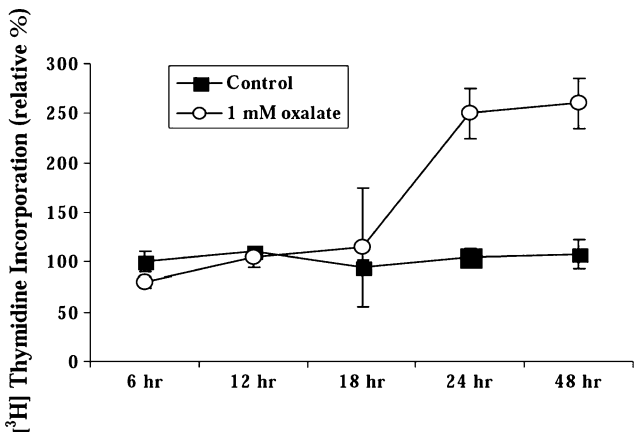

Fig. 6 Effect of oxalate on DNA synthesis: for these studies, confluent, quiescent, growth-arrested, serum-starved LLC-PK1 cells were exposed to oxalate (1 mM) for various times (6–48 h). At the end of the experimental period, DNA synthesis was assessed by [^{3}H]-thymidine incorporation into the TCA precipitable material. Each point represents mean ± SD of 6–12 observations. *$P < 0.05$ versus control

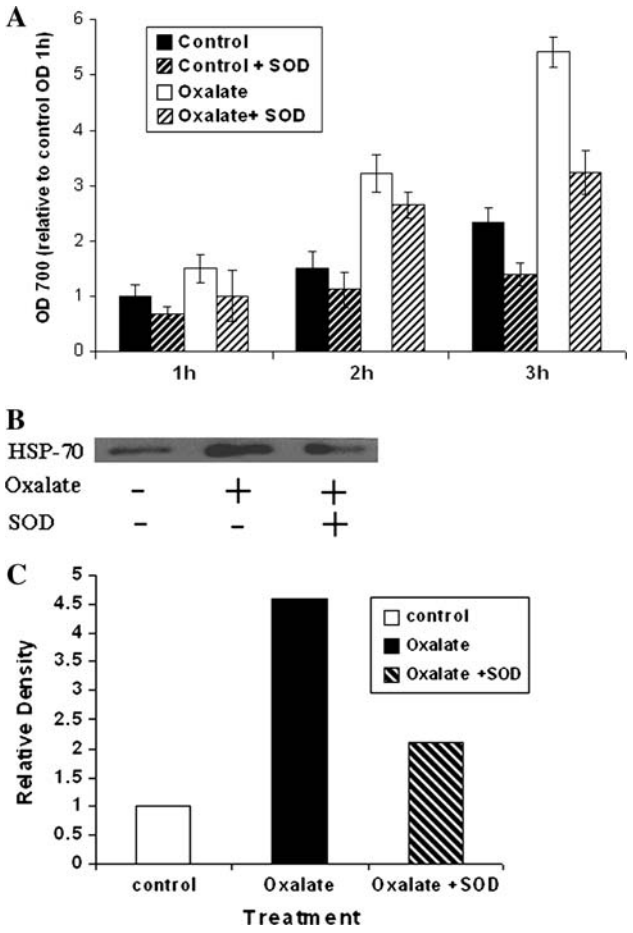

Fig. 7 Oxalate exposure is associated with increased free-radical production: for these experiments, confluent monolayers of LLC-PK1 cells were exposed to DMEM containing 25 μg/ml NBT plus varying oxalate (0 or 1 mM total) alone or in presence of SOD or catalase

starting at 4 h postexposure, and the elevated levels continued up to 24 h postexposure (Fig. 8a). Results also show that induction of HSP 70 prior to oxalate exposure resulted in protecting the cells against oxalate toxicity as measured by Trypan blue exclusion (Fig. 8b). These data suggest that HSP 70 expression may indeed serve as a protective role in response to oxalate toxicity.

Discussion

The present studies on renal epithelial cells with characteristics of the S1–S3 segment of the proximal tubule characterized several early genetic events associated with oxalate exposure. Oxalate exposure selectively increased the levels of mRNA encoding IEGs c-*myc* and c-*jun* as well as stress protein HSP 70. Furthermore, oxalate-induced c-*myc*, c-*jun*, and *HSP 70* expression was accompanied by delayed induction of generalized transcription as well as reinitiation of the DNA synthesis. Oxalate exposure was also associated with

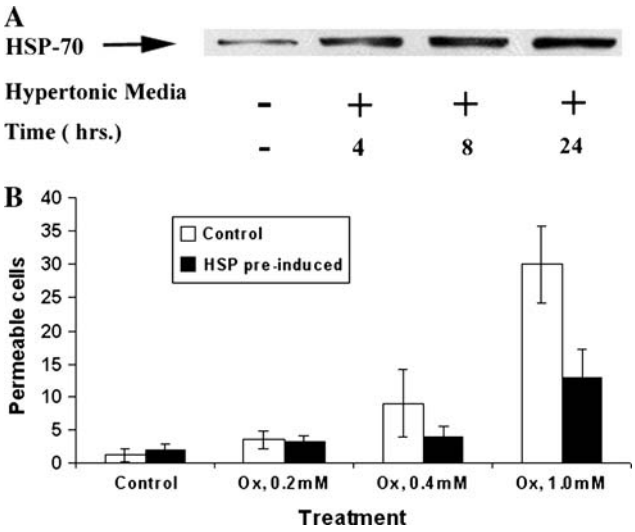

Fig. 8 Prior induction of HSP 70 protects renal cells against oxalate toxicity: for these experiments, HSP 70 was induced in LLC-PK1 cells by exposure to hypertonic media (osmolarity of 325 mM vs. 300 mM) and cells were exposed to oxalate at least 8 h after exposure to hypertonic media. **a** Effect of hypertonic media on HSP 70 induction. **b** Effect of hypertonicity on oxalate toxicity

increased free radical production that could be abolished by treatment with superoxide dismutase. Abolishing the free radical production partially abolished HSP 70 expression. We also observed that prior induction of HSP 70 was protective against oxalate toxicity.

Previous studies have shown that oxalate is toxic to renal epithelial cells [20, 21, 24–28, 41]. Adaptations that renal cells have to make with elevated levels of oxalate are complex and remain poorly understood at the genetic level. Several studies, including preliminary report of the present study, have shown that exposure of renal cells to oxalate or COM-crystals results in expression of mRNA for Egr-1, Osteopontin, c-myc, NUR-77, PAI-1, IL-6, and bikunin [7, 12, 14–16, 24, 28, 29, 32]. We demonstrated that oxalate- and COM-crystal-stimulated reinitiation of the DNA synthesis in growth-arrested LLC-PK1 cells requires new gene expression as well as new protein synthesis and is mediated by activation of p38 MAP kinase signal transduction pathway [7, 29, 30]. These findings further strengthen the notion that renal cellular adaptation to the oxalate toxicity involves new gene expression as well as protein synthesis. Previous studies demonstrated that renal cells respond to several divergent stress stimuli including hypertonicity and heat shock by overexpressing HSP 70 [9, 42]. In the present study, we used LLC-PK1 cells as model system to evaluate the potential involvement of IEG and HSP 70 expression in this adaptive process. Renal cells selectively increased the levels of mRNA encoding IEGs c-*myc* and c-*jun* within 15 min to 2 h of oxalate exposure. c-Myc and c-Jun are nuclear binding proteins and serve as transcription factors and are known to regulate expression of several genes. Moreover, c-*myc* and c-*jun* expression does not require any new protein synthesis; they provide a link between signal transduction and initiation of coordinated program of gene expression [17, 38].

Oxalate exposure also resulted in increased expression of *HSP 70* (Fig. 3). The heat shock response is a highly conserved genetic program and is induced under a variety of stress conditions, including heat shock, oxidative stress, heavy metals, and ethanol exposure [2, 13, 43]. It has been proposed that heat shock response is provoked by damage to the cytoplasmic proteins, and it has been demonstrated that intracellular proteins are unfolded by heat stress [3, 43]. We and others have previously shown that oxalate exposure results in an oxidant stress [21, 24, 25, 41]. It is therefore conceivable that oxalate exposure may also damage intracellular proteins, which could provoke HSP 70 expression. Other studies have shown that antioxidants do prevent renal cells from oxalate toxicity and thus block adaptive responses following oxalate exposure. Since there is reduced toxicity in presence of SOD and catalase, it is expected that adaptive responses are not needed. In light of these data, we also evaluated effects of SOD on oxalate-induced free radical production and HSP response. To this end, we observed that scavenging the free radicals, which were generated following oxalate exposure by pretreatment of the cells with superoxide dismutase, resulted in decreased HSP 70 response (Fig. 7). These data suggest that free radical generation associated with oxalate exposure provokes HSP response. These data support the previously established notion that oxalate toxicity in part involves free radical generation and strengthen the notion that some cellular genetic responses following oxalate exposure may indeed be adaptive responses to oxalate toxicity.

HSP 70 protein serves as a molecular chaperon and helps in the maintenance of activation-competent state of a key number of regulatory proteins in response to diverse stress stimuli [4]. Moreover, HSP 70, normally not expressed in renal epithelial cells, has been shown to be upregulated in renal epithelial cells upon adaptation to hyperosmolarity [5] and may be playing a role in cell survival during adaptation to hypertonicity. Previous studies have shown that oxalate exposure of the renal epithelial cells is associated with lipid peroxidation products, indicative of considerable oxidative stress. Besides lipid peroxidation, it is possible that oxalate-induced oxidative stress can result in protein misfolding as well as DNA adduct formation. Given the established role of HSP 70 as a molecular chaperone, it is conceivable that HSP 70 could be playing a critical role in protein stabilization in the face of oxalate-induced oxidant stress. These considerations suggest that HSP 70 may serve in protective capacity even before a broad response/adaptation is made

by the cells. To test this possibility, we induced HSP 70 expression in the cells by exposing them to mildly hypertonic media (by addition of 25 mM NaCl to the culture media). Results presented in Fig. 8 demonstrate that prior induction of HSP 70 indeed protected the cells against oxalate-induced cell damage as monitored by changes in membrane permeability. Moreover, we confirmed the protective role of HSP 70 against oxalate toxicity by directly transecting the cells with HSP 70 expression vector system (data not shown). These data further support the protective role of HSP 70 against oxalate nephrotoxicity.

Our results demonstrate that while the expression of c-*myc* and c-*jun* was rapid and transient, HSP 70 expression was delayed and stable. This selective expression of c-myc, c-jun, and HSP 70 by renal epithelial cells following oxalate exposure was confirmed by lack of generalized transcription (as seen by [^{3}H]-uridine incorporation) at these time points tested (Fig. 5). Therefore, sequential activation of IEGs and HSP 70 would be ideal for cell proliferation. However, in several stress conditions, IEG expression as well as HSP 70 expression has been observed despite marked inhibition of the DNA synthesis [10]. In the present study, oxalate exposure resulted in sequential expression of IEG and HSP 70 followed by reinitiation of the DNA synthesis. These data strengthen the notion that renal cellular adaptations to oxalate toxicity involve specific genetic component.

The regulatory mechanisms that govern c-myc, c-jun, and HSP 70 expression are complex [18, 19, 34, 40]. These genes are not only induced in response to cellular stress, but are also upregulated by growth factors [47, 48]. In addition, it has been shown that HSP 70 and c-Myc protein can induce expression of each other [6, 22]. In the present series, however, c-jun and c-myc expression was observed with 15 min to 2 h of oxalate exposure, while HSP 70 expression was not observed until 6 h after oxalate exposure, suggesting that early c-myc expression in this case was not induced by HSP 70 expression. Previous studies have shown that oxalate exposure was associated with free radical production. We and others have also shown that free radical scavengers protected renal cells against oxalate toxicity. In the present study, we clearly demonstrate that HSP 70 induction can be partially abolished by scavenging the free radicals, thus suggesting that HSP 70 induction may indeed in part be governed by free-radical-induced changes to cellular macromolecules. Clearly, additional studies are needed to understand the molecular mechanisms that regulate c-*myc*, c-*jun*, and *HSP 70* expression following oxalate nephrotoxicity.

In conclusion, we have for the first time demonstrated that oxalate exposure to the renal epithelial cells provokes heat shock response and that heat shock response protected the renal cells against oxalate toxicity. Transcriptional activation of c-*myc* and c-*jun* may govern the program of gene expression in response to oxalate exposure, while HSP 70 expression serves as an interim protective measure and may reflect adaptations of renal cells to oxalate toxicity.

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