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Checkpoint kinase (Chk1) and reactive oxygen species induced oncotic cell death during the cell cycle in LLC-PK1 cells

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Abstract

2,3,5–*tris*-(Glutathion-S-yl)hydroquinone (TGHQ) is a reactive oxygen species (ROS) generating metabolite of the nephotoxicant, hydroquinone. In renal proximal tubule epithelial cells (LLC-PK1), TGHQ catalyzes the formation of single strand breaks in DNA, followed by growth arrest and oncotic/necrotic death during the cell cycle. Treatment of synchronized LLC-PK1 cells with TGHQ causes an initial decrease in the expression of the DNA damage inducible protein kinase chk1. The degradation of chk1 in highly synchronized LLC-PK1 cells might induce mitotic catastrophe, a potential mechanism of cell death in this model.

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Introduction

The eukaryotic cell cycle is a tightly regulated process controlled by various checkpoint mechanisms, and tightly coupled to cell growth and DNA damage repair. Checkpoint control mechanisms are key for maintaining genomic integrity, failure of which may result in cell transformation or apoptosis (Nurse, 2000). One of the most important regulatory cell cycle checkpoint kinases is Chk1, the activity of which fluctuates during the cell cycle.

Quinones and their thioether metabolites are reactive electrophiles capable of redox cycling with the concomitant generation of reactive oxygen species (ROS) (Monks and Lau, 1998). 2,3,5-*tris*-(Glutathion-S-yl)hydroquinone (TGHQ), a redox active metabolite of hydroquinone, is a potent nephrotoxicant (Monks and Lau, 1998) and nephrocarcinogen (Yoon *et al.,* 2002). The mutation spectra induced by TGHQ is consistent with the participation of hydroxyl radicals in this process (Jeong *et al.,* 1999). Quinol-thioethers also catalyze the formation of single strand breaks in DNA (Mertens *et al.,* 1995) , accompanied by an immediate growth arrest of renal epithelial cells (LLC-PK1) characterized by rapid inhibition of DNA synthesis, coupled to increased expression of *gadd153* mRNA, decreases in histone mRNA and ultimately oncotic/necrotic cell death (Jeong *et al.,* 1997b).

TGHQ induced ROS also activate all three major subfamilies (ERK, JNK/SAPK and p38) of mitogen–activated protein kinases (MAPK) (Ramachandiran *et al.,* 2002). While JNK activation does not play role in cell death (Ramachandiran *et al.,* 2002), TGHQ induced ERK1/2 and p38 MAPK activation appear to contribute to oncotic cell death, partially via histone H3 phosphorylation and subsequent premature chromatin condensation and cell death (Tikoo *et al.,* 2001). However, since inhibition of ERK1/2 or p38 MAPK with PD098059 or SB202190 alone does not completely protect LLC-PK1 cells against TGHQ induced oncotic cell death, additional pathways are thereby likely to be operative in this model of ROS induced cell death (Ramachandiran *et al.,* 2002). In this respect, appropriate post-translational modification of proteins is important for maintaining cellular homeostasis and phosphorylation/dephosphorylation mechanisms are particularly well studied in this regard. Protein phosphorylation/dephosphorylation contributes to the regulation of cell metabolism, gene expression, cellular architecture and cell survival/cell death in eukaryotic cells.

To better define the role of phophorylation/dephosphorylation networks in response to cell stress we need to define these events at various stages of the cell cycle. Indeed, cell cycle checkpoints represent a critical component regulating progression through the cell cycle. Using functional cell cycle analysis we now demonstrate that following treatment of synchronized LLC-PK1 cells with TGHQ, Chk1 is degraded in a time dependent manner, which therefore appears to contribute to ROS-induced oncotic cell death of renal epithelial LLC-PK1 cells.

Materials and methods

Caution

TGHQ is nephrotoxic and nephrocarcinogenic in rats and therefore must be handled with protective clothing and in a ventilated hood.

Chemicals

Unless specified otherwise, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). TGHQ was synthesized in the laboratory according to established methodology. All reagents were of the highest grade commercially available.

Cell Culture Conditions and synchronization

LLC-PK1 cells, a renal proximal tubule epithelial cell line derived from the New Hampshire mini-pig (used between passage 210-240) was obtained from the American Type Culture Collection (Rockville, MD).

Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (high glucose without pyruvate) containing 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA) at 37°C in a humidified incubator containing 5% CO2. After growth in complete medium for 22-24 h, cells were synchronized by serum starvation for 48 h and released by addition of FBS. Experiments were performed 8 hr after addition of FBS. All treatments were performed in DMEM containing 25 mM HEPES (pH7.4).

Alternatively, to obtain active synchronization, LLC-PK1 cells were sequentially exposed to thymidine-aphidicolin. In brief, 2mM of thymidine (Sigma, USA) was applied to cells at 50% confluency for 16 hours and washed (3X) with the same media followed by release to the same media for another 8 hours. Aphidicolin (5 µg/ml) of (Sigma, USA) was added to the culture and cells were grown for another 16 hours to block the cells in S-phase. Cells were then washed with the same media three times, all with sterile solutions, and released in to the same media followed by cell cycle analysis or TGHQ (200 µM) application.

Western blot analysis

After specified treatments, LLC-PK1 cells were washed twice with phosphate buffered saline (PBS) and homogenized in ice-cold lysis buffer (Cell Signaling Technology, Inc.- 20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 1µg/ml leupeptin). Homogenates were centrifuged at 10,000 g for 10 min at 4°C. Supernatants were collected, and 30 µg of total protein electrophoresed on 10% SDS-polyacrylamide gels and transferred to a PVDF membrane. After blocking in TBS-T containing 5% dried milk or 5% BSA, membranes were incubated with appropriate primary antibodies (1:1000 dilution) overnight at 4°C (Cell Signaling Technology, Beverly, MA). After Washing in TBS-T, membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:2000 dilutions) (Santa Cruz Biotechnology). After thoroughly washing in TBS-T, bound antibodies were visualized using standard chemilumiscence on autoradiographic film.

Neutral red assay for cell viability

LLC-PK1 cells were seeded at a density of 0.25 X 10^5 cells/well in 24-well plates. After growth in complete medium for 22-24 h, cells were synchronized by serum starvation for 48 h and released by addition of FBS. Experiments were performed 8 h after addition of FBS. All treatments were performed in DMEM containing 25mM HEPES (pH 7.4). Cells were washed and treated with TGHQ in DMEM containing 25 mM HEPES pH 7.4. Neutral Red assays were then performed as previously described (Mertens *et al.,* 1995).

Flow cytometry

For flow cytometric analysis, cells were collected at hourly intervals, washed twice with cold PBS and treated with 0.25% w/v trypsin solution prewarmed to 37°C. When sheets of cells monolayers were lifting off the flask (3-5 min.), approximately 10 volumes of PBS were added and centrifuged for 5 min at 1200 rpm. Cells were then fixed with 70% cold ethanol for at least 30 minutes at -20°C for further analysis by flow cytometry. Cells were washed twice with PBS and filtered (20 µM) to yield cell suspensions which were subsequently treated with RNAse A and stained with propidium iodide (50 µg/ml). Cell cycle analysis was performed using a FACS scan flow cytometer (Beckman Coulter) followed by Wincycle software. Chk1 Si-RNA was purchased from Upstate,USA and plasmid transfection was performed according to manufacturer's instructions. Mitotic index was determined as described (Ehsan *et al.,* 1998).

Results

Characterization of thymidine-aphidicolin synchronized LLC-PK1 cells.

The sequential application of thymidine-aphidicolin to LLC-PK1 cells provided a better synchronization procedure than serum starvation (data not shown). Flow cytometric analyses and determination of mitotic index were used to assess the degree of synchronization. When cells were released from the aphidicolin block (S-phase), more than 80% of cells accumulated in S-phase (2 hours after release) and subsequently proceeded to the next phase of the cycle (Fig: 1b). As cells progressed further, G2/M phase cells (4n) exhibited a lower degree of synchronization consistent with the fact that cells lose synchrony as they proceed further through the cycle (Fig. 1c & 1d). However, flow cytometric analysis revealed a satisfactory S-phase and G2/M peak at 2 hours and 10 hours respectively (Fig. 1b, c & e). Stationary cells were used as a positive control for this analysis (Fig. 1a). Mitotic figures were subsequently visualized by fluorescent microscopy at hourly intervals. During the S and G2- phases, not a single metaphase or anaphase chromosome was observed (Fig. 2a upper two images), whereas, at 10 hours, approximately 20% of the cells contained metaphase-anaphase chromosomes (Fig. 2a, lower two images & b).

Cell cycle regulatory protein expression and ROS mediated cell death in synchronized LLC-PK1 cells

The expression of cell cycle regulatory proteins such as cyclin E, Cdk7 and Chk1 fluctuate during the cell cycle, as does their phosphorylation status (Fig. 3a). As anticipated, the constitutive expression of Cdc2, Cdc25c, cyclin B1, wee1 and cyclin A2 did not change throughout the cell cycle (Fig. 3 b). TGHQ induces oncotic cell death in synchronized LLC-PK1 cells in a dose and time dependent manner, as determined by decreases in Neutral Red absorbance (Fig. 4). TGHQ stimulated oncotic cell death was determined in both synchronized LLC-PK1 cells. In serum starved synchronized cells, decreases in Neutral Red were observed as early as 2h after TGHQ treatment and reached a maximum at 4h (Fig 4 a). Similar results were obtained in thymidine-aphidicolin synchronized LLC-PK1 cells (Fig. 4b).

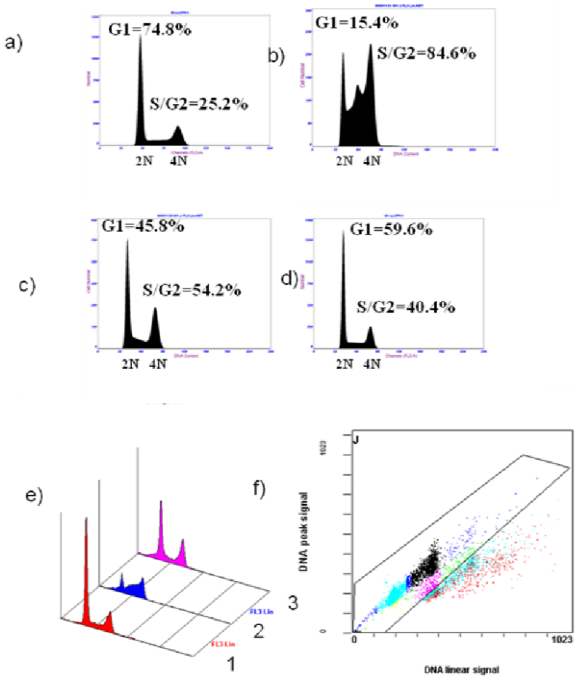

Fig. 1. Flow cytometric analysis of LLC-PK1 synchronized cells. a) Statistical analysis of stationary LLC-PK1 cells. b) Thymidine-aphidicolin synchronized cells at time point 2 hours after release. Approximately 84% diploid cells are in S-phase obtained from the analysis. c) At 8 hours after release from aphidicolin, approximately 54% cells showed up in G2/M (4n) channels. d) 20 hours later, most of the cells are accumulate in G1 phase (2n) of the cycle. e) 3-D overlay images of the FCM analysis of stationary culture (lane 1), synchronized S-phase cells (lane 2) and G2/M phase cells (lane 3). f) Only the gated regions were taken for statistical analysis. The gated region excluded the doublets or debris.

DNA damage induced protein kinase Chk1 is degraded in LLC-PK1 Cells by TGHQ

Since quinol thioethers induce DNA damage and growth arrest in LLC-PK1 cells (Jeong *et al.,* 1997), we determined the effects of TGHQ on the status of cell cycle checkpoint proteins. The Chk1 protein kinase is a central component of a conserved checkpoint pathway

activated by DNA damage or replication stress (Walworth and Bernards, 1996). Since ROS induce DNA damage, we examined the Chk1 protein in synchronized cells. Western blot analysis of Chk1 protein levels in TGHQ treated synchronized cell culture revealed that decreases in Chk1 expression occurred as early as 1 hour after TGHQ treatment, followed by a constant gradual decrease as time progressed (Fig. 5, upper panel). There were essentially no changes in total Cdc2 protein levels in these samples (Fig. 5, lower panel). Taken together, the results suggest that the premature degradation of Chk1 did not alter the expression of downstream regulators, such as Cdc2 in synchronized LLC-PK1 cell lines, and that the effects on Chk1 degradation are selective.

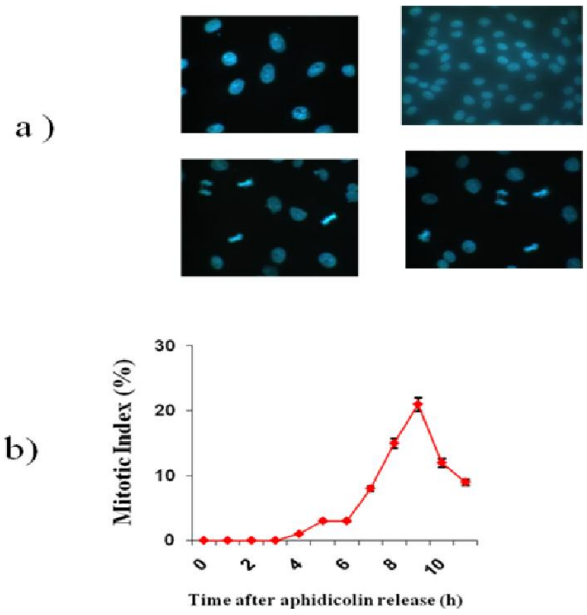

Fig. 2. Mitotic index profile of synchronized LLC-PK1 cells. a) Cells were synchronized with the thymadine-aphidicolin regime and release into new media. Mitotic index were monitored each hour interval under the floroscence microscope after DAPI staining. Bottom two images represent the time point 8 and 9 respectively where metaphase-anaphase cells were observed. Upper two panels: not a single cell showed metaphase-anaphase after release from aphidicolin. Approximately, 300 cells were counted for mitotic index. b) Percentage of mitotic index from aphidicolin release cells.

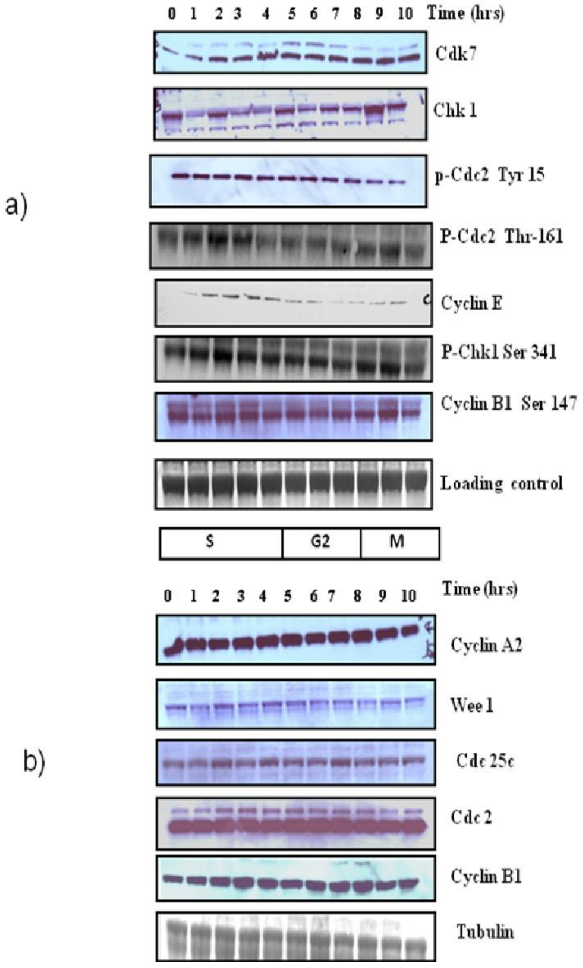

Fig. 3. Western analysis of cell cycle regulatory protein in synchronized LLC-PK1 cell lines: Cells were synchronize in S-phase with the thymadine-aphidicolin regime and release into media containing 10% FBS. Cells were collected every hour interval, lysed the cells with SDS sample buffer. Equal amount of protein were resolved on SDS-PAGE, transfer to PVDF membrane and immunoblotted with different antibody at different dilution rate. Panel a) cell cycle regulatory proteins which express differentially throughout the cell cycle and panel b) cell cycle proteins those expressed constitutively throughout the cell cycle. Note that, each of the blots was followed by loading control, for simplicity, only one loading control is shown in the figure. Also, anti-tubulin western was performed as a loading control.

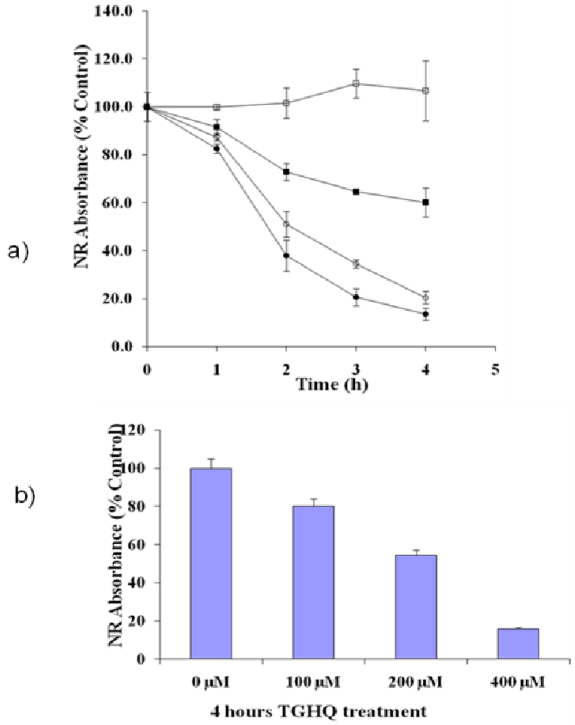

Fig. 4. TGHQ induces oncotic cell death in synchronized LLC-PK1 cells. a) LLC-PK1 cells were synchronized by 48 h serum starvation and released by the addition of FBS. LLC-PK1 cells were synchronized by 48 h serum starvation and released by the addition of FBS. After 8 h they were either untreated or treated with 100 (■), 200 (o), or 400 mM (●) TGHQ. Cell viability was assessed with the Neutral Red assay. b) TGHQ induces oncotic cell death in LLC-PK1 cells. Stationary cells were released into new media containing 10% FBS and waited until cells get 70% confluent (32 hours) and synchronized with thymadine-aphidicolin system. TGHQ treatment with different concentration for 4 hours shows a similar cell death as describe above. Cell viability was assessed with the Neutral Red assay.

Discussion

Genomic stability is monitored at four checkpoints during the cell cycle, whose job it is to ensure that damaged or unreplicated DNA is repaired before they go for next cell cycle progression. So, the checkpoint is the most important traffic mechanism where the progression/inhibition of cell division will monitor by "checkpoint proteins". However, the gap of our understanding and the knowledge of specific pathways how cells drive the division or cell death remain to be elucidated. One of the most valuable regulatory checkpoint protein is Chk1, whose activity dramatically fluctuates and function is to monitor the checkpoint during the cell cycle. Chk1 is an effector kinase whose upstream component is a kinase and downstream components are several different protein. Over the past decade there has been a large increase evidence of Chk1 protein functions in the DNA replication checkpoint (Boddy *et al.,* 1998: Francesconi *et al.,* 1997; Lindsay *et al.,* 1998; Walworth *et al.,* 1996; Zeng *et al.,* 1998).

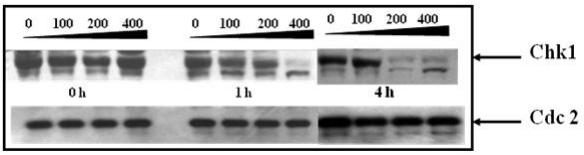

Fig. 5. Chk1 differentially expressed in synchronized LLC-PK1 cell lines. a) Synchronized LLC-PK1 cells were release from aphidicolin and subsequently cells were collected at different phases of the cycle followed by western analysis with Chk1 antibody. b) Thymadine-aphidicolin synchronized cells were treated with TGHQ at different concentrations and cells were collected for hourly interval for immuno-blot analysis on Chk1. The degradation of Chk1 protein levels were observed as early as one hour with the higher concentration of TGHQ (400 µM). At four hours after treatment with the concentration of 200 µM, Chk1 protein levels were greatly reduced. All the blots were stripped for Cdc2 western as a loading control.

Upstream of Chk1 kinases ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3 related) play a central role in the cellular response to replication stress and DNA damage such as double-strand breaks (Zhou *et al.,* 2000; Abraham, 2001). In a broader context, this checkpoint pathway promotes DNA repair, recombination, or cell death and transduce

genomic stress signals to halt cell cycle progression (Zhou and Elledge, 2000; Cortez 2001). In response to stimuli, ATR involves in phosphorylation of specific protein substrates such as p53, Brca1, Chk1 and Rad 17 (Zhou and Elledge, 2003). After the DNA damage, activating phosphorylation of Chk1 leads to the phosphorylation of specific protein substrates and direct towards the repair mechanisms or cell cycle halt. Recent studies in human cells (A549 cells) have shown the degradation of Chk1 by the ubiquitin-proteasome pathway upon genotoxic stress (Zhang *et al.,* 2005). Another available finding suggests that, a phosphatase (PPM1D) interact with Chk1and eventually de-phosphorylated Chk1 Ser-345 leading towards inactive kinase (Lu *et al.,* 2005). Another group has recently addressed the cell death issue by depleting Chk1 leading to premature activation of Cdc2-cyclin B complexes (Niida *et al.,* 2005).

We initially investigate the cell cycle regulatory protein and other biochemical and cell biological analysis in synchronized LLC-PK1 cells in order to understand the cell cycle events. This study is the first to our knowledge to test the effect of TGHQ during the cell cycle in particular S-phase of the cycle. In this study we have found that the TGHQ induces depletion/degradation of Chk1, which is sufficient to, drives the cells towards necrotic cell death. While a number of studies have shown that Chk1 inhibition enhances cytotoxicity (Morgan *et al.,* 2006) in different cell lines, our findings have shown the importance of Chk1 towards cell death in a synchronized environment. Perhaps, synchronized cells are more sensitive to study the cell death mechanism compare to a study on mixed populations, as many of them are non-cycling cells.

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