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Quinone Thioether-Mediated DNA Damage, Growth Arrest, and *gadd153* Expression in Renal Proximal Tubular Epithelial Cells

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SUMMARY

Although the conjugation of quinones with glutathione is associated with the process of detoxication, the reaction frequently facilitates quinone-induced toxicity. Thiol conjugates of quinones retain the ability to redox cycle and generate reactive oxygen species (ROS), contributing to the biological (re)activity of a variety of polyphenolic compounds. 2-Bromo-bis(glutathion-*S*-yl)hydroquinone (2-Br-bis(GSyl)HQ) and 2-bromo-6-(glutathion-*S*-yl)hydroquinone [2-Br-6-(GSyl)HQ] are potent nephrotoxicants in rats, inducing rapid karyolysis *in vivo* and DNA single-strand breaks in cultured renal proximal tubular epithelial cells (LLC-PK$_1$). We investigated the cellular and molecular responses initiated after exposure of LLC-PK$_1$ cells to 2-Br-bis(GSyl)HQ and 2-Br-6-(GSyl)HQ. Both quinone thioethers cause the concentration-dependent formation of DNA single-strand breaks, rapidly (2–10 min) inhibit DNA synthesis, and increase the expression of *gadd153*, a gene responsive to growth arrest and DNA damage. The addition of catalase to LLC-PK$_1$ cells exposed to 2-Br-6-(GSyl)HQ or 2-Br-bis(GSyl)HQ effectively prevents *gadd153* induction, which is consistent with findings that the *gadd153* gene is subject to redox modulation and that ROS play an important role in quinone thioether-mediated cytotoxicity. Deferoxamine pretreatment also diminishes *gadd153* induction, suggesting that in renal proximal tubular epithelial cells, decreased expression of *gadd153* is not dependent on the removal of hydrogen peroxide *per se* but rather on preventing the generation of hydroxyl radical. Chelation of intracellular calcium with ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid-acetoxymethyl ester also reduces *gadd153* induction by 2-Br-6-(GSyl)HQ and 2-Br-bis(GSyl)HQ, suggesting a role for calcium in the signaling process. Thus, 2-Br-6-(GSyl)HQ and 2-Br-bis(GSyl)HQ activate a genomic stress response via a signaling pathway that may include ROS, Ca^{2+}, and DNA damage.

ROS and oxidative stress have been implicated in an increasing number of human diseases and in the *modus operandi* of a variety of cytotoxic chemicals. A role for ROS as cellular messengers (1) and for redox signaling in the control of cell growth and cell death has recently been articulated (2). For example, hydrogen peroxide is required for platelet-derived growth factor signal transduction in rat vascular smooth muscle cells (3). The importance of ROS in regulating cell function is underscored by the finding that the proto-oncogene *bcl-2* prevents apoptotic and necrotic neuronal cell death by decreasing the generation of ROS (4), implicating ROS as mediators of apoptosis (5). Oxidants induce the transcription of a particular subclass of stress response genes, including the heat shock (6), hemeoxygenase (7), GSH-*S*-transferase (8), and DNA damage-inducible (9) genes. Stress genes induced by oxidants and a variety of physical and chemical stimuli (10–13) play a vital role in the response to toxic cell injury (14–19). Genes activated by stress may function to protect cells from damage, such as *hsp70*, or actively participate in a cells demise, such as c-*fos* and c-*myc*. However, the genomic signals that determine the balance between protection and cell death are poorly understood.

ROS produce a spectrum of structural changes in DNA, including base pair mutations, deletions, rearrangements, and insertions. DNA damage is produced by a variety of environmental chemicals (20) and from endogenous processes involving activated oxygen species and other reactive agents (21). Quinones are an ubiquitous class of environmen-

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ABBREVIATIONS: ROS, reactive oxygen species; 2-Br-bis(GSyl)HQ, 2-bromo-bis(glutathion-*S*-yl)hydroquinone; 2-Br-6-(GSyl)HQ, 2-bromo-6-(glutathion-*S*-yl)hydroquinone; C/EBP, CCAAT/enhancer-binding protein; DCVC, (1,2-dichlorovinyl)-L-cysteine; DMEM, Dulbecco's modified Eagle's medium; EBSS, Earl's balanced salt solution; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid-acetoxymethyl ester; GSH, glutathione; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; LB, Luria-Bertani.

tal chemicals and are formed as reactive metabolites of innumerable drugs, environmental pollutants, and food additives (22). The biological reactivity of quinones is attributed to their ability to redox cycle and generate ROS and to alkylate tissue macromolecules (22, 23). Although the conjugation of quinones with GSH has customarily been associated with the process of detoxication, the reaction frequently facilitates quinone-induced toxicity (24–26). In particular, thiol conjugates of quinones retain the ability to redox cycle and generate ROS (27, 28). ROS can cause DNA strand breaks. For example, menadione causes single- and double-strand breaks in DNA in human MCF-7 breast carcinoma cells (29, 30) and DNA fragmentation in isolated hepatocytes (31). Furthermore, 2-Br-bis(GSyl)HQ causes DNA single-strand breaks in renal tubular epithelial cells (32) and karyolysis 2–4 hr after administration to rats (33). The initial response to DNA-damaging agents is a transient growth arrest, which permits the cell to assess the location and extent of DNA damage. Cell cycle arrest is characteristic of both the bacterial and mammalian cell response to DNA damage (34, 35).

A battery of genes are expressed in Chinese hamster ovary cells after growth arrest or DNA damage (9). One of these genes, *gadd153*, encodes a 19-kDa protein related to the C/EBP family of transcription factors. Although the GADD153 protein forms stable heterodimers with other C/EBP proteins, it lacks a functional DNA-binding domain (36), and the GADD153 protein-C/EBP heterodimer is unable to interact with known C/EBP-binding sites. Thus, GADD153 may function as a negative regulator of C/EBP transcription factors and as a negative regulator of the activity of other transcription factors, playing an important role in cell cycle or cell growth control. *gadd153* is induced by a broad range of DNA-damaging agents and a variety of growth arrest signals, including methylmethanesulfonate (9, 37), hydrogen peroxide (37), UV radiation (37), DNA cross-linking agents, DNA intercalators, topoisomerase inhibitors, cadmium chloride (38), the calcium ionophore A23187 (39), the cyclopentenone prostaglandin A_2 (40), and a number of different growth arrest conditions, including serum starvation and medium depletion (9, 37, 40, 41). *gadd153* is also induced in response to the sulfhydryl agent dithiothreitol and nephrotoxic cysteine conjugates in LLC-PK_1 cells (19). In addition, GSH depletion by a specific inhibitor of γ-glutamyl-cysteine synthetase, L-buthionine-(*SR*)-sulfoximine, also induces *gadd153* (42), suggesting that oxidative stress may induce *gadd153* expression. In a model of quinone thioether-mediated toxicity, we have shown that 2-Br-bis(GSyl)HQ causes DNA single-strand breaks in renal tubular epithelial cells and that cytotoxicity is dependent on the formation of hydrogen peroxide (32). Because the nature of the cellular stress response to quinone thioethers has not been previously investigated, the current study was initiated to obtain insights into the mechanism of quinone thioether-mediated cytotoxicity and the cellular response to this toxicity. Using a renal tubular epithelial cell line (LLC-PK_1) as a model, we report that 2-Br-bis(GSyl)HQ and 2-Br-6-(GSyl)HQ cause the concentration-dependent formation of DNA single-strand breaks, rapidly inhibit DNA synthesis, and increase the expression of *gadd153*.

Materials and Methods

Chemicals and probes. 2-Br-6-(GSyl)HQ (43) and 2-Br-bis-(GSyl)HQ (44) were synthesized according to established methodology. [^{3}H]Thymidine (specific activity, 58 Ci/mmol) was purchased from ICN Biochemicals (Cleveland, OH). EGTA-AM was purchased from Molecular Bioprobes (Eugene, OR). Catalase, deferoxamine, actinomycin D, and cycloheximide were obtained from Sigma Chemical (St. Louis, MO). Unless specified otherwise, other chemicals were obtained from Sigma. Plasmid pBluescript containing a 600-bp *gadd153* cDNA was kindly provided by Dr. Niki J. Holbrook (National Institute on Aging, Gerontology Research Center, Bethesda, MD). Dr. Sue Fischer (M. D. Anderson Cancer Center, Smithville, TX) provided 7*S* ribosomal RNA cDNA.

Cell culture and treatment conditions. LLC-PK_1 cells were obtained from American Type Culture Collection (Rockville, MD) at passage 181. LLC-PK_1 cells are a renal tubule epithelial cell line derived from the New Hampshire minipig. Cells were maintained in DMEM containing 10% fetal bovine serum and high glucose without pyruvate (GIBCO BRL, Baltimore, MD) at 37° in a humidified incubator containing 5% CO_2. Cells were seeded at a density of 2×10^6 cells/100-mm dish and were used after overnight culture. Cultures were washed twice with EBSS (0.8 mM $MgSO_4 \cdot 7H_2O$, 116.3 mM NaCl, 10.0 mM $NaHCO_3$, 1.0 mM $NaH_2PO_4 \cdot H_2O$, 5.4 mM KCl, 5.5 mM D-glucose, 20 mM HEPES, and 1.8 mM $CaCl_2 \cdot 2H_2O$) and treated with 2-Br-6-(GSyl)HQ or 2-Br-bis(GSyl)HQ in EBSS or DMEM without added serum. With the exception of EGTA-AM (50 μM), which was added to cultures 30 min before drug treatment, all inhibitors were incubated with LLC-PK_1 cells for 1 hr before drug exposure.

DNA fragmentation. Analysis of DNA strand breaks was performed as described previously (45, 46). LLC-PK_1 cells were seeded into 25-cm^2 culture flasks in 10 ml of media that contained 0.1 μCi of [^{3}H]thymidine and grown for 48 hr before exposure to the quinone thioethers. L1210 cells (2.5×10^5 cells/ml) were incubated with [^{14}C]thymidine (0.02 μCi/ml) for 24 hr. These labeled cells were then irradiated with 5 Gy of ^{137}Cs γ rays in the cold and used as an internal standard. LLC-PK_1 cells were exposed to 2-Br-6-(GSyl)HQ or 2-Br-bis(GSyl)HQ in EBSS and harvested at various times by gentle scraping into 3 ml of cold medium. L1210 cells were mixed with quinone thioether-treated cells immediately before alkaline elution. Results of the 12-hr elution are plotted on a double-log scale in which the fraction of [^{14}C]DNA retained on filter corrects for variations in flow rate, thereby providing a corrected time scale.

cDNA probe preparation and Northern blot analysis. The plasmid containing the *gadd153* cDNA insert (20 ng) was mixed with 50 μl of a competent cell line (DH5α; GIBCO BRL). After transformation, cells (200 μl) were spread onto LB agar plates containing ampicillin (100 μg/ml) and incubated overnight at 37°. One single colony from the LB agar plate was selected and cultured in 3 ml of LB (ampicillin; 100 μg/ml) at 37° overnight with shaking (225 rpm). Plasmid DNAs were isolated in a miniscale preparation using the QIA prep-spin kit (Qiagen, Studio City, CA) and digested with appropriate restriction enzymes to verify the presence of the *gadd153* cDNA. Large-scale plasmid preparations were performed using the Qiagen plasmid maxikit. Plasmids were digested by restriction enzymes (*Xho*/*Pst*I) at 37° for 1 hr or overnight. Digested DNAs were separated by electrophoresis using 0.8% agarose gels. The correct size inserts (0.6 kb) were extracted from agarose gels and purified using the QIAEX kit (Qiagen).

Total RNA was isolated using RNAzol (Cinna/Biotech Laboratory, Friendswood, TX) and following the procedure of Chomczynski and Sacchi (47). For Northern blot analysis, RNA was electrophoresed in 1.0% agarose gel containing formaldehyde and transferred to Zeta Probe membranes (BioRad, Richmond, CA). cDNA probes were labeled with [^{32}P]dCTP (ICN) by the random primer method (48) using a kit (Boehringer-Mannheim Biochemica, Mannheim, Germany). All blots were hybridized in 0.25 M Na_2HPO_4, pH 7.2, and 7% SDS overnight at 65°. Blots were washed twice with 25 mM Na_2HPO_4, pH

7.2, and 5% SDS at 65° for 30 min and twice with 25 mM Na_2HPO_4, pH 7.2, and 1% SDS at 65° for 30 min. All blots were rehybridized with the cDNA probe for 7*S* rRNA after stripping the original probe with 0.1× standard saline citrate (15 mM NaCl and 1.5 mM citrate, final concentration) and 0.5% SDS at 95° for 0.5–1 hr to correct for potential variations in RNA loading. Autoradiography was performed using Kodak XAR-5 film (Sigma) exposed for 1 hr to 5 days, depending on the probe, at −70° with intensifying screens. Northern blots were quantified by densitometery (Imaging Research, St. Catherine's, Ontario, Canada). All densitometric values were normalized to 7*S* rRNA obtained from the same blot. Levels of 7*S* rRNA remained constant during all experimental manipulations.

Determination of DNA synthesis. Cells were seeded at a density of 2 × 10^5 cells/well onto six-well plates and were used after overnight culture. After exposure to 2-Br-bis(GSyl)HQ or 2-Br-6-(GSyl)HQ for varying lengths of time, cultures were washed twice with DMEM containing HEPES without fetal bovine serum. Each well was pulsed with 5 μCi/ml [^{3}H]thymidine (specific activity, 58 Ci/mmol). After incubation with [^{3}H]thymidine for 4 hr, cells were washed three times with phosphate-buffered saline, pH 7.4, and 5% trichloroacetic acid was added. Cells were harvested by gentle scraping and centrifuged for 5 min at 14,000 rpm. The supernatant was removed, and the pellet was washed by the addition of 5% trichloroacetic acid to remove unincorporated [^{3}H]thymidine. The same procedure was repeated three times. A final washing with absolute ethanol was performed to remove residual trichloroacetic acid, and the pellet was dried. Dried pellets were dissolved in 1 ml of 0.1 N NaOH and used for the determination of protein concentration and radioactivity by liquid scintillation spectroscopy. Protein concentrations were determined by the BioRad protein method using bovine serum as the standard.

Results

Cellular and molecular response to 2-Br-bis(GSyl)HQ and 2-Br-6-(GSyl)HQ-mediated DNA single-strand breaks. Both 2-Br-bis(GSyl)HQ and 2-Br-6-(GSyl)HQ induced the concentration-dependent formation of single-strand breaks in DNA in cultured renal tubular epithelial cells (Fig. 1). Higher concentrations of 2-Br-bis(GSyl)HQ (200 μM) were required to produce single-strand breaks equivalent to the level seen with 50 μM 2-Br-6-(GSyl)HQ, consistent with the finding that 2-Br-6-(GSyl)HQ is more cytotoxic than 2-Br-bis(GSyl)HQ in LLC-PK_1 cells (32). We subsequently investigated the efficiency of the cellular response to DNA damage by examining the expression of *gadd153* mRNA (one of several genes that are activated as a consequence of growth arrest and DNA damage) by Northern blot analysis. To avoid interaction of the substrate with serum proteins, we initially used EBSS as a vehicle for the pulsed treatment of cell cultures. However, deprivation of the cells of serum and nutrients was sufficient to induce *gadd153* expression (data not shown). Supplementation of EBSS with glucose failed to overcome *gadd153* induction, but DMEM, with or without added serum, prevented the increase in *gadd153* (data not shown). Therefore, in subsequent experiments, cultures were pulse-treated with substrate dissolved in DMEM (with HEPES, pH 7.4). When LLC-PK_1 cells were treated with either 2-Br-bis(GSyl)HQ (Fig. 2) or 2-Br-6-(GSyl)HQ (Fig. 3) a concentration- and time-dependent increase in *gadd153* mRNA was observed. Because the levels of 7*S* rRNA did not change under these experimental conditions, we conclude that the *gadd153* gene was specifically activated by the conjugates. *gadd153* expression was detected 30 min after 50 μM 2-Br-6-(GSyl)HQ (Fig. 3B) or 200 μM 2-Br-bis(GSyl)HQ (Fig. 3C) or 60 min after 50 μM 2-Br-bis(GSyl)HQ (Fig. 2B). Thus, induction of *gadd153* was seen at concentrations of 2-Br-bis(GSyl)HQ (50 μM) lower than those required to observe DNA single-strand breaks and cytotoxicity (200 μM), indicating that the induction of *gadd153* is an early and sensitive cellular response to these conjugates. Maximum *gadd153* expression occurred at 200 μM 2-Br-6-(GSyl)HQ (Fig. 3A) and 500 μM 2-Br-bis(GSyl)HQ (Fig. 2A), which is consistent with the relative ability of the two conjugates to cause single-strand breaks (Fig. 1).

Growth arrest and/or DNA damage as the signal for *gadd153* induction? Because *gadd153* can be induced by conditions of growth arrest and DNA damage, either or both

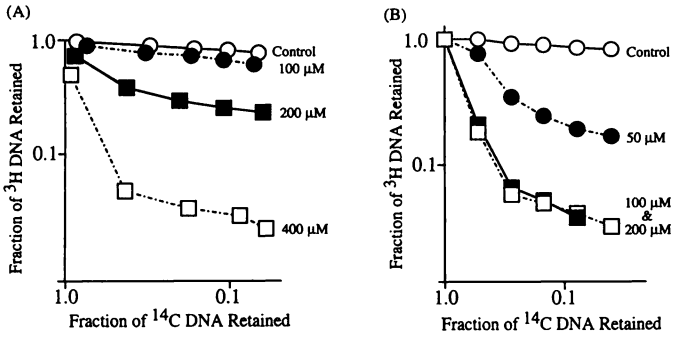

Fig. 1. Concentration-dependent formation of DNA single-strand breaks in renal tubular epithelial cells after exposure to 2-Br-bis-(GSyl)HQ (100–400 μM) (A) or 2-Br-6-(GSyl)HQ (50–200 μM) (B). Cells were exposed to varying substrate concentrations for 2 hr, and DNA single-strand breaks were determined by alkaline elution (see Materials and Methods). Experiments were repeated a minimum of three times and produced similar results.

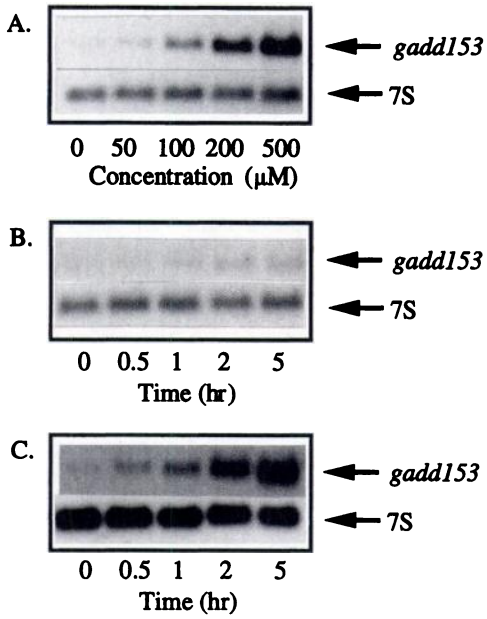

Fig. 2. Concentration- and time-dependent effects of 2-Br-bis-(GSyl)HQ on *gadd153* mRNA expression in renal tubular epithelial cells. A LLC-PK_1 cells were exposed for 2 hr to either 50, 100, 200, or 500 μM concentrations of 2-Br-bis(GSyl)HQ (A). Alternatively, LLC-PK_1 cells were exposed to either 50 μM (B) or 200 μM 2-Br-bis(glutathion-*S*-yl)hydroquinone (C) for either 0.5, 1.0, 2.0, or 5 hr. Total RNA was extracted, separated, and examined by Northern blot analysis. The level of 7*S* rRNA was used as the internal control. Experiments were repeated a minimum of three times and produced similar results.

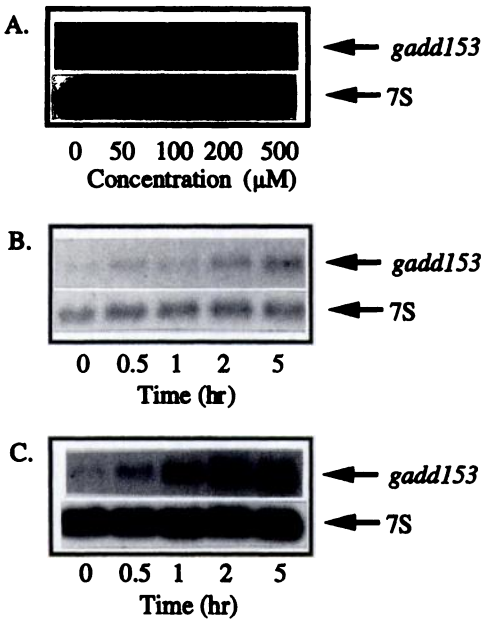

Fig. 3. Concentration- and time-dependent effects of 2-Br-6-(GSyl)HQ on *gadd153* mRNA expression in renal tubular epithelial cells. LLC-PK$_1$ cells were exposed for 2 hr to either 50, 100, 200, or 500 μM concentrations of 2-Br-6-(GSyl)HQ (A). Alternatively, LLC-PK$_1$ cells were exposed to either 50 μM (B) or 200 μM 2-Br-6-(GSyl)HQ (C) for either 0.5, 1.0, 2.0, or 5 hr. Total RNA was extracted, separated, and examined by Northern blot analysis. The level of 7S rRNA was used as the internal control. Experiments were repeated a minimum of three times and produced similar results.

of these conditions might provide the signal for 2-Br-bis-(GSyl)HQ- and 2-Br-6-(GSyl)HQ-mediated *gadd153* expression. To examine this question in more detail, the ability of the conjugates to induce growth arrest, indicated by the incorporation of [^{3}H]thymidine into newly synthesized DNA, was determined. Remarkably, DNA synthesis was significantly inhibited within 2 min of exposure of LLC-PK$_1$ cells to 2-Br-6-(GSyl)HQ and by 10-min exposure to 2-Br-bis-(GSyl)HQ (Fig. 4). We prelabeled DNA with [^{3}H]thymidine for 4 hr before exposing the cells for 60 min to 200 μM

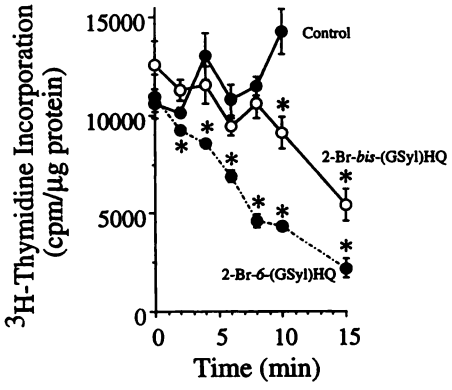

Fig. 4. 2-Br-bis(GSyl)HQ- (200 μM) and 2-Br-6-(GSyl)HQ- (200 μM) mediated inhibition of DNA synthesis in renal tubular epithelial cells. Cells were exposed to the conjugates for various periods of time and then pulsed with [^{3}H]thymidine (see Materials and Methods). Incorporation of [^{3}H]thymidine into newly synthesized DNA was determined by liquid scintillation spectroscopy. Protein concentrations were determined by the BioRad protein method using bovine serum as the standard. Data represent the mean ± standard error of four experiments. *, Significantly different from control cells at $p < 0.05$.

2-Br-bis(GSyl)HQ. Because neither the amount of radioactivity recovered in DNA (control, 216,600 ± 13,327 cpm; treated, 221,517 ± 9,248 cpm; mean ± standard error, $n = 3$) nor the absolute amount of protein recovered changed, and the specific activity (dpm/μg) over 60 min remained constant, these data argue that significant release of thymidine from either intact or fragmented DNA does not occur over the 15-min time course of the experiment, and thus the thymidine pool is not diluted in treated cells. Interestingly, although incubation of cells in DMEM in the absence of serum caused a significant reduction (~98%) in the incorporation of [^{3}H]thymidine into newly synthesized DNA, indicative of a state of growth arrest, *gadd153* was not induced under these conditions (data not shown).

The role of transcription and translation in *gadd153* expression. To determine whether the induction of *gadd153* by 2-Br-6-(GSyl)HQ and 2-Br-bis(GSyl)HQ is regulated at the transcriptional and/or post-transcriptional level, we investigated the effects of inhibiting RNA synthesis on gene expression (Fig. 5). LLC-PK$_1$ cells were preincubated for 1 hr with actinomycin D (10 μg/ml) and then exposed to 2-Br-6-(GSyl)HQ or 2-Br-bis(GSyl)HQ. Under these conditions, *gadd153* mRNA levels were completely suppressed. To examine whether the induction of *gadd153* requires protein synthesis (possibly transcription factors), LLC-PK$_1$ cells were incubated with 2-Br-6-(GSyl)HQ or 2-Br-bis(GSyl)HQ after a 1-hr pretreatment with cycloheximide (50 μg/ml). Inhibition of protein synthesis with cycloheximide had little effect on constitutive *gadd153* mRNA levels but augmented the induction of *gadd153* by 2-Br-6-(GSyl)HQ and 2-Br-bis(GSyl)HQ (Fig. 5).

The role of iron, hydrogen peroxide, and calcium in 2-Br-6-(GSyl)HQ-mediated *gadd153* expression. The cytoxicity of 2-Br-bis(GSyl)HQ and 2-Br-6-(GSyl)HQ to LLC-PK$_1$ cells involves the generation of ROS (32), most likely via

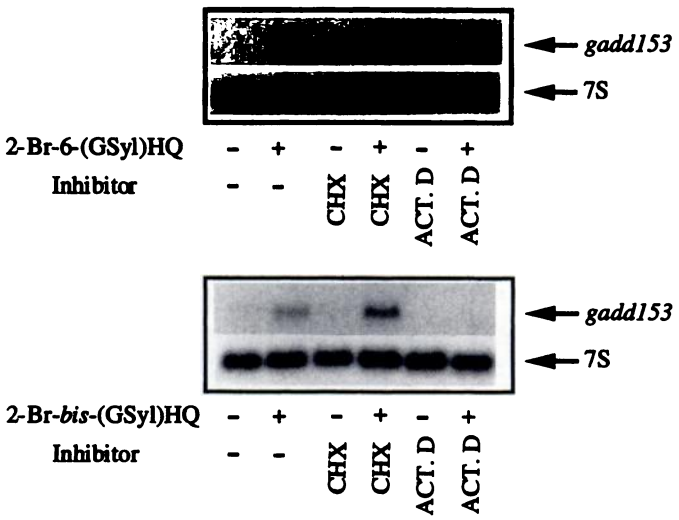

Fig. 5. Effect of cycloheximide and actinomycin D on *gadd153* mRNA expression in response to 2-Br-6-(GSyl)HQ (*top*) and 2-Br-bis(GSyl)HQ (*bottom*) renal tubular epithelial cells. LLC-PK$_1$ cells were incubated in DMEM-HEPES (control) with cycloheximide (*CHX*) (50 μg/ml) or actinomycin D (*ACT. D*) (10 μg/ml) for 1 hr before treatment for 2 hr with 200 μM 2-Br-6-(GSyl)HQ or 2-Br-bis(GSyl)HQ. Total RNA was extracted, separated, and examined by Northern blot analysis. The level of 7S rRNA was used as the internal control. Experiments were repeated a minimum of three times and produced similar results.

the iron-catalyzed Haber-Weiss reaction, in which $O_2^{\cdot-}$ undergoes dismutation to form hydrogen peroxide and reduces Fe^{3+} to Fe^{2+}. The hydrogen peroxide then reacts with Fe^{2+} to generate the highly reactive hydroxyl radical, which may be the species responsible for the DNA damage. To determine whether iron-dependent events are related to the induction of *gadd153*, we investigated the effects of deferoxamine on *gadd153* expression. Consistent with the finding that both catalase and deferoxamine protect LLC-PK_1 cells from 2-Br-bis(GSyl)HQ- and 2-Br-6-(GSyl)HQ-mediated cytotoxicity (31), pretreatment with catalase or deferoxamine decreases 2-Br-6-(GSyl)HQ-mediated *gadd153* induction (Fig. 6). Treatment of LLC-PK_1 cells with EGTA-AM to chelate intracellular calcium also suppresses the induction of *gadd153* by 2-Br-6-(GSyl)HQ (Fig. 7). Experiments on the effects of catalase and deferoxamine on the induction of *gadd153* by 2-Br-bis(GSyl)HQ produces similar results (data not shown).

Discussion

Cells respond to stress in a highly coordinated and well-regulated fashion. However, when the stress response fails, or becomes overwhelmed, the cell dies. The cellular and molecular responses that determine whether a cell survives or dies after a chemical-induced stress are unclear. 2-Br-bis-(GSyl)HQ and 2-Br-6-(GSyl)HQ cause the concentration-dependent formation of single-strand breaks in DNA, as determined by alkaline elution (Fig. 1). Cells respond to DNA damage by initiating the transcription of a battery of genes involved in the DNA repair process (9). The initial response includes the arrest of cell cycle progression, presumably to facilitate DNA repair before DNA replication (G_1 arrest) and cell division (G_2 arrest). We subsequently investigated the efficiency of the cellular response to DNA damage in renal epithelial cells by examining the expression of *gadd153*, a gene activated as a consequence of growth arrest and DNA damage (9). Both 2-Br-bis(GSyl)HQ and 2-Br-6-(GSyl)HQ cause a concentration- and time-dependent increase in *gadd153* mRNA (Figs. 2 and 3). Furthermore, induction of *gadd153* is rapid, detectable within 30 min of exposure of cells to either 2-Br-bis(GSyl)HQ or 2-Br-6-(GSyl)HQ, and persists for ≥5 hr. DNA synthesis is also rapidly inhibited in LLC-PK_1 cells exposed to 2-Br-bis(GSyl)HQ and 2-Br-6-(GSyl)HQ (Fig. 4), although the immediacy of this response may be due to a direct inhibitory effect of the quinone thioethers on DNA synthesis. Quinones inhibit DNA polymerase (49–51), thymidylate synthase (52), and ribonucleotide reductase (53).

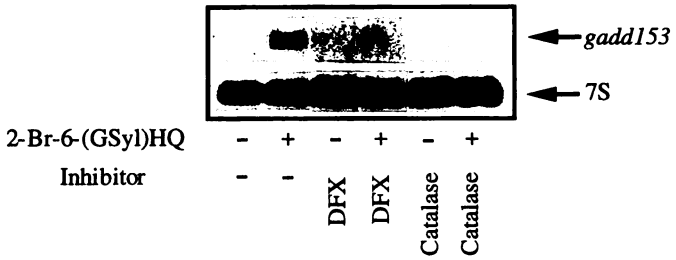

Fig. 6. Effect of catalase and deferoxamine on *gadd153* mRNA expression in response to 2-Br-6-(GSyl)HQ in renal tubular epithelial cells. LLC-PK_1 cells were coincubated for 2 hr in either DMEM-HEPES (control) with catalase (10 units/ml) and 200 μM conjugate or pretreated with deferoxamine (*DFX*) (10 mM) for 1 hr before treatment for 2 hr with 200 μM 2-Br-6-(GSyl)HQ. Total RNA was extracted, separated, and examined by Northern blot analysis. The level of 7*S* rRNA was used as the internal control. Experiments were repeated a minimum of three times and produced similar results.

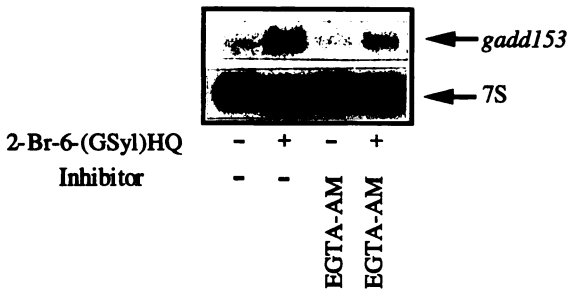

Fig. 7. Effect of EGTA-AM on *gadd153* mRNA expression in response to 2-Br-6-(GSyl)HQ in renal tubular epithelial cells. LLC-PK_1 cells were incubated in DMEM-HEPES (control) with EGTA-AM (50 μM) for 0.5 hr before treatment for 2 hr with 200 μM 2-Br-6-(GSyl)HQ. Total RNA was extracted, separated, and examined by Northern blot analysis. The level of 7*S* rRNA was used as the internal control. Experiments were repeated a minimum of three times and produced similar results.

The cytotoxicity of quinone thioethers in LLC-PK_1 cells involves the generation of ROS because chelation of cellular iron with deferoxamine and the addition of catalase afford significant cytoprotection (32). ROS exhibit both positive (enhancer) and negative (inhibitor) effects on gene transcription and may act as second messengers in a number of intracellular signal transduction pathways (54–56); they inhibit protein phosphatases (57) and activate protein kinases (58). The *gadd153* gene may also be subject to redox modulation (19) and is induced in cells exposed to hydrogen peroxide, which may be important in the *modus operandi* of the quinone thioethers (32). Consistent with this view, the addition of catalase to LLC-PK_1 cells exposed to 2-Br-6-(GSyl)HQ prevents *gadd153* induction (Fig. 6). Although hydrogen peroxide inhibits DNA synthesis in mouse osteoblastic MC3T3 cells (59) and is a known inducer of *gadd153* (38, 60), deferoxamine also significantly attenuates *gadd153* induction in LLC-PK_1 cells (Fig. 6). Thus, in LLC-PK_1 cells, decreased expression of *gadd153* is not dependent on the removal of hydrogen peroxide *per se* but rather on prevention of the generation of hydroxyl radical. Quinone thioether-mediated *gadd153* induction therefore seems to be a consequence of reactive oxygen generation, either directly, or of DNA damage subsequent to reactive oxygen generation. Oxidative stress and DNA damage may regulate the transcription of *gadd153* through different pathways (61). However, the rapid induction of *gadd153* and the lack of a requirement for protein synthesis (Fig. 5) indicate that the factors required to engage *gadd153* transcription in response to DNA damage are readily accessible to the transcriptional machinery.

mRNA levels can be regulated at many points (62), and we examined transcriptional and/or post-transcriptional regulation in 2-Br-6-(GSyl)HQ- and 2-Br-bis(GSyl)HQ-mediated induction of *gadd153*. Actinomycin D completely blocks the increase in *gadd153* mRNA (Fig. 5), indicating that active transcription is necessary for the induction of *gadd153*. However, cycloheximide augments the induction of *gadd153* caused by 2-Br-6-(GSyl)HQ and 2-Br-bis(GSyl)HQ (Fig. 5), perhaps because constitutively expressed proteins decrease the stability of *gadd153* mRNA or cycloheximide prevents the interaction of a repressor protein with the promoter region of the gene. In support of the former view, *gadd153*

mRNA has a short half-life in log-phase cells (9), indicative of rapid degradation. Interestingly, the induction of *gadd153* by DCVC in LLC-PK$_1$ cells was decreased by treatment of cells with cycloheximide (19). The reasons for such differences in the regulation of chemical-induced *gadd153* induction are unclear but probably relate to differences in the mechanism of action of the chemicals. Thus, DCVC requires bioactivation by cysteine conjugate β-lyase and targets mitochondria (63). In contrast, β-lyase does not seem to play a major role in the bioactivation of 2-Br-bis(GSyl)HQ (64), which redox cycles and generates ROS that target the nucleus and DNA. Our studies therefore indicate that chemicals with diverse mechanisms of action may activate signal transduction pathways that channel into a common genomic stress response. Indeed, Gately *et al.* (65) recently reported that cisplatin, which forms both intrastrand and interstrand cross-links in DNA, and taxol, which inhibits mitosis by disrupting microtubule formation, activate different signal pathways leading to the expression of GADD153.

Treatment of HeLa cells with A23187, a calcium ionophore, increases the transcription of *gadd153*, implicating a role for calcium in the regulation of this gene (39). Furthermore, the induction of *gadd153* by methyl methanesulfonate is inhibited by buffering of intracellular and extracellular calcium with the acetoxymethyl ester of bis(amino-phenoxy)ethane-*N*,*N*′-tetraacetic acid and EGTA, supporting a role for calcium in the induction of *gadd153* after DNA damage (39). Treatment of LLC-PK$_1$ cells with EGTA-AM to chelate intracellular calcium suppresses the induction of *gadd153* by 2-Br-6-(GSyl)HQ (Fig. 7) and 2-Br-bis(GSyl)HQ (data not shown), suggesting that increased concentrations of intracellular free calcium play a role in signaling gene induction after quinone thioether-induced stress in these cells. Furthermore, it is unlikely that the response to EGTA-AM in this system is due to nonspecific effects on RNA polymerase II-catalyzed mRNA synthesis. Thus, in LLC-PK$_1$ cells, EGTA-AM does not affect quinone thioether-mediated increases in *hsp70* expression,[2] DCVC (500 μM)-mediated *gadd153* (19) and *hsp70* (66) expression, or HSF-1 DNA binding activity (67). Buffering of DCVC-induced increases in cytosolic free calcium also had no effect on c-*fos* mRNA, although it did partially block c-*myc* mRNA induction (68). In addition, activators of cAMP-dependent protein kinase or protein kinase C do not induce *gadd153* in LLC-PK$_1$ cells,[3] indicating a degree of specificity in terms of the signaling pathway. Further studies are required to determine the relative role of transcriptional and post-transcriptional processes in 2-Br-6-(GSyl)HQ- and 2-Br-bis(GSyl)HQ-mediated increases in *gadd153* mRNA.

In summary, 2-Br-6-(GSyl)HQ and 2-Br-bis(GSyl)HQ activate a genomic stress response, which includes the induction of *gadd153*, via a signaling pathway that may include ROS, Ca^{2+}, and DNA damage. *gadd153* is induced by conditions of growth arrest and DNA damage, and both of these conditions occur in LLC-PK$_1$ cells exposed to 2-Br-bis(GSyl)HQ and 2-Br-6-(GSyl)HQ. However, because growth arrest induced by serum deprivation is insufficient to induce *gadd153* and because DNA synthesis is inhibited within minutes of exposure of the cells to the quinone thioethers, it seems likely that the DNA damage, either directly or indirectly, provides the initial signal that results in changes in *gadd153* gene expression.

[2] J. K. Jeong, S. S. Lau, and T. J. Monks, unpublished observations.
[3] T. Weber, S. S. Lau, and T. J. Monks, unpublished observations.

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