Reduction of \textit{trans}-4,5-Dihydroxy-1,2-dithiane by Cellular Oxidoreductases Activates \textit{gadd153/chop} and \textit{grp78} Transcription and Induces Cellular Tolerance in Kidney Epithelial Cells*

(Received for publication, April 7, 1997)

Margarettann M. Halleck‡, Hong Liu, Jason North, and James L. Stevens§

From the W. Alton Jones Cell Science Center, Lake Placid, New York 12946

\textit{trans}-4,5-Dihydroxy-1,2-dithiane, the intramolecular disulfide form of dithiothreitol (DTTox) transcriptionally activates the stress-responsive genes \textit{gadd153/chop} and \textit{grp78}. Herein, we used a renal epithelial cell line, LLC-PK1, to investigate the mechanism(s) whereby DTTox activates a molecular stress response. DTTox activated both \textit{grp78} and \textit{gadd153} transcriptionally, but \textit{gadd153} mRNA stability also increased suggesting that both transcriptional and posttranscriptional mechanisms are involved. DTTox did not activate \textit{hsp70} transcription indicating that a heat shock response was not induced. Structure-activity studies showed that DTTox analogues lacking the intramolecular disulfide were inactive. Furthermore, the ring-open intermolecular disulfide form of DTTox, 2-hydroxyethyl disulfide, was only a weak inducer of \textit{grp78} and \textit{gadd153} but was a strong inducer of \textit{hsp70} mRNA and a potent oxidant that lowered the NADPH/NADP$^+$ ratio and depleted reduced glutathione (GSH). DTTox had little effect on the overall GSH and NADPH levels; thus cells were not undergoing oxidative stress; however, the NADPH/NADP$^+$ ratio decreased slightly indicating that reducing equivalents were consumed. LLC-PK1 cells reduced DTTox to DTT, and the kinetics as well as the concentration dependence for reduction correlated with induction of both \textit{grp78} and \textit{gadd153} mRNA. Prior treatment with DTTox rendered cells tolerant to the potent nephrotoxic S-(1,1,2,2-tetrafluoroethyl)-L-cysteine. Bacitracin, an inhibitor of plasma membrane oxidoreductases, blocked DTTox reduction and gene activation as well as DTTox-induced tolerance. Thus, activation of stress genes and induction of cellular tolerance by DTTox is mediated by a novel mechanism involving cellular oxidoreductases.

Mounting evidence indicates that perturbation of the cellular thiol-disulfide redox potential activates gene expression. For example, depletion of cellular GSH causes protein thiol oxidation and activation of \textit{hsp70} transcription (1, 2). Likewise, perturbing cellular Ca$^{2+}$ with ionophores or toxicants that cause oxidative stress activates \textit{c-fos}, \textit{c-myc}, and the growth arrest and DNA damage-inducible gene, \textit{gadd153} (3–7). In some cases, oxidants may directly regulate transcription factors, such as oxyR, by protein thiol oxidation (8). On the other hand, oxidation of a cysteinyl residue in Fos or Jun blocks DNA binding (9). Other transcription factors including NFkB, p53, Egr-1, Usf, and Srf, to name a few, are also regulated directly or indirectly by oxidation (10–14).

Cellular sensors capable of transducing redox signals to the nucleus include protein phosphatases, protein kinases, hormone-responsive calcium pools, and cellular glutathione pools, all of which respond to oxidant exposure (15–18). In particular, oxidation or reduction of cellular protein and nonprotein thiols may be an important link between toxicant exposure and altered gene expression (1, 2). Although oxidative stress has been studied more extensively, reductive stress also activates stress-response genes. For example, organic thiols increase expression of the glucose-regulated protein \textit{grp78} and \textit{gadd153} genes (19–22). \textit{gadd153}, also called \textit{chop} for C/EBP homologous protein, is a member of the C/EBP gene family of transcription factors (23, 24) and is particularly interesting because, unlike \textit{grp78} (20), it is activated by either oxidative or reductive stress (22, 25, 26).

The LLC-PK1 cell line, a porcine renal epithelial cell line, retains many characteristics of the proximal tubule epithelium (27, 28) and has been used to investigate mechanisms of oxidative and reductive toxicity as well as stress-gene activation in the kidney (1, 6, 21, 22, 29–33). Dithiothreitol (DTT),\textsuperscript{1} a powerful disulfide reducing agent, is toxic to LLC-PK1 cells and activates both \textit{grp78} and \textit{gadd153} (20, 22). In preliminary structure-activity studies we found that the nontoxic intramolecular disulfide form of DTT, \textit{trans}-4,5-dihydroxy-1,2-dithiane (DTTox), also induced \textit{gadd153} and \textit{grp78} mRNA. Since both genes are activated by thiol-induced reductive stress (20, 22), it was surprising that DTTox, an intramolecular disulfide with a very high reduction potential, was a good inducer. Herein, we show that reduction of DTTox by cellular oxidoreductases mediates transcriptional activation of \textit{gadd153} and \textit{grp78} as well as induction of cellular tolerance to chemical toxicants. In a related study, we show that induction of cellular tolerance by DTTox depends on its ability to activate an endoplasmic reticulum stress response and to increase expression of chaperone proteins located in the endoplasmic reticulum (34). Thus, DTTox is a novel activator of stress-gene expression and will be useful in probing the role of the thiol/disulfide redox status in the cellular and molecular responses to stress.

EXPERIMENTAL PROCEDURES

Cell Culture and Experimental Treatment—LLC-PK1 cells (27) were obtained from American Tissue Type Culture (Rockville, MD) and were used between passages 205 and 215. Cells were grown to confluence in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies, Inc. or Upstate Biotechnology, Lake

\textsuperscript{1}The abbreviations used are: DTT, dithiothreitol; DTTox, oxidized dithiothreitol; TFE, S-(1,1,2,2-tetrafluoroethyl)-L-cysteine.
Placid, NY) in an atmosphere of 5% CO₂, 95% air in a humidified 37 °C incubator. Cells (1.25 × 10⁶) were plated in 100-mm dishes 5 days prior to experimental treatment. The medium was routinely changed 3 days after seeding. Confluent cultures of LLC-PK₁ cells were rinsed with phosphate-buffered saline and treated with DTT or TFE prepared fresh in Earle’s balanced salt solution (1.8 mM CaCl₂, 5.4 mM KCl, 1.7 mM MgSO₄, 26.2 mM NaHCO₃, 1.0 mM NaH₂PO₄, 5.6 mM glucose, and 25 mM HEPES). Actinomycin D was dissolved in absolute ethanol at a concentration of 5.0 mg/ml, and 1 µl of the ethanol stock solution was added to 1 ml of Earle’s balanced salt solution to achieve a final working concentration of 5 µg/ml.

**Northern Analysis—** Total RNA was isolated from cells using RNAzol supplied by Cinna/Biotex (Houston, TX). Poly(A)⁺ RNA was isolated using oligo(dT)-cellulose, a standard technique. Northern analysis was performed on blots on which either total RNA (40 µg) or poly(A)⁺ RNA (15 µg) had been size separated by electrophoresis in 1.4% agarose denaturing gels. After prehybridization, blots were hybridized (21) overnight with cDNA probes for gadd153 (35) or grp78 (36), a gift of Dr. Amy Lee, and labeled with [α-³²P]dCTP by random priming (37) using a kit (Boehringer Mannheim). Blots were allowed to decay before reprobing with a 200-bp pair cDNA insert for mouse β-actin cDNA excised from pMACTE4, an SK-based plasmid (a gift of Dr. D. Eisinger). Bands were visualized by autoradiography using Du Pont Cronex film and an intensifying screen and were quantitated using a BioImage Densitometer (Bio-Rad Laboratories, CA) and a BioImage 1520 Intensity Scanner. The run-on data collection served as internal controls, and pBluescript SK (Stratagene, La Jolla, CA) was used to assess nonspecific binding. Run-on transcription was quantitated using a BioImage Densitometer. The run-on data presented in the results are a summary of three separate experiments but were not normalized to β-actin since DTTox and cycloheximide both perturbed β-actin transcription somewhat.

**Biochemical Assays—** Protein and nonprotein thiols were determined spectrophotometrically using Ellman’s reagent (5,5'-dithiobis(2-nitrobenzoic acid)) (40) as described previously (41). Intracellular GSH was measured by the high performance liquid chromatography method of Reed et al. (42). Nonprotein thiol values measured with Ellman’s reagent were very similar to GSH values measured directly by high performance liquid chromatography analysis. Pyridine nucleotides were determined using the enzymatic cycling assay of Passonneau and Lowry (43). Protein was determined using the Bio-Rad protein assay determined using the enzymatic cycling assay of Passonneau and Lowry (43). Protein was determined using the Bio-Rad protein assay developed by Bradford (21). The intermolecular disulfide was a necessary functional group because of its ability to deplete cellular glutathione (see below), a known stimulus for hsp70 transcription (1, 2). Thus, the intramolecular disulfide was a necessary functional group for activation of grp78 and gadd153 by DTTox.

**Cytotoxicity Assays—** Cytotoxicity was assessed by measuring release of lactate dehydrogenase into the medium as described (44). Unlabeled or ³⁵S-labeled S-[1,2,12,2-tetrafluorohylthio]-l-cysteine (TfEC) were synthesized as before (45). When covalent binding of reactive TFEC molecules to proteins and the thiol oxidation state, we first characterized the induction of gadd153 and grp78 mRNA by DTTox and then compared the data to induction by DTT (Fig. 1). DTTox induced both gadd153 and grp78 mRNA expression in a time- and concentration-dependent manner. At 10 mM, DTT and DTTox were equally effective inducers of gadd153 and grp78 mRNA. However, DTT was effective at lower concentrations and at earlier times compared with DTTox. We also tested analogues of DTTox (Fig. 2). trans-1,2-Cyclohexanediol lacks the intramolecular disulfide bond and was ineffective. 2-Hydroxyethyl disulfide, the ring-open intermolecular disulfide analogue of DTTox, proved to be extremely toxic but induced grp78 and gadd153 only modestly. However, 2-hydroxyethyl disulfide was a potent inducer of hsp70 because of its ability to deplete cellular glutathione (see below), a known stimulus for hsp70 transcription (1, 2). Thus, the intramolecular disulfide was a necessary functional group for activation of grp78 and gadd153 by DTTox.

**Transcriptional and Posttranscriptional Mechanisms of grp78 and gadd153 mRNA Induction—** To determine if DTTox treatment transcriptionally activated gadd153 and grp78 in LLC-PK₁ cells, nuclear run-on analyses were performed (Fig. 3). Since hsp70 is more responsive to oxidative than reductive stress (20) and is not activated by DTT (22, 29), we also analyzed hsp70 transcription. Basal grp78 transcription was much higher than gadd153; however, treatment with 10 mM DTTox transcriptionally activated grp78 and gadd153 16- to 9- and 9- to 4-fold, respectively (n = 3), but failed to activate hsp70 transcription. DTT increased transcription of gadd153 and grp78 markedly, 13 ± 6- and 18 ± 14-fold, respectively (n = 3). There was also a significant and reproducible decrease in β-actin transcription in cells treated with DTTox, but the mechanism underlying this effect is not clear at present.

**Transcriptional activation can be classified as a primary or secondary response based on cycloheximide sensitivity (46).** Cycloheximide treatment caused only a modest inhibition of DTT-mediated induction of gadd153 and grp78 mRNA (Fig. 4). In contrast, cycloheximide completely blocked induction of gadd153 and grp78 (94% inhibition) by DTTox. Similar results were obtained by nuclear run-on analysis; cycloheximide blocked DTT-mediated transcriptional activation of gadd153 and grp78 by 58 and 21%, respectively, whereas transcriptional activation induced by DTTox was completely blocked by cycloheximide for both genes (Fig. 3). These results indicate that transcriptional activation of grp78 and gadd153 by DTTox is dependent on protein synthesis.
Although DTTox induction of gadd153 and grp78 mRNA appeared to be due in part to transcriptional activation, run-on experiments do not exclude effects on posttranscriptional processes (47). Therefore, we used actinomycin D, an inhibitor of transcription, to examine the contribution of mRNA stability to the induction of both mRNAs by DTT (Fig. 5) or DTTox (data not shown). When we first induced gadd153 mRNA by DTT and then added actinomycin D to prevent further transcription, there was no observable decrease in grp78 mRNA in the presence of actinomycin D alone or actinomycin plus DTTox (data not shown), indicating that grp78 mRNA was extremely stable ($t_{1/2} \sim 18$ h). However, gadd153 mRNA was very unstable ($t_{1/2} = 2$ h) in the presence of actinomycin D (Fig. 5); DTTox dramatically increased gadd153 mRNA stability ($t_{1/2} > 18$). Thus, post-transcriptional processes contribute to the increase in gadd153 but not grp78 mRNA after DTTox treatment.

*Fig. 4. Effect of cycloheximide on induction of gadd153 and grp78 mRNA by DTTox.* LLC-PK1 cells were treated with 10 mM DTTox or DTT in the presence and absence of cycloheximide (CXM, 50 µg/ml) and poly(A)$^+$ RNA prepared for Northern analysis to assess gadd153 (A) and grp78 (B) relative mRNA expression. The data are the average ± the range of two separate experiments ($n = 2$).

Induction of grp78 and gadd153 by DTTox Correlates with Reduction—Exogenous disulfides are reduced by GSH- and/or NAD(P)H-dependent cellular reductase systems (48). As a consequence, disulfides can induce oxidative stress by depleting cellular GSH and NAD(P)H. Although the redox potential of DTTox does not favor reduction (49), LLC-PK1 cells reduced DTTox in a time- and concentration-dependent fashion (Figs. 6 and 7). The concentration dependence for DTTox reduction correlated with induction of grp78 and gadd153 mRNA (Fig. 7). However, DTTox treatment did not alter the levels of GSH or protein thiols (Table I). The only significant change was in the nicotinamide adenine dinucleotide phosphate pool where a modest, yet significant, decrease in the NADPH/NADP$^+$ ratio indicated that reducing equivalents were consumed by DTTox reduction (Table I). Although 2-hydroxyethyl disulfide was a poor inducer of grp78 and gadd153, it was reduced to a much greater extent than DTTox (666 ± 155 versus 174 ± 66 nmol/h for 10 mM 2-hydroxyethyl disulfide and DTTox, respectively). Accordingly, the NADPH/NADP$^+$ ratio dropped dramatically after 2-hydroxyethyl disulfide treatment as did GSH (Table I), consistent with a severe oxidative stress. Severe GSH depletion activates the heat shock response (1, 2) and 2-hydroxyethyl disulfide was a strong inducer of hsp70 transcription (Fig. 2).

Bacitracin inhibits disulfide reduction by oxidoreductases at the plasma membrane (50) and inhibited DTTox reduction by LLC-PK1 cells almost completely (Fig. 8). Bacitracin also blocked 2-hydroxyethyl disulfide reduction (data not shown). To determine if reduction of DTTox was linked to gadd153 and

FIG. 3. Nuclear run-on analysis of grp78 and gadd153 transcription. RNA newly transcribed in isolated nuclei in the presence of $\alpha$-[32P]UTP was extracted and hybridized with cDNA inserts immobilized on nylon membranes. Nuclei were prepared from cells treated for 2.5 h with Earle’s balanced salt solution alone (Control) or with 10 mM DTT or DTTox. The + designation indicates that cells were treated in the presence of cycloheximide (CXM, 50 µg/ml) to determine the dependence of transcription on protein synthesis. The data are from a single experiment representative of three ($n = 3$). Means with standard deviations for induction are reported in the text.
grp78 expression, LLC-PK₁ cells were treated with DTTox in the presence and absence of bacitracin, and Northern blot analyses were performed. As shown in Fig. 9 bacitracin attenuated the induction of gadd153 mRNA by DTTox treatment. The data are from a single experiment representative of two separate experiments (n = 2). The ti₅₀ values for grp78 and gadd153 mRNA are reported in the text.

DTTox Treatment Causes Cytoprotection—Although DTTox did not activate a heat shock response, induction of other stress-response genes, including grp78, has been shown to protect cells against subsequent toxicant treatment (94, 51–54). Therefore we determined if DTTox treatment rendered cells tolerant to subsequent toxic insults. TFEC is a potent nephrotoxicant that kills renal epithelial cells after activation to a reactive acylating species (55). When cells were treated with TFEC immediately after DTTox treatment, there was no protection (Fig. 10). However, when cells were allowed to recover for 6 h, tolerance developed despite the fact that the cells were still able to activate [³⁵S]TFEC to reactive species which covalently bound to cellular macromolecules indicating that there was no change in the uptake or metabolism of [³⁵S]TFEC (Fig. 10, inset). Tolerance was maintained up to 24 h after DTTox removal. Simultaneous treatment with DTTox and bacitracin blocked induction of tolerance to TFEC even though bacitracin was removed during the recovery period (Fig. 11). Therefore, DTTox induces cellular tolerance in a manner consistent with activation of gene expression and by a signaling pathway linked to cellular oxidoreductase activity. DTTox itself at a concentration of 10 mM did not produce any increase in lactate dehydrogenase release compared with LLC-PK₁ cells treated with EBSS alone under any conditions tested. In addition, DTTox treatment did not cause collapse of the domes (data not shown), an indication of active transport, under the conditions used here (22). DTTox itself activates grp78 and gadd153 in LLC-PK₁ cells (22). To determine if the ability of DTTox to activate gene expression and induce tolerance depended merely on the DTTox produced by reduction of the oxidized form, we carried out two experiments. First, we added DTTox to the cells and then washed the cells and applied fresh DTTox every 30 min throughout the induction period (repeated addition protocol). In this way, DTTox was never allowed to accumulate over a
Finally, we showed that adding DTT at the culture medium for the full 3-h induction period. DTT treatment of five separate experiments (\(n = 5\)) mM DTTox or 10 mM 2-hydryoxyethyl disulfide (2-HED) in a 10-ml volume. After 5 h the total nicotinamide adenine dinucleotide phosphate pool (Total) and the individual pools of NADPH and NADP\(^+\), in pmol/mg cellular protein, and the ratio were calculated for each experiment. Cellular GSH was determined by HPLC, and protein thiols (PSSH) were determined using Ellman’s reagent (see “Experimental Procedures”) and are expressed as nmol of DTNB reactive substance per 100-mm dish. Significant differences were determined by analysis of variance. The data are the mean \(\pm S.D\). of data from 6 (\(n = 6\)) and 3 (\(n = 3\)) separate experiments for the controls and treatment groups, respectively.

Table I

|               | Control | DTTox | 2-HED |
|---------------|---------|-------|-------|
| Total (pmol/mg) | 388 \(\pm 46\) | 414 \(\pm 36\) | 605 \(\pm 127^a\) |
| NADPH | 373 \(\pm 49\) | 366 \(\pm 49\) | 367 \(\pm 83\) |
| NADP\(^+\) | 23 \(\pm 5\) | 37 \(\pm 6\) | 174 \(\pm 4^a\) |
| NADPH/NADP\(^+\) | 17 \(\pm 4\) | 10 \(\pm 1^a\) | 2 \(\pm 1^a\) |
| GSH (nmol) | 30 \(\pm 7\) | 29 \(\pm 3\) | 11 \(\pm 2^a\) |
| PSSH (nmol) | 256 \(\pm 84\) | 262 \(\pm 79\) | 209 \(\pm 30\) |

\(^a\) Means that were different from other treatment or control means.

Concentration dependence for bacitracin inhibition of DTTox reduction by LLC-PK1 cells. LLC-PK1 cells were incubated for 5 h with various concentrations of bacitracin in the presence or absence of 10 mM DTTox. After 5 h, an aliquot of the medium was taken to determine the amount of nonprotein thiols (NPSH) present. Bacitracin itself reacted with Ellman’s reagent weakly accounting for the slight rise in the base-line media nonprotein thiol concentration in the absence of DTTox. The entire concentration range was performed in only one experiment; however, the data at 10 mM bacitracin are representative of five separate experiments (\(n = 5\)).

FIG. 8. Concentration dependence for bacitracin inhibition of DTTox reduction by LLC-PK1 cells. LLC-PK1 cells were incubated for 5 h with various concentrations of bacitracin in the presence or absence of 10 mM DTTox. After 5 h, an aliquot of the medium was taken to determine the amount of nonprotein thiols (NPSH) present. Bacitracin itself reacted with Ellman’s reagent weakly accounting for the slight rise in the base-line media nonprotein thiol concentration in the absence of DTTox. The entire concentration range was performed in only one experiment; however, the data at 10 mM bacitracin are representative of five separate experiments (\(n = 5\)). Using LDH release as an index of tolerance, the repeated addition protocol for DTTox treatment produced a reduction in LDH release, from 63% in unconditioned cells to 29% in DTTox-pretreated cells (average of two experiments) compared with 31% LDH release when DTTox was left in for the full 3-h pretreatment period. Second, we added DTT at concentrations of 10, 25, 50, and 100 µM to the culture medium for the full 3-h induction period. DTT treatment under these conditions did not induce tolerance to TFEC (data not shown). Finally, we showed that adding DTT at the above concentrations in the presence of 10 mM DTTox did not increase or decrease the development of tolerance (data not shown). Thus, it would appear that the induction of tolerance is not due simply to the reduction product of DTTox, i.e. DTT.

DISCUSSION

Cells maintain a reducing thiol-disulfide redox potential in the cytoplasm and an oxidizing potential in the endoplasmic reticulum (56). Perturbation of the thiol-disulfide redox potential in either compartment activates stress-response genes (1, 2, 19, 56). The molecular response to DTTox resembles the reductive stress response induced by DTT and was differenti-
since it was less effective in preventing cannot be attributed solely to inhibition of protein synthesis factors suggest this is not the case. First, the effect of bacitracin possible that cycloheximide prevents grp78

**Figure 11. Bacitracin inhibits DTTox induction of cellular tolerance.** Cells were treated with DTTox as in Fig. 10 in the presence (DTTox/BAC) or absence of 10 mM bacitracin. A control, cells were also pretreated with bacitracin alone (BAC). After the 3-h induction period, both bacitracin and DTTox were removed, and the cells were allowed to recover for 6 h and then challenged with TFEC (0.5 mM for 3 h). Lactate dehydrogenase release (% LDH Release) was determined as described in the legend to Fig. 10. Means that were not significantly different (p < 0.05) are denoted by a common letter designation. Means with different letter designation are significantly different (p < 0.05) as determined by analysis of variance.

blocked induction of gadd153 and grp78. However, several factors suggest this is not the case. First, the effect of bacitracin cannot be attributed solely to inhibition of protein synthesis since it was less effective in preventing grp78 induction compared with gadd153, yet both genes were equally sensitive to cycloheximide. Second, induction of grp78 and gadd153 by DTTox occurred even in a balanced salt solution which is already devoid of amino acids. Third, addition of bacitracin prevented reduction of DTTox and cellular tolerance even after the two compounds were removed and the cells returned to complete medium for 6–12 h. Thus, when taken together, the dose response, time course, structure-activity, and bacitracin inhibition data indicate that reduction of DTTox by cellular oxidoreductases is a prerequisite for gene activation.

The efficacy of DTTox in a particular cell type will depend on the presence of appropriate oxidoreductases since nonenzymatic reduction of DTTox is not favored (49). However, the nature of the oxidoreductase that mediates gene activation in LLC-PK1 cells is not clear. Either protein disulfide isomerase or thioredoxin-like enzymes might reduce DTTox using GSH and/or nicotinamide adenine dinucleotide pools as reducing equivalents (48, 57, 58). DTTox was not reduced in cell lysates supplemented with NADPH alone (data not shown). Thus, an oxidoreductase system that utilizes the reducing potential of both GSH and NADPH may reduce DTTox, but further work will be necessary to determine the nature of this oxidoreductase activity. Since bacitracin inhibits exofacial redox activity at the plasma membrane (50), some DTTox reduction may take place outside the cell. Such an enzyme system may also serve as a redox-sensitive sensor between the extracellular and intracellular environments.

Either the mild stress on the NADPH/NADP⁺ ratio caused by DTTox reduction or the presence of DTTox, the reduction product, could play a role in DTTox activation of gene expression. DTTox is a potent reducing agent that inhibits intramolecular disulfide formation in the endoplasmic reticulum causing unfolded polypeptides to accumulate (59, 60). Agents that block protein processing in the endoplasmic reticulum activate both grp78 and gadd153 expression (61–63). On that note, it is possible that cycloheximide prevents grp78 and gadd153 activation because protein synthesis is inhibited, thus preventing nascent polypeptide chains from accumulating in the endoplasmic reticulum. However, the ability of DTTox to induce grp78 and gadd153 is not simply due to the reduction to DT since DT at the concentrations expected from reduction of DTTox were not effective in inducing tolerance. It seems that the ability of DTTox to induce grp78 and gadd153 depends on a complex combination of the enzymatic reduction coupled to use of cellular reducing equivalent in addition to the production of DT.

DTT and other classic endoplasmic reticulum stress inducers not only disrupt protein processing but also inhibit protein synthesis generally and can be quite toxic (64, 65). Unlike DTT, DTTox does not inhibit protein synthesis, did not induce dome collapse in LLC-PK1 cells, as does DTT (22), nor did it cause lactate dehydrogenase release from LLC-PK1 cells, yet paradoxically, it activated grp78 expression. It seems likely that the answer to this apparent paradox lies in the fact that cells compensate for the mild stress caused by DTTox treatment by increasing expression of chaperones, like grp78, thus maintaining endoplasmic reticulum protein processing. When DTTox is removed, the cell is left with a high level of chaperones in the endoplasmic reticulum and cell injury is prevented. [35S]Met-thionine metabolic labeling studies show that GRP78 and GRP94 synthesis occur during the first 4 h after DTTox removal, a time course that correlates with the induction of tolerance (34). In addition, expression of an antisense grp78 mRNA in LLC-PK1 cells blocked the ability of DTTox to produce a tolerant phenotype to another toxicant, iodoacetamide (34). Therefore, the ability of DTTox to induce tolerance depends on induction of grp78 expression. Induction of gadd153 by endoplasmic reticulum stress has been reported by others (22, 62, 63). However, it seems unlikely that gadd153 plays a role in tolerance given that ectopic expression of gadd153 induces apoptosis in myeloblastic leukemia cells (66).

In conclusion, DTTox is a novel pharmacological tool to investigate the relationship between stress protein induction and cell death. Since DTTox is a potent radioprotective agent in mice in vivo under conditions that cause no apparent adverse effect to the animals (67), its utility may extend to whole animals. Moreover, a novel mechanism that links cellular oxidoreductases with the signaling pathways which activate transcription of two stress-responsive genes, grp78 and gadd153, has been elucidated. Given that redox regulation of gene expression is an important physiological mediator of genomic stress responses, our data suggest that cellular oxidoreductase activity may be an important determinant of cellular responsiveness. When taken in context with our observations that induction of tolerance by DTTox depends on grp78 induction (34), the data suggest that DTTox may be a very useful tool to study the physiological role of endoplasmic reticulum stress in cellular tolerance in vivo and in vitro.

Acknowledgments—We thank Dr. Amy Lee for providing the grp78 cDNA probe, for helpful discussions, and for sharing unpublished data. Critical reading of the manuscript by Dr. Nikki Holbrook was greatly appreciated.

REFERENCES
1. Liu, H., Lightfoot, D. L., and Stevens, J. L. (1996) J. Biol. Chem. 271, 4805–4812
2. Freeman, M. L., Borrelli, M. J., Syed, K., Senisterra, G., Stafford, D. M., and Lepock, J. R. (1995) J. Cell. Physiol. 16, 356–366
3. Maki, A., Berezovsky, I. K., Fargnoli, J., Holbrook, N. J., and Trump, B. F. (1992) PASER J. 6, 919–924
4. Bartlett, J. D., Luethy, J. D., Carlson, S. G., Sollot, S. J., and Holbrook, N. J. (1992) J. Biol. Chem. 267, 20465–20470
5. Luethy, J. D., and Holbrook, N. J. (1992) Cancer Res. 52, 5–10
6. Yu, K., Chen, Q., Liu, H., Zhan, Y., and Stevens, J. L. (1994) J. Cell. Physiol. 151, 303–311
7. Roy, B., and Lee, A. S. (1995) Mol. Cell. Biol. 15, 2263–2274
8. Storz, G., Tartaglia, L. A., and Ames, B. N. (1990) Science 248, 189–194
9. Ahate, C., Patel, L., Rauscher, F. J., and Curran, T. (1990) Science 249,
Activation of Stress Genes by DTTox

1157–1161
10. Huang, R. P., and Adamson, E. D. (1993) DNA Cell Biol. 12, 265–273
11. Hainaut, P., and Milner, J. (1993) Cancer Res. 53, 4469–4473
12. Pognoone, P., Kato, H., and Roeder, R. G. (1992) J. Biol. Chem. 267, 25450–25457
13. Schmidt, K. N., Amstad, P., Ceratti, P., and Baeuerle, P. A. (1995) J. Biol. Chem. 270, 13–22
14. Datta, R., Taneja, N., Wadington, V. P., Quersey, S. A., Weichselbaum, R., and Kufe, D. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2419–2422
15. Bauskin, A. R., Alkalay, I., and Ben-Neriah, Y. (1991) Cell 66, 885–896
16. Guy, G. R., Cairns, J., Ng, S. B., and Tan, Y. H. (1993) Cancer Res. 53, 6574–6579
17. Malter, J. S., and Hong, Y. (1991) J. Biol. Chem. 266, 3167–3171
18. Nakamura, K., Hori, T., Sato, N., Stieg, K., Kawakami, T., and Yodoi, J. (1993) Oncogene 8, 3133–3139
19. Kim, Y. K., and Lee, A. S. (1987) Mol. Cell. Biol. 7, 2974–2976
20. Whelan, S. A., and Hightower, L. E. (1986) J. Cell. Physiol. 125, 251–258
21. Chen, Q., Yu, K., Holbrook, N. J., and Stevens, J. L. (1992) J. Biol. Chem. 267, 8207–8212
22. Halleck, M. M., Holbrook, N. J., Skinner, J., Liu, H., and Stevens, J. L. (1997) Cell Stress and Chaperones 2, 31–40
23. Ron, D., and Habener, J. F. (1992) Genes Dev. 6, 439–453
24. Sylvester, S. L., ap Rhy, C. M. J., Leuthy-Martindale, J. D., and Holbrook, N. J. (1994) J. Biol. Chem. 269, 20119–20125
25. Luethy, J. D., Fargnoli, J., Park, S. J., Fornace, A. J., Jr., and Holbrook, N. J. (1990) J. Biol. Chem. 265, 16521–16526
26. Luethy, J. D., and Holbrook, N. J. (1994) Cancer Res. 54, 19025–19038
27. Hull, R. N., Cherry, W. R., and Weaver, G. W. (1976) In Vitro 12, 660–677
28. Stevens, J., Hayden, P., and Taylor, G. (1986) J. Biol. Chem. 261, 3325–3332
29. Chen, Q., Yu, K., and Stevens, J. L. (1992) J. Biol. Chem. 267, 24322–24327
30. Ueda, N., and Shah, S. V. (1995) J. Clin. Invest. 90, 2593–2597
31. Ueda, N., and Shah, S. V. (1992) Am. J. Physiol. 263, F214–F221
32. Matsuoka, M., and Call, K. M. (1995) Kidney Int. 48, 383–389
33. Vamvakas, S., Bitner, D., and Koster, U. (1995) Toxicon. Lett. (Amst.) 67, 161–172
34. Liu, H., Bowes, R. C., van de Water, B., Silfence, C., Nagelkerke, J. F., and Stevens, J. L. (1997) J. Biol. Chem. 272, 21751–21759
35. Fornace, A. J., Jr., Alamo, I. M., Baker, V., and Chow, P. C. (1983) J. Biol. Chem. 258, 597–603
36. Fornace, A. J., and Vogelstein, B. (1984) Annu. Rev. Biochem. 53, 137–226
37. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1993) Current Protocols in Molecular Biology, pp. 4.10.1–4.10.4, John Wiley and Sons, Inc., New York
38. Celano, P., Berchtold, C., and Casero, R. A. J. (1989) BioTechniques 7, 942–943
39. Geddes, J., and Lindsay, R. (1968) Annu. Rev. Biochem. 37, 192–205
40. Chen, Q., and Stevens, J. L. (1992) Arch. Biochem. Biophys. 291, 442–450
41. Reed, D. J., Baarman, J. R., Beatty, P. W., Brodie, A. E., Ellis, W. W., and Potter, D. W. (1980) Annu. Rev. Biochem. 56, 55–62
42. Passonneau, J. V., and Lawry, O. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed) pp. 2059–2075, Academic Press, New York
43. Chen, Q., Jones, T. W., Brown, P. C., and Stevens, J. L. (1990) J. Biol. Chem. 265, 21603–21611
44. Haydon, P. J., and Stevens, J. L. (1990) Mol. Pharmacol. 37, 468–476
45. Herschman, H. (1991) Annu. Rev. Biochem. 60, 281–319
46. Jackman, J., Alamo, I., and Fornace, A. J., Jr. (1994) Cancer Res. 54, 5656–5662
47. Reed, D. J. (1990) Chem. Res. Toxicol. 3, 495–502
48. Rothwarf, D. M., and Scheraga, H. A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7944–7948
49. Mandoz, R., Rytzer, H. J.-P., Ghani, F., Wu, M., and Peak, D. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4112–4116
50. Li, L.-J., Liu, X., Ferrario, A., Rucker, N., Liu, E. S., Wong, S., Gomer, C. J., and Lee, A. S. (1992) J. Cell. Physiol. 153, 575–582
51. Sugawara, S., Takeda, K., Lee, A., and Dennert, G. (1993) Cancer Res. 53, 6001–6005
52. Little, E., and Lee, A. S. (1995) J. Biol. Chem. 270, 9526–9534
53. Gomer, C. J., Ferrario, A., Rucker, N., Wong, S., and Lee, A. S. (1991) Cancer Res. 51, 6574–6579
54. Hayden, P. J., Ichinura, T., McCann, D. J., Pohl, L. R., and Stevens, J. L. (1991) J. Biol. Chem. 266, 18415–18418
55. Huang, C., Sinskey, A. J., and Lodish, H. F. (1992) Science 257, 1496–1502
56. Bardwell, J. C. A., and Beckwith, J. (1993) Cell 74, 769–771
57. Fasol, C. R., Brostrom, M. A., Malara, E. M., and Brostrom, C. O. (1992) FEBS Lett. 313, 53–58