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Endoplasmic Reticulum Stress Proteins Block Oxidant-induced Ca^{2+} Increases and Cell Death*

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Oxidants are important human toxicants. Increased intracellular free Ca^{2+} may be critical for oxidant toxicity, but this mechanism remains controversial. Furthermore, oxidants damage the endoplasmic reticulum (ER) and release ER Ca^{2+}, but the role of the ER in oxidant toxicity and Ca^{2+} regulation during toxicity is also unclear. *tert*-Butylhydroperoxide (TBHP), a prototypical organic oxidant, causes oxidative stress and an increase in intracellular free Ca^{2+}. Therefore, we addressed the mechanism of oxidant-induced cell death and investigated the role of ER stress proteins in Ca^{2+} regulation and cytoprotection after treating renal epithelial cells with TBHP. Prior ER stress induces expression of the ER stress proteins Grp78, Grp94, and calreticulin and rendered cells resistant to cell death caused by a subsequent TBHP challenge. Expressing antisense RNA targeted to *grp78* prevents *grp78* induction sensitized cells to TBHP and disrupted their ability to develop cellular tolerance. In addition, overexpressing calreticulin, another ER chaperone and Ca^{2+}-binding protein, also protected cells against TBHP. Interestingly, neither prior ER stress nor calreticulin expression prevented lipid peroxidation, but both blocked the rise in intracellular free Ca^{2+} after TBHP treatment. Loading cells with EGTA, even after peroxidation had already occurred, also prevented TBHP-induced cell death, indicating that buffering intracellular Ca^{2+} prevents cell killing. Thus, Ca^{2+} plays an important role in TBHP-induced cell death in these cells, and the ER is an important regulator of cellular Ca^{2+} homeostasis during oxidative stress. Given the importance of oxidants in human disease, it would appear that the role of ER stress proteins in protection from oxidant damage warrants further consideration.

Because we exist in an oxygenated atmosphere, oxygen radicals and organic oxidants are arguably the most important class of exogenous and endogenous human toxicants. Accordingly, oxidant toxicity has been implicated in many disease processes including ischemia reperfusion injury, aging, cancer, and neurodegenerative diseases, to name only a few (1–4). An important role for intracellular Ca^{2+} in oxidant-induced cell death has been suggested by some, and the subject has been reviewed (5–10); yet this proposal remains controversial. On the one hand, it has been suggested that an increase in free Ca^{2+} is nothing more than a late event associated with loss of membrane integrity and does not contribute appreciably to oxidant-induced cell killing (7–9). On the other hand, it has been proposed that an early increase in intracellular free Ca^{2+} exacerbates oxidative stress, damages mitochondria, activates Ca^{2+}-dependent degradative enzymes, and disrupts the cytoskeleton, all of which play a central role in oxidant-induced cell death (5, 10, 11). Thus, the contribution of Ca^{2+} deregulation to oxidant-induced cell death remains unclear.

In addition, in cases where Ca^{2+} appears to be involved in cell killing, the contribution of intracellular *versus* extracellular Ca^{2+} is not clear (5, 10). The endoplasmic reticulum (ER)[1] is the major intracellular Ca^{2+} storage site (12, 13). The ER Ca^{2+} pool plays an important role in the folding and post-translational processing of secreted and cell surface proteins (14). ER chaperones, including Grp78/BiP (where Grp is named for *g*lucose-*r*egulated *p*rotein), Grp94, calnexin, and calreticulin, are Ca^{2+} binding proteins (15–17) and regulate ER Ca^{2+} accumulation and release (18–21). Inhibiting the ER Ca^{2+}-ATPase with thapsigargin or adding ionophores releases the ER Ca^{2+} pool, blocks ER protein processing causing partially folded proteins to accumulate, and activates transcription of ER chaperone genes, *e.g. grp78* and *grp94* (21–24). Loss of ER Ca^{2+} also activates eIF2α kinases causing a general inhibition of translation, effects that are attenuated by prior induction of ER stress proteins (25–29). Overexpression of calreticulin and Grp78, both of which are ER Ca^{2+}-binding proteins and chaperones, increases the capacity of intracellular Ca^{2+} stores (20, 21) and prevents Ca^{2+} toxicity (30, 31). Oxidants, sulfhydryl active agents, and free radicals also inhibit Ca^{2+}-ATPases and release Ca^{2+} from the ER (32–36), suggesting that ER Ca^{2+} could play a role in oxidant toxicity. Nonetheless, despite the fact that the ER is the major intracellular Ca^{2+} store, that ER stress proteins regulate the ER Ca^{2+} pool, and that increased cellular free Ca^{2+} may contribute to oxidant-induced cell killing, there is no direct evidence linking regulation of cellular Ca^{2+} by the ER to cell death after oxidant exposure.

Prior induction of ER chaperones imparts tolerance to the translational block caused by ER Ca^{2+} depletion and protects against the toxicity of Ca^{2+} ionophores and other toxic insults (22, 37, 38). Protection depends in part on expression of *grp78* and *grp94* because preventing an increase in their expression

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[1] The abbreviations used are: ER, endoplasmic reticulum; TBHP, *t*-butylhydroperoxide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; EBSS, Earle's balanced salt solution; TBARS, thiobarbituric acid-reactive substances; pkNEO, neomycin-selected cells; pkAS*grp78*, LLC-PK1 cells expressing antisense to *grp78*; pkCRT, LLC-PK1 cells overexpressing calreticulin; DTTox, *trans*-4,5-dihydroxy-1,2-dithiane; EGTA-AM, acetoxymethyl esters of EGTA; LDH, lactate dehydrogenase.

sensitizes cells to injury (30, 31, 39, 40). Recently, we demonstrated that increasing the expression of ER stress protein genes protects renal epithelial cells against a subsequent challenge with the alkylating and acylating agents, iodoacetamide, or nephrotoxic cysteine conjugates, respectively (31, 41). With iodoacetamide, protection required *grp78* induction and was linked both to inhibition of lipid peroxidation and maintenance of low intracellular free Ca^{2+} (31). Furthermore, overexpression of calreticulin, an ER Ca^{2+}-binding chaperone that increases ER calcium retention (19, 42, 43), also protected cells from iodoacetamide toxicity (31). These and related studies (21) indicate that control of intracellular Ca^{2+} by the ER may be important in preventing toxicant-induced cell death. However, the possibility that ER stress proteins blocked oxidative stress directly, thus preventing the rise in Ca^{2+}, could not be excluded in these studies.

TBHP is a prototypical organic oxidant and has been used extensively to study the role of Ca^{2+} in oxidant-induced cell death. TBHP treatment causes peroxidation of cellular lipids, oxidation of glutathione, loss of protein thiols, release of ER Ca^{2+}, a general increase in cytosolic free Ca^{2+}, a permeability transition in the mitochondrial inner membrane, and lipid peroxidation (36, 44–47). However, the role of these perturbations in TBHP-induced cell death and in particular the role of Ca^{2+} remains unclear (5, 8, 9, 44). Our previous studies on the ER stress response provided new insights into cell death induced by alkylating and acylating agents (31). Therefore, we examined the effect of ER stress on TBHP toxicity. The results indicate that prior ER stress or overexpression of calreticulin prevented TBHP-induced cell death and blocked the increase in cellular Ca^{2+}. Notably, neither manipulation blocked lipid peroxidation pointing to a central role for Ca^{2+} and regulation of cellular Ca^{2+} levels by the ER in oxidant-induced cell death.

MATERIALS AND METHODS

The acetoxymethyl esters of EGTA (EGTA-AM) was purchased from Molecular Probes (Eugene, OR). Sigma provided the TBHP. Other common chemical and cell culture reagents were obtained from commercial sources.

LLC-PK1 cells were obtained from American Type Culture Collection (Manassas, VA) at passage 195 and were used from passage 205–215. LLC-PK1 cells expressing either an antisense *grp78* construct (pkASgrp78 cells) or overexpressing calreticulin (pkCRT cells) as well as their counterparts transfected with the same pcDNA3 based plasmid containing no insert (pkNEO cells) were all selected for neomycin resistance and cloned as described (31). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (complete medium) as described (31). TBHP was added to Earle's balanced salt solution (EBSS), and cultures were treated with TBHP for 40 min in EBSS. Following treatment, cells were washed with phosphate-buffered saline and then returned to complete medium. An ER stress response was produced by treating cells with DTTox (10 mM), A23187 (7 μM), thapsigargin (0.3 μg/ml), or tunicamycin (1.5 μg/ml) as described (31).

Cell death was assessed by measuring the release of lactate dehydrogenase (LDH) into the medium (48). Formation of thiobarbituric acid-reactive substances (TBARS) was used as a measure of lipid peroxidation (49). In general, cells selected for neomycin resistance alone, *i.e.* pkNEO cells, may differ in their responsiveness to toxicant relative to wild type LLC-PK1 cells, an effect that is likely to be due to the selection process itself as noted before (50), and have slightly lower levels of lipid peroxidation after TBHP treatment (see Table II). Intracellular free Ca^{2+} measurements were carried out using Fura-2 as described (31). Briefly, cells were treated with TBHP and then loaded with Fura-2 for 1 h, and the intracellular free Ca^{2+} concentration was determined using a spectrofluorometer.

Significant differences ($p < 0.05$) were determined using a one-way analysis of variance followed by a Student-Newman-Keul's test for multiple comparisons. When analysis of variance was performed, letter designations are used in the figures and tables to indicate significant differences. Means designated with a common letter are not different, but different letter designations indicate a significant difference from other means. If more than one letter designation is shown, it indicates that the mean is not different from other means with either letter designation. As an example, means designated C and D are different from each other, but neither is significantly different from a mean designated CD.

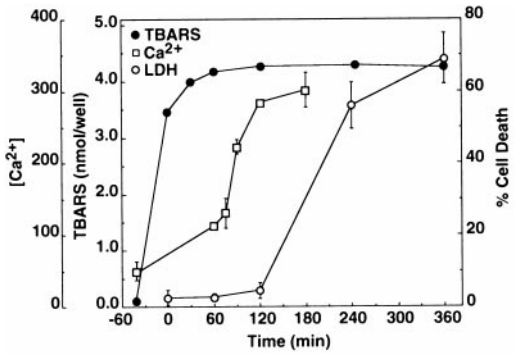

FIG. 1. **Temporal dependence of lipid peroxidation, increased cellular Ca^{2+} and cell death.** Cells were treated with TBHP (1 mM) for 40 min in EBSS and then washed and returned to DMEM containing 10% FBS. The abscissa indicates the time when TBHP treatment was initiated (−40 min) or after cells were returned to DMEM (0 min). Lipid peroxidation was determined by measuring formation of TBARS. Ca^{2+} was determined by spectrofluorometry in cells loaded for 1 h with Fura-2 immediately after TBHP treatment. The time delay due to Fura-2 loading is accounted for in the Ca^{2+} data shown; thus, the first Ca^{2+} data were collected 60 min after TBHP removal. Cell death was measured by determining the release of LDH into the medium. The cell death and TBARS data are the averages ± the range of data from two separate experiments. Standard deviations on the TBARS data were smaller than the symbols for each data point. For the Ca^{2+} determination, data points with standard deviation (−40, 75, 90,and 120 min) reflect the averages ± the range of data from two separate experiments ($n = 2$). Two additional points were collected, one in each of the two experiments (60 and 120 min), to further define the shoulders on the curve.

RESULTS

Exposing cells to oxidants, including TBHP, increases cellular free Ca^{2+} and causes lipid peroxidation (44, 48). To establish the temporal relationship between these events and cell death in LLC-PK1 cells, we determined the time courses for all three events following TBHP exposure (Fig. 1). Lipid peroxidation increased within the initial TBHP treatment period (40 min) and continued to rise after TBHP removal. An increase in cellular free Ca^{2+} followed the increase in lipid peroxidation, but both events preceded cell death, which occurred between 2 and 4 h. Adding antioxidants prevented the rise in Ca^{2+} (data not shown) as reported previously (44), suggesting that lipid peroxidation contributes to the Ca^{2+} increase. These temporal relationships are consistent with the proposal that oxidative stress preceded the rise in Ca^{2+}.

We next examined the effect of conditioning cells with prior ER stress on TBHP-induced cell death. Prior treatment with four different ER stress inducers, all of which activate expression of ER stress proteins in these cells (31), prevented cell death caused by a subsequent TBHP pulse treatment (Table I). However, with the exception of A23187, none of these agents blocked lipid peroxidation, even though they blocked the rise in cellular Ca^{2+} that was observed 2 h after TBHP treatment. The induction of *grp78* and the protective effect of prior ER stress in iodoacetamide toxicity are disrupted in pkASgrp78 cells by forced expression of an 0.5-kilobase antisense *grp78* construct (31). Therefore, we tested the TBHP sensitivity of three pkASgrp78 clones relative to pkNEO cells that carry only the neomycin resistance marker (Fig. 2). pkASgrp78 cells were more sensitive to TBHP relative to pkNEO cells. Pretreating pkASgrp78 cells with ER stress inducers was less effective in preventing TBHP toxicity compared with the pkNEO counter-

TABLE I
ER stress blocks cell death and Ca^{2+} perturbations but not lipid peroxidation following TBHP treatment

Control cells or cells conditioned with DTTox, A23187, thapsigargin, or tunicamycin to cause ER stress were treated with TBHP (1 mM) for 40 min, then washed, and returned to DMEM plus 10% FBS. Intracellular free Ca^{2+} was determined 2 h after removal of TBHP. TBARS were measured before washing the cells and then again 2 h later as an index of lipid peroxidation. The values shown are nmol/well from cells cultured in 6-well dishes and are the sum of the TBARS that accumulated during the TBHP treatment and that which accumulated during the 2-h recovery period (see Fig. 1). Cell death was determined by measuring LDH release 6 h after removal of TBHP. There was no LDH release during the 40-min TBHP treatment. The data represent the means ± S.D. of data from three independent experiments ($n = 3$). Significant differences ($p < 0.05$) among the means were determined by a one-way analysis of variance with multiple comparisons using the Student-Neuman-Keul's test and are indicated by different letters (see "Materials and Methods").

Inducer	TBHP	$[Ca^{2+}]$	TBARS	Cell Death
		nM	*nmol*	*%*
None	−	44 ± 11^a	0.2 ± 0.1^a	0.7 ± 0.9^a
None	+	281 ± 6^b	3.9 ± 0.2^b	54 ± 3^b
DTTox	+	82 ± 35^a	3.1 ± 0.2^b	6.4 ± 0.7^a
A23187	+	109 ± 22^a	1.9 ± 0.5^c	2.1 ± 3^a
Thapsigargin	+	64 ± 17^a	3.2 ± 0.1^b	10 ± 7^a
Tunicamycin	+	138 ± 68^a	3.5 ± 0.3^b	23 ± 2^c

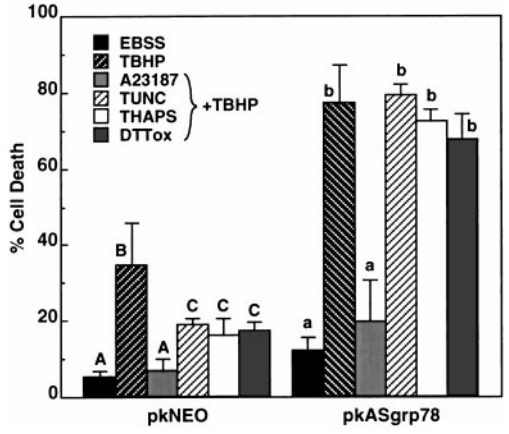

FIG. 2. **pkAS*grp78* cells are more sensitive to TBHP and do not develop tolerance.** The pkASgrp78 cells, which express a 0.5-kilobase antisense *grp78* construct, and their pkNEO counterparts were treated with EBSS or TBHP (1 mM) in EBSS for 40 min with or without prior induction of ER stress with A23187, the oxidized form of dithiothreitol (DTTox), tunicamycin (*TUNC*),or thapsigargin (*THAPS*) as described under "Materials and Methods." Cells were returned to DMEM with 10% FBS after TBHP cell death was determined 6 h later. The data are the means ± S.D. of data collected from three separate pkNEO clones and three separate pkAS*grp78* clones of LLC-PK1 cells ($n = 3$). Significant differences ($p < 0.05$) among means within the pkNEO or pkASgrp78 treatment groups were determined separately by a one-way analysis of variance with multiple comparisons using the Student-Neuman Keul's test and are indicated by different letters (uppercase for pkNEO cells, and lowercase for pkASgrp78 cells; see "Materials and Methods"). However, the pkASgrp78 cells were significantly more sensitive ($p < 0.05$) to TBHP treatment compared with the pkNEO cells when all the means from both the pkNEO and pkAS*grp78* treatment groups were analyzed together by a one-way analysis of variance (not shown).

parts. A23187 was again the exception. Thus, the ability of prior ER stress to prevent TBHP toxicity depended on expression of ER chaperones.

Previously we found that pkCRT cells, which overexpress the ER chaperone and calcium binding protein calreticulin, were also resistant to iodoacetamide damage (31). Therefore, we evaluated their response to TBHP toxicity (Table II). pkCRT cells were also less sensitive to TBHP compared with pkNEO cells, and the protection correlated with maintenance of low intracellular free Ca^{2+} 2 h after TBHP treatment, but at the same time, lipid peroxidation was unaffected. Therefore, increasing the ER chaperone content by two separate mechanisms, prior ER stress or forced expression of calreticulin, attenuated cell death and the increase in cellular free Ca^{2+} after TBHP treatment without affecting lipid peroxidation.

TABLE II
Overexpression of calreticulin blocks cell death and Ca^{2+} disturbances but not lipid peroxidation

Three separate clones of pkCRT cells and pkNEO cells were treated with TBHP (1 mM) for 40 min. Immediately following treatment, cells were washed and returned to DMEM plus 10% FBS. Intracellular free Ca^{2+} was determined 2 h after washing the cells. TBARS, as a measure of lipid peroxidation, and LDH release, as a measure of cell death, were determined as indicated under "Materials and Methods" and the legend to Table I. In general, TBARS levels are lower after TBHP treatment in cells that have been selected for neomycin resistance (see "Materials and Methods"). The data are the means ± S.D. of the values for each parameter determined for the three individual clones ($n = 3$). Significant differences were determined using a one-way analysis of variance with the Student-Neuman-Keul's test for multiple comparisons ($p < 0.05$) and are indicated by different letters (see "Materials and Methods").

Cells	Treatment	$[Ca^{2+}]$	TBARS	Cell death
		nM	*nmol*	*%*
pkCRT	none	48 ± 5^a	0.04 ± 0.01^a	4 ± 1^a
pkCRT	TBHP	95 ± 7^a	1.94 ± 0.16^b	21 ± 2^b
pkNEO	none	45 ± 9^a	0.01 ± 0.01^a	3 ± 2^a
pkNEO	TBHP	219 ± 59^b	2.24 ± 0.05^b	55 ± 7^c

If Ca^{2+} is important in TBHP toxicity, then loading cells with EGTA should prevent TBHP toxicity. However, EGTA may chelate iron, thus making it difficult to separate effects on Ca^{2+} from interference with iron-dependent Fenton generation of free radicals (51, 52). To get around this problem, we exploited the time difference between lipid peroxidation (early) and the increase in Ca^{2+} (later). Cells were treated with TBHP, then washed, and returned to medium containing the cell-permeable form of EGTA, EGTA-AM. Under these conditions, the majority of the peroxidation had already occurred; yet adding EGTA-AM still prevented cell death (Table III). There was only a modest, albeit significant, effect on lipid peroxidation, an effect that is probably associated with the decrease in cell death because in these experiments lipid peroxidation was measured at 6 h, a time when maximal cell death had already occurred. Thus the EGTA experiments further support a role for Ca^{2+} in TBHP-induced cell death.

DISCUSSION

These studies provide the first evidence that ER stress proteins protect cells from organic oxidants and provide new insights into the roles of lipid peroxidation and intracellular free Ca^{2+} and ER Ca^{2+} handling in cell killing. When taken in context with prior literature (22, 31, 37), it appears that the role of ER stress proteins in cytoprotection can now be generalized to several distinct classes of toxicants and multiple pathways of cell death. In addition, preventing Ca^{2+} disturbances during cell injury may be a general mechanism underlying the ability of the ER to protect against toxicants. This hypothesis is in general agreement with the observations that ER stress protects against Ca^{2+} ionophore toxicity and that the stressed ER is able to accumulate an increased load of Ca^{2+} (21, 30, 40, 53). Notably, ER stress also prevents apoptosis induced by iodoacetamide and thapsigargin,[2] indicating that protection by ER stress can be extended to multiple pathways of cell death.

By inducing ER stress proteins, we were also able to dissociate lipid peroxidation from TBHP toxicity by preventing the

[2] B. van de Water, H. Liu, E. Miller, and J. L. Stevens, unpublished data.

TABLE III
EGTA-AM prevents cell death caused by TBHP

Cell were treated with TBHP (1 mM) for 40 min and then washed and returned to DMEM plus 10% FBS for 6 h in the presence or absence of EGTA-AM (50 μM). TBARS were determined as noted in the legend to Table I with the exception that cells were allowed to recover for 6 h before the second TBARS determination. Cell death was measured as described in the legend to Table I. The data represent a summary of three independent experiments ($n = 3$). Significant differences were determined by a one-way analysis of variance with multiple comparisons using the Student-Neuman-Keul's test for multiple comparisons ($p < 0.05$) and are indicated by different letters (see "Materials and Methods").

Treatment	Cell death	TBARS
	%	*nmol*
EBSS	4 ± 4^a	0.4 ± 0.5^a
TBHP	47 ± 8^b	4.4 ± 0.4^b
TBHP + EGTA-AM	7 ± 2^a	3.2 ± 0.5^c

Ca^{2+} disturbance. Importantly, this was accomplished without pharmacological agents, which have ancillary effects, such as iron chelation or antioxidant properties (52). TBHP causes an early accumulation of oxidized glutathione, due to TBHP metabolism (51), and lipid peroxidation, due to radical formation, both of which precede the increase in cellular free Ca^{2+} (44, 47, 48, 54). Both TBHP metabolism to radical species and/or accumulation of oxidized glutathione can damage Ca^{2+}-ATPases in the plasma membrane and the endoplasmic reticulum, disabling the major cellular Ca^{2+} buffering systems and allowing intracellular Ca^{2+} to increase (34, 36, 54–57). Thus, oxidative stress and lipid peroxidation are upstream events that initiate the rise in Ca^{2+}. Because an ER stress response blocks the Ca^{2+} increase downstream of the oxidant stress, cell death is prevented without an effect on lipid peroxidation caused by TBHP. Although we do not know the mechanisms coupling increased Ca^{2+} to cell death, there are several clear possibilities including induction of a permeability transition in the mitochondrial inner membrane and collapse of the membrane potential (45, 46, 58–60), activation of degradative enzymes such as phospholipases and proteases (10), and further stimulation of oxidant production (61, 62), all of which occur after TBHP treatment. This mechanism could differ with much higher concentrations of TBHP or in other cell types but provides a reasonable explanation for the observations reported here and elsewhere. Importantly, ER stress fits neatly into this model because ER chaperones contribute to Ca^{2+} buffering by the ER (21), the major intracellular Ca^{2+} storage site.

This mechanism resembles the model suggested for iodoacetamide injury with an important exception; after iodoacetamide treatment, lipid peroxidation is a late event relative to the increase in Ca^{2+} and is blocked by prior ER stress (31, 48). Unlike TBHP, iodoacetamide is not an oxidant *per se* but induces lipid peroxidation after depletion of glutathione, loss of protein thiols, and increased cellular free Ca^{2+} (31, 48, 63). Consequently, ER stress blocks lipid peroxidation after iodoacetamide treatment because it prevents the rise in Ca^{2+}, that is, in conjunction with the loss of glutathione, necessary to cause lipid peroxidation. Thus, in the iodoacetamide and TBHP models different primary events initiate the Ca^{2+} increase, *i.e.* thiol depletion in the case of iodoacetamide and free radical production in the case of TBHP, accounting for the difference in the effect of ER stress on lipid peroxidation in the two models.

To our knowledge, this is the first report that calreticulin plays a role in protection against oxidant toxicity. Cells that overexpress calreticulin have increased ER Ca^{2+} buffering capacity and/or resist Ca^{2+} toxicity (18–20, 31). Thus, the protective effect of calreticulin may be due to better ER Ca^{2+} buffering, decreased Ca^{2+} release, or an indirect mechanism involving cooperation between Ca^{2+} uptake by the ER and/or extrusion across the plasma membrane. Elucidating the mechanism whereby calreticulin expression prevents Ca^{2+} disturbances during injury may provide novel insights into general mechanisms of cellular Ca^{2+} handling during stress. In this regard, it is interesting to note that calreticulin is also induced by thapsigargin treatment and protects cells from thapsigargin-induced apoptosis.[3]

The results with A23187 are also worth noting. A23187, ionomycin, and thapsigargin are all used to perturb cellular Ca^{2+} and induce ER stress. Clearly, A23187 differs from the other agents used in this study because treatment with A23187 prevented lipid peroxidation and protected pkASgrp78 cells. If Ca^{2+} ionophore toxicity induces oxidative stress, A23187 treatment may also induce cellular antioxidant defense proteins, in addition to activating ER stress response genes. Because antioxidants block TBHP-induced cell death in LLC-PK1 cells (48), activating antioxidant defense systems would be expected to protect cells.

In conclusion, these studies highlight the importance of Ca^{2+} and the ER stress response in oxidant-induced cell injury. The results could have broad implications in our understanding of how cells exploit an ER stress response to prevent cell injury. For example, a defect in this type of endogenous protective response could, in conjunction with defects in other antioxidant defense systems or an increase in oxidative stress (1–4), predispose cells and organs to injury. In this regard, it has already been shown that cells from aging rats are less able to upregulate expression of heat shock proteins relative to their younger counterparts (64). Given the importance of oxidants in human disease, it would seem that further investigation of the role of molecular stress responses in regulating oxidative injury is warranted.

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