Protection of Renal Epithelial Cells against Oxidative Injury by Endoplasmic Reticulum Stress Preconditioning Is Mediated by ERK1/2 Activation*

Cheng-Chieh Hung\‡, Takaharu Ichimura\‡ ‡‡, James L. Stevens\¶, and Joseph V. Bonventre\‡**

From \¶† Medical Services, Massachusetts General Hospital and Brigham and Women’s Hospital, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, \¶Lilly Research Laboratories, Greenfield, Indiana 46146, and **Harvard-Massachusetts Institute of Technology Divisions of Health, Sciences, and Technology, Cambridge, Massachusetts 02139

We investigated the role of the endoplasmic reticulum (ER) stress response in intracellular Ca\(^{2+}\) regulation, MAPK activation, and cytoprotection in LLC-PK\(_1\) renal epithelial cells in an attempt to identify the mechanisms of protection afforded by ER stress. Cells preconditioned with trans-4,5-dihydroxy-1,2-dithiane, tunicamycin, thapsigargin, or A23187 expressed ER stress proteins and were resistant to subsequent H\(_2\)O\(_2\)-induced cell injury. In addition, ER stress preconditioning prevented the increase in intracellular Ca\(^{2+}\) concentration that normally follows H\(_2\)O\(_2\) exposure. Stable transfection of cells with antisense RNA targeted against GRP78 (pkASgrp78 cells) prevented GRP78 induction, disabled the ER stress response, sensitized cells to H\(_2\)O\(_2\)-induced injury, and prevented the development of tolerance to H\(_2\)O\(_2\) that normally occurs with preconditioning. ERK and JNK were transiently (30–60 min) phosphorylated in response to H\(_2\)O\(_2\). ER stress-preconditioned cells had more ERK and less JNK phosphorylation than control cells in response to H\(_2\)O\(_2\) exposure. Preincubation with a specific inhibitor of JNK activation or adenoviral infection with a construct that encodes constitutively active MEK1, the upstream activator of ERKs, also protected cells against H\(_2\)O\(_2\) toxicity. In contrast, the pkASgrp78 cells had less ERK and more JNK phosphorylation upon H\(_2\)O\(_2\) exposure. Expression of constitutively active ERK also conferred protection on native as well as pkASgrp78 cells. These results indicate that GRP78 plays an important role in the ER stress response and cytoprotection. ER stress preconditioning attenuates H\(_2\)O\(_2\)-induced cell injury in LLC-PK\(_1\) cells by preventing an increase in intracellular Ca\(^{2+}\) concentration, potentiating ERK activation, and decreasing JNK activation. Thus, the ER stress response modulates the balance between ERK and JNK signaling pathways to prevent cell death after oxidative injury. Furthermore, ERK activation is an important downstream effector mechanism for cellular protection by ER stress.

When the kidney is rendered ischemic or is obstructed, it is protected against subsequent ischemia for at least 15 days from the time of the initial insult (1, 2). The mechanisms of the protection afforded by prior ischemic stress are not known; however, many of the downstream signals that mediate ischemia/reperfusion injury have been well characterized. For example, reactive oxygen species (ROS)\(^\dagger\) are major mediators of ischemia/reperfusion injury (3–5). Although cells contain antioxidant defenses that minimize susceptibility to ROS, ROS generation and oxidative stress often exceed the cell’s antioxidant capacity (6). Oxidative stress causes a rapid increase in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in a number of cell types, including cells derived from the renal tubule (7, 8). Increases in [Ca\(^{2+}\)]\(_i\) can result in enhanced Ca\(^{2+}\) influx into mitochondria, disrupting mitochondrial metabolism and leading to cell death (9, 10). Changes in [Ca\(^{2+}\)]\(_i\) also modulate gene transcription and proteases and nucleases that control cell apoptosis (9, 10). Recent investigations using renal epithelial cells indicate that the endoplasmic reticulum (ER) stress response can modulate both oxidative stress and [Ca\(^{2+}\)]\(_i\), after treatment with organic hydroperoxides and alkylating agents (11, 12). Likewise, increased attention has been paid recently to the possibility that ER stress influences the pathophysiology of acute ischemia in the brain, heart, or kidney (13–15). Given the association between ROS, oxidative stress, and ischemic injury in the kidney, we investigated the association between the ER stress response and H\(_2\)O\(_2\) in renal epithelial cells. ROS-induced cell injury has been attributed, in part, to the change in activation of intracellular signaling molecules, including mitogen-activated protein kinases (MAPKs). MAPKs, which include extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK), and p38 subfamilies, are important regulatory proteins that transduce various extracellular signals into intracellular events (16, 17). The ERK, JNK/SAPK, and p38 subfamilies are all activated in response to oxidative injury. In 1996, Guyton et al. (18) implicated ERK activation as a survival factor following oxidative injury. Subsequent studies from a number of laboratories confirmed these findings in other cell

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\dagger Exchange Scholar and supported in part by Chang-Gung Memorial Hospital, Taiwan. Present address: Dept. of Nephrology, Chang-Gung Memorial Hospital, 199 Tun-Hwa North Rd., Taipei, Taiwan.

\¶† To whom correspondence should be addressed: Renal Div., Brigham and Women’s Hospital, Medical Research Bldg., 4th Floor, 75 Francis St., Boston, MA 02115. Tel.: 617-732-6020; Fax: 617-582-6010; E-mail: tichimura@partners.org.

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Endoplasmic Reticulum Stress and ERK Signaling

![Graph](image_url)

**Fig. 1. Temporal relationship between increased [Ca\(^{2+}\)], and cell injury in LLC-PK1 cells.** Cells were treated with H\(_2\)O\(_2\) (1 mM) for 15 min in EBSS and then returned to DMEM containing 10% fetal calf serum (FCS). The abscissa indicates the time after cells were returned to DMEM (0 min). [Ca\(^{2+}\)], was determined by spectrophotometry after loading cells for 1 h with Fura-2/AM immediately following H\(_2\)O\(_2\) treatment. Cell injury was measured by determining the release of LDH into the medium. The data represent the means ± S.E. of duplicate measurements from two independent experiments for [Ca\(^{2+}\)], measurement or three independent experiments for LDH assay. Significant differences (*, p < 0.05) at various time points versus time 0 were determined by Student’s t test.

The signals that activate the ER stress response are well characterized, as is the link between ER Ca\(^{2+}\) release and cell injury. The ER is involved in both Ca\(^{2+}\) signaling and post-translational protein folding and maturation. Release of Ca\(^{2+}\) from the ER may contribute to the ischemia/reperfusion injury of the brain, heart, and kidney (15, 23, 24). The response of the ER to unfolded proteins, known as the unfolded protein response (UPR), is currently the best understood model of ER stress signaling. The UPR has been shown to modulate expression of ER chaperones, allowing the cell to tolerate the accumulation of unfolded proteins (15, 25–27). In mammalian cells, the UPR is activated by agents that prevent protein glycosylation (tunicamycin) and disulfide bond formation (DTT/tox) and by agents that deplete ER Ca\(^{2+}\) stores such as thapsigargin and the Ca\(^{2+}\)-ionophore A23187. The UPR also regulates many genes that affect diverse aspects of cell physiology. In the yeast *Saccharomyces cerevisiae*, 381 of 6000 genes were found to participate in the UPR, including 208 genes for which some functional information is available (28). Thus, the current view of the ER stress pathway has broadened from a pathway that simply regulates ER molecular chaperones to one that impacts many aspects of cell physiology (29). It is not entirely clear, however, how ER stress protects cells. In particular, it is not known whether the cellular MAPK and Akt/protein kinase B (PKB) pathways are effectors of the ER stress response (30, 31).

Although the ER stress response has been implicated in the pathophysiology of ischemic injury (13–15, 32), the role of individual ER stress proteins has not been addressed. Glucose-regulated proteins are the prototypical ER chaperones induced by ER stress (33). Induction of glucose-regulated proteins has been associated with protection against an increase in [Ca\(^{2+}\)]\(_i\), (11, 12, 15) and facilitation of protein folding (25, 27, 34). Overexpression, antisense, and ribozyme approaches in tissue culture systems have led to the conclusion that GRP78, GRP94, and Adapt78 protect cells against cell death (11, 35–38). Overexpression of the ER molecular chaperones also correlates with increased survival of renal epithelial cells subjected to ATP depletion (39). Therefore, glucose-regulated proteins might also be involved in ER protection from ischemic injury.

In this study, we investigated the effect of preconditioning the renal epithelial cell line LLC-PK\(_1\) with different inducers of ER stress to determine whether ER stress can protect against injury caused by H\(_2\)O\(_2\), the prototypical ROS thought to contribute to ischemia/reperfusion injury. Our results demonstrate that ER stress and GRP78 protect epithelial cells against oxidative stress by preventing the increase in [Ca\(^{2+}\)]\(_i\) that normally follows H\(_2\)O\(_2\) exposure. In naive renal epithelial cells, H\(_2\)O\(_2\) treatment activated both ERK1/2 and JNK phosphorylation. With prior ER stress, cells survived H\(_2\)O\(_2\) challenge and had increased ERK1/2 relative to JNK phosphorylation. The results indicate that ERK1/2 activation is downstream of the ER stress response in the cell protective pathway. Accordingly, modulating the balance between JNK and ERK1/2 phosphorylation with inhibitors or genetic manipulations that alter upstream MAPK signaling shows that cell survival by ER stress
preconditioning is linked to an increase in ERK1/2 and a decrease in JNK activation. These results place ERK as a distal mediator of the ER stress response in protection against oxidative injury and may be important for understanding how renal preconditioning affects mechanisms of ischemia/reperfusion injury in vivo.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fetal calf serum and Dulbecco’s modified Eagle’s medium (DMEM) were obtained from Invitrogen. The acetoxymethyl ester of Fura-2 (Fura-2/AM) and Pluronic F-127 (20%) at a 1:1000 (v/v) dilution was added to Fura-2/AM to avoid bias that might have occurred randomly through selection of individual clones.

**Construction of Recombinant Adenoviral Vectors**—MEKI-DD, a constitutively active mutant of MAPK/ERK kinase-1 (MEK1), the upstream activator of ERK1/2, was created by PCR using primers to substitute serines 218 and 222 with aspartic acid residues as previously described (42). This mutant has been shown to activate ERK1/2 when expressed in COS-7 cells as well as in NIH3T3 cells (42, 43). A recombinant adenoviral vector carrying the MEKI-DD cDNA (AdMEKI-DD) was treated with 250 μM H2O2 by directly adding H2O2 to the medium without changing the medium to prevent an effect of medium change on signaling pathways during oxidative stress. The later protocol was used when activation of members of the MAPK family was determined to avoid serum stimulation upon refeeding. Specific inhibitors were added 1 h before H2O2 treatment. Cell injury was determined by measurement of LDH release as a percent of total LDH as described previously (11).

In some experiments, the effect of altering expression of specific ER stress proteins was examined. LLC-PK1 cells, expressing an antisense RNA targeted to GRP78 (pkASgrp78 cells) or overexpressing human calreticulin (pkCRT cells) as well as controls transfected with the same pcDNA3 plasmid (used to construct both cell lines) containing no insert (pkNEO cells) were established as described previously (11, 12). When the effects of ER stress on H2O2-induced cell injury and MAPK activation were tested using pkASgrp78 or pkCRT cells, three independent clones of both lines as well as three pkNEO lines were compared to avoid bias that might have occurred randomly through selection of individual clones.

**Measurement of Intracellular Free Calcium Concentration**—[Ca2+]i was determined with the Ca2+-sensitive fluorescent dye Fura-2/AM according to Chen et al. (40) with modifications. Cells grown on coverslips coated with bovine collagen type I were rinsed with phosphate-buffered saline and loaded with 3 μM Fura-2/AM for 1 h at 37°C. After incubation with Fura-2/AM for 1 h at 37°C, cells were washed two to three times with EBSS in the presence of probenecid. The coverslips were positioned in a quartz cuvette, containing 2.5 ml of EBSS with probenecid, for fluorescence analysis using a Shimadzu RF-5000 spectrofluorophotometer. [Ca2+]i was calculated as equal to $K_c$ ($224 \text{ nmol} × (R - R_{\text{min}})/(R_{\text{max}} - R)$) according to Grynkiewicz et al. (41). Fluorescence emission was monitored at 505 nm. R is the ratio of the fluorescence at 340 nm excitation to that at 380 nm excitation.
was constructed as previously described (44). Protein expression was confirmed by immunoblotting and by assay of ERK activity in mouse mesangial cells. The recombinant adenovirus carrying the Escherichia coli LacZ gene (AdLacZ) encoding β-galactosidase was kindly provided by Dr. Roger Hajjar (Cardiovascular Research Center, Massachusetts General Hospital, Boston, MA).

Western Blot Analysis—Cells were lysed in a solution containing 70 mM β-glycerophosphate (pH 7.2), 0.1 mM sodium orthovanadate, 2 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Triton X-100, 10% glycerol, and protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride) for 30 min. Lysates were centrifuged at 14,000 rpm for 10 min at 4°C. Supernatants (30–50 μg) were boiled in 1× sample buffer (500 mM Tris-HCl (pH 6.8), 10% SDS, 2% glycerol, 0.05% bromphenol blue, and 1% 2-mercaptoethanol) for 5 min and separated on 10% polyacrylamide gels. Proteins were electrotransferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) and blotted with the indicated antibodies at 4°C overnight in Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat milk. Membranes were then incubated with horseradish peroxidase-conjugated antibodies at room temperature for 45 min, and the signal was detected using chemiluminescence (ECL, Amersham Biosciences), followed by exposure to X-Omat AR film (Eastman Kodak Co.). Blots were stripped and rebotted with the indicated antibodies to determine equal loading of samples. Stripping of the initial antibody probe was accomplished by submerging the membrane in buffer containing 100 mM 2-mercaptoethanol, 20% SDS, and 62.5 mM Tris-HCl (pH 6.8) at 55°C for 50 min, followed by washing twice with Tris-buffered saline and 0.1% Tween 20 for 10 min each.

Statistical Analyses—Student’s t test was used to determine whether there was a significant difference between two groups (p < 0.05). When multiple means were compared, significance (p < 0.05) was determined by analysis of variance, followed by Fisher’s protected least significant difference test. For analysis of variance, letter designations are used to indicate significant differences. Means with a common letter designation are not different, and those with a different letter designation are significantly different from all other means with different letter designations. StatView software (SAS Institute, Cary, NC) was used as a statistical tool in this study.

RESULTS

H₂O₂ Increases [Ca²⁺], prior to Injury to LLC-PK₁ Cells—To establish the temporal relationship between an increase in [Ca²⁺], and cell injury in LLC-PK₁ cells under oxidative stress, we examined [Ca²⁺], at various times after H₂O₂ treatment. Cells were treated with 1 mM H₂O₂ for 15 min in EBSS and then returned to EBSS and loaded for 1 h with Fura-2/AM. An increase in [Ca²⁺], was detected as early as 1 h after H₂O₂ treatment and reached a maximum at 2 h. In contrast, a significant amount of H₂O₂-induced cell death, as measured by

FIG. 4. Prevention of GRP78 expression disrupts the effect of ER stress preconditioning. A, pkASgrp78 cells, which express a 0.5-kb antisense grp78 construct, and their pkNEO counterparts were treated with EBSS or different concentrations of H₂O₂ in EBSS for 15 min. B, cells with or without prior induction of ER stress by DTTox (10 mM, 3 h), tunicamycin (1.5 μg/ml, 16 h), thapsigargin (THAPS; 0.5 μg/ml, 16 h), or A23187 (1 μM, 16 h) were treated with EBSS or H₂O₂ (1 mM) in EBSS for 15 min. Cells were returned to DMEM with 10% FCS after H₂O₂. Cell injury was determined 4 h later by measuring LDH release. The data are means ± S.E. of results with three separate pkNEO clones and three separate pkASgrp78 clones of LLC-PK₁ cells. *p < 0.05 between two groups (pkASgrp78 versus pkNEO cells). Groups are distinguished statistically (p < 0.05) by different letters.

FIG. 5. Overexpression of calreticulin blocks cell injury and [Ca²⁺], increases after H₂O₂ treatment. A, two clones each of pkNEO and calreticulin-overexpressing pkCRT cells were treated with various concentrations of H₂O₂ in EBSS for 15 min. Immediately following treatment, cells were washed and returned to DMEM plus 10% FCS. LDH release, as a measure of cell injury, was determined 4 h later after removal of H₂O₂. B, pkNEO and pkCRT cells were treated with H₂O₂ (1 mM) in EBSS for 15 min. Cells were washed and loaded with Fura-2/AM for 1 h immediately after H₂O₂ treatment. [Ca²⁺], was determined 2 h after removal of H₂O₂ by spectrofluorometry. The data are the means ± S.E. from two independent experiments with two different clones of each cell line. Significant differences (*) between two groups (pkCRT versus pkNEO cells) were determined by Student’s t test.
LDH release, was not observed until 2 h and increased steadily through 4 h (Fig. 1). Thus, the increase in [Ca\textsuperscript{2+}]\textsubscript{i} preceded significant cell injury in LLC-PK\textsubscript{i} cells treated with H\textsubscript{2}O\textsubscript{2}.

**ER Stress Preconditioning Prevents H\textsubscript{2}O\textsubscript{2}-induced Cell Injury and Ca\textsuperscript{2+} Accumulation**—We next examined the effect of prior ER stress on H\textsubscript{2}O\textsubscript{2}-induced cell injury. LLC-PK\textsubscript{i} cells preconditioned with different ER stress inducers were protected against subsequent H\textsubscript{2}O\textsubscript{2}-induced cell injury at 4 h (Fig. 2A). Prior ER stress induced by DTTox, tunicamycin, thapsigargin, or A23187 prevented the rise in [Ca\textsuperscript{2+}]\textsubscript{i}, normally occurring 2 h after H\textsubscript{2}O\textsubscript{2} treatment (Fig. 2B). To confirm the biological significance of the protection afforded by ER stress preconditioning, we assessed cell injury at later time points under two different conditions of H\textsubscript{2}O\textsubscript{2} exposure. Under conditions of transient (Fig. 3, A and B) or continuous (C and D) H\textsubscript{2}O\textsubscript{2} exposure, the protective effect afforded by previous ER stress persisted up to 24 h. Thus, ER stress preconditioning modified the magnitude of oxidant-induced cell injury and not just the kinetics of the process.

**Blocking Expression of GRP78 Disrupts the Effect of ER Stress Preconditioning**—Because prior ER stress prevented both the increase in [Ca\textsuperscript{2+}]\textsubscript{i} and cell death after H\textsubscript{2}O\textsubscript{2}, we determined whether disruption of the ER stress response alters the sensitivity to oxidant injury and prevents adaptation. We tested the H\textsubscript{2}O\textsubscript{2} sensitivity of pkASgrp78 cells, which express antisense grp78 and are unable to increase grp78 gene expression after ER stress, relative to pkNEO cells, which carry only the neomycin resistance marker. Cell death was much greater in pkASgrp78 cells compared with pkNEO cells (Fig. 4A). ER stress did not result in protection of pkASgrp78 cells subsequently exposed to H\textsubscript{2}O\textsubscript{2}, in contrast to pkNEO cells (Fig. 4B). Thus, the ability of ER stress preconditioning to prevent H\textsubscript{2}O\textsubscript{2}-induced cell injury depended on an intact ER stress response and expression of GRP78.

**Overexpression of Calreticulin Blocks Cell Injury and Ca\textsuperscript{2+} Disturbance**—If one mechanism underlying ER stress preconditioning is to prevent increased [Ca\textsuperscript{2+}]\textsubscript{i}, then increasing expression of Ca\textsuperscript{2+}-binding proteins in the ER might produce a similar effect. Previously, we demonstrated that overexpression of the ER calcium-binding protein calreticulin in LLC-PK\textsubscript{i} (pkCRT) cells prevents deregulation of Ca\textsuperscript{2+} in response to alkylating agents and organic hydroperoxides (11, 12). pkCRT cells were also more resistant to H\textsubscript{2}O\textsubscript{2}-induced cell injury relative to pkNEO cells (Fig. 5A). This protection was associated with maintenance of low [Ca\textsuperscript{2+}]\textsubscript{i} after H\textsubscript{2}O\textsubscript{2} treatment. Without H\textsubscript{2}O\textsubscript{2} treatment, there was no difference in [Ca\textsuperscript{2+}]\textsubscript{i} in pkCRT and pkNEO cells. However, 2 h after H\textsubscript{2}O\textsubscript{2} treatment, there was a significant increase in [Ca\textsuperscript{2+}]\textsubscript{i} in pkNEO cells, but not in pkCRT cells (Fig. 5B). Therefore, calreticulin attenuates cell injury and prevents the increase in [Ca\textsuperscript{2+}]\textsubscript{i}, normally observed after H\textsubscript{2}O\textsubscript{2} treatment.

**Akt/PKB and MAPK Signaling Pathways in LLC-PK\textsubscript{i} Cells Treated with H\textsubscript{2}O\textsubscript{2}**—Because various members of the MAPK family and Akt/PKB signaling pathways have been implicated in cell survival signaling in response to various toxic influences (45–47), we examined the activation of ERK, JNK, p38, and Akt/PKB in LLC-PK\textsubscript{i} cells treated with H\textsubscript{2}O\textsubscript{2}. To avoid the effect of medium changes on MAPK signaling, cells were serum-deprived for 3–4 h after ER stress preconditioning and then treated with 250 \( \mu \)M H\textsubscript{2}O\textsubscript{2} by directly adding H\textsubscript{2}O\textsubscript{2} to the medium to minimize the effect of changing the medium on kinase activation. At the various time points, cell injury as measured by LDH release was determined. The data are the means ± S.E. of duplicate measurements from three different experiments. Significant differences (*, p < 0.05) at various time points (versus time 0) were determined by Student’s t test. B, shown is the time course of Akt/PKB, ERK, JNK, and p38 MAPK activation in LLC-PK\textsubscript{i} cells treated with 250 \( \mu \)M H\textsubscript{2}O\textsubscript{2} for the indicated times before significant cell injury. We used phospho-specific antibodies that recognize phosphorylation (p) of Akt/PKB, p38, and dually phosphorylated activated ERK and JNK. Total (T) Akt/PKB, ERK, JNK, and p38 were equivalent in cells before and after H\textsubscript{2}O\textsubscript{2} exposure, confirming equal protein loading. The blots shown are representative of three separate experiments in which similar results were observed.

**ER Stress Modulates Cellular MAPK Activation**—We investigated whether prior ER stress modulates the MAPK signaling pathways and their response to H\textsubscript{2}O\textsubscript{2}. When cells were pretreated with tunicamycin and then exposed to H\textsubscript{2}O\textsubscript{2} after serum starvation, the ER stress-preconditioned cells were more resistant to H\textsubscript{2}O\textsubscript{2} (Fig. 7A) and had much stronger and persistent phosphorylation of ERK1/2 (Fig. 7C). The phosphorylation of MEK1/2, an upstream kinase of ERK, was increased in tunicamycin-pretreated cells relative to control cells (Fig. 7C), indicating that ER stress preconditioning results in activation of the ERK pathway by regulating kinases upstream of ERK.
Prior induction of ER stress in LLC-PK1 cells also resulted in a diminished level of JNK phosphorylation in response to H$_2$O$_2$ exposure (Fig. 7C). Total ERK and JNK proteins were equivalent in ER-stressed and control cells before and after H$_2$O$_2$ treatment, confirming equal protein loading (Fig. 7C). Expression of GRP94, another prototypical ER stress protein, is shown in the lower panel of Fig. 7C to confirm that the ER stress response was activated in response to tunicamycin. These effects of ER stress on MAPKs are not specific to tunicamycin because pretreatment of LLC-PK1 cells with another ER stress inducer, thapsigargin, protected cells against oxidative injury (Fig. 7B) and, like tunicamycin, up-regulated the MEK/ERK and down-regulated the JNK pathways in response to H$_2$O$_2$ (Fig. 7D). Therefore, ERK phosphorylation may promote cell survival in LLC-PK1 cells exposed to H$_2$O$_2$. It was not possible to use U0126, an inhibitor of MEK1, because U0126 is a free radical scavenger and also inhibited the activation of JNK that occurred at 30–60 min upon H$_2$O$_2$ exposure (data not shown).

We next examined whether JNK activation is linked to cell injury of H$_2$O$_2$-treated LLC-PK1 cells. Preincubation of LLC-PK1 cells with a specific JNK inhibitor (SP600125, 40 μM) 1 h prior to addition of H$_2$O$_2$ completely abolished the phosphorylation of JNK (Fig. 8E) and protected cells from oxidative injury (Fig. 8F). Thus, ERK1/2 activation protected against oxidative injury, whereas JNK activation potentiated cell death.

Cells Overexpressing Calreticulin Have More ERK Phosphorylation in Response to H$_2$O$_2$ Treatment—As indicated previously by the dose response to H$_2$O$_2$ (Fig. 5A) and by the time course of LDH release after exposure to 500 μM H$_2$O$_2$ (Fig. 9A), pkCRT cells were more resistant to H$_2$O$_2$-induced cell injury than were pkNEO cells. To clarify whether the protective effect afforded by calreticulin overexpression is through the modulation of MAPK pathways, we examined the activation pattern of ERK and JNK in pkCRT cells in response to H$_2$O$_2$ treatment. In comparison with pkNEO cells, there was more phosphorylation of ERK in pkCRT cells after H$_2$O$_2$ exposure. The phosphorylation of JNK was not significantly different between pkNEO and pkCRT cells (Fig. 9B). Thus, overexpression of calreticulin resulted in enhanced ERK activation in response to H$_2$O$_2$ treatment in LLC-PK1 cells.
GRP78 May Play a Role in Modulating Cell Signaling Pathways in ER Stress-preconditioned Cells—As indicated previously by the dose response to H2O2 (Fig. 4A) and by the time course of LDH release after exposure to 250 μM H2O2 (Fig. 10A), pkASgrp78 cells were more susceptible to H2O2-induced cell injury. The phosphorylation of ERK in pkASgrp78 cells after 30 min of H2O2 exposure was less than that in pkNEO cells (Fig. 10B). After pretreatment with tunicamycin, the pkNEO cells also had a significant increase in phosphorylation of ERK in response to H2O2, similar to wild-type LLC-PK1 cells (Fig. 7C). In contrast, there was only a mild increase in ERK phosphorylation in response to H2O2 in pkASgrp78 cells (Fig. 10B). In contrast to the effect of GRP78 in decreasing ERK activation, the phosphorylation of JNK in response to H2O2 was much greater in pkASgrp78 cells in comparison with pkNEO cells. After pretreatment with tunicamycin, the activation of JNK in both cells decreased, but remained greater in pkASgrp78 cells than in pkNEO cells (Fig. 10B).

To evaluate whether ERK activation can rescue the pkASgrp78 cells exposed to H2O2, we infected them with adenovirus carrying constitutively active MEK1-DD to enhance phosphorylation of ERK in these cells (Fig. 10C). After adenoviral infection, the pkASgrp78 cells with constitutively active ERK were more resistant to oxidative injury compared with cells infected with adenovirus carrying the LacZ gene only (Fig. 10D). Thus, the enhanced sensitivity of pkASgrp78 cells to oxidative stress (Figs. 4A and 10A) was associated with less phosphorylation of ERK in response to H2O2 (Fig. 10B), an effect that could be overcome by expression of constitutively active MEK1 (Fig. 10D). Therefore, ERK pathway activation is a downstream effector of the protective response mediated by ER stress and GRP78 expression.

**Fig. 8.** Influences of signaling pathways on cell survival in H2O2-treated cells. A, LLC-PK1 cells were pretreated with 10 μM LY294002 for 4 h before addition of 250 μM H2O2. Cells were lysed at different times, and Akt/PKB activation in cell lysates was determined using phospho-specific antibody recognizing phosphorylation (p) at Ser473 of Akt/PKB. Total (T) Akt/PKB was also measured with anti-total Akt/PKB antibody to confirm equal protein loading. B, at different times, cell injury was measured by LDH release. C, 80% confluent LLC-PK1 cells in DMEM with 2% FCS were infected with a recombinant adenoviral vector carrying MEK1-DD cDNA (AdMEK-DD) that constitutively activates ERK. Other cells were infected with the adenovirus carrying the E. coli LacZ gene (AdLacZ) encoding β-galactosidase as a negative control. The multiplicity of infection was 100. After infection, cells were allowed to recover in DMEM with 10% FCS for 24 h and then serum-deprived for 3 h before addition of 250 μM H2O2. Cells were lysed at different times, and ERK activation in cell lysates was determined with phospho-specific antibody. Total ERK was determined to confirm equal protein loading. D, at times indicated, cell injury was determined by measurement of LDH release. E, LLC-PK1 cells were pretreated with 40 μM SP600125 for 1 h before addition of 250 μM H2O2. Cells were lysed at times indicated, and JNK activation in cell lysates was determined with phospho-specific antibody recognizing dually phosphorylated activated JNK. Total JNK was also measured using anti-total JNK antibody to confirm equal protein loading. F, at different times, cell injury was determined by measurement of LDH release. The LDH data are the means ± S.E. from results with three separate pkNEO clones and three separate pkCRT clones of LLC-PK1 cells. Significant differences (*, p < 0.05) between groups at various time points were determined by Student’s t test. The plots shown are representative of the results from three separate clones of pkCRT or pkNEO cells. Confirmation of overexpression of calreticulin (CRT) is also shown.

**Fig. 9.** MAPK signaling pathways in calreticulin-overexpressing LLC-PK1 cells treated with H2O2. A, confluent pkCRT and pkNEO cells were serum-deprived for 3 h before adding 500 μM H2O2. At time points indicated, cell injury was determined by LDH release. The LDH data are means ± S.E. from results with three separate pkNEO clones and three separate pkCRT clones of LLC-PK1 cells. Significant differences (*, p < 0.05) between groups at various time points were determined by Student’s t test. B, at time points indicated after H2O2 treatment, cells were lysed, and phosphorylated (P) and total (T) ERKs and JNKs were determined with specific antibodies. The plots shown are representative of the results from three separate clones of pkCRT or pkNEO cells. Confirmation of overexpression of calreticulin (CRT) is also shown.
DISCUSSION

Increased amounts of RNA transcripts for GRP78 and other ER stress proteins are characteristic of rat models of brain, kidney, and heart ischemia/reperfusion (13–15, 48). Overexpression of the ER molecular chaperones correlates with increased survival of cells subjected to conditions modeling ischemia/reperfusion (39) as well as injury by other agents (27, 49, 50). Despite efforts of a number of laboratories, however, the mechanism of cytoprotection due to ER stress remains unclear. The results from this investigation demonstrate that the protection that ER stress affords against oxidative injury is mediated by enhanced ERK pathway activation.

The ability of prior ER stress to prevent a rise in \([\text{Ca}^{2+}]_\text{i}\) in response to \(H_2O_2\) is consistent with the facts that the ER serves as a major storage site of intracellular calcium (51) and that several of the ER molecular chaperones bind calcium (38, 52, 53). Our data are consistent with previous studies showing that induction of ER stress moderates rises in \([\text{Ca}^{2+}]_\text{i}\), that occur in \(H_2O_2\)-induced cell injury as well as oxidative stress caused by glutathione depletion and organic oxidants, hence reducing the threat of oxidative stress to the cell (11, 12, 54, 55). Although other ER proteins may contribute to this cytoprotection, our evidence suggests that GRP78 is a critical aspect of the integrated ER stress response. Blocking induction of GRP78 sensitizes the epithelial cell to oxidative stress. There are previous reports that the protective effects of prior ER stress are disrupted in pkASgrp78 cells (11, 12). The levels of expression of other stress proteins in pkASgrp78 cells, including GRP94 and another KDEL protein, increase when cells express the antisense GRP78 RNA (56). Despite the increases in these other ER stress proteins, they cannot overcome the effect of disabling the ER stress response by GRP78 depletion.

Calreticulin is one of the major ER \(\text{Ca}^{2+}\)-binding proteins in non-muscle cells and has been shown to modulate ER \(\text{Ca}^{2+}\) release (57). Cells that overexpress calreticulin have increased ER \(\text{Ca}^{2+}\)-buffering capacity and/or resist \(\text{Ca}^{2+}\) toxicity (11, 12, 58). Calreticulin is increased markedly when NIH3T3 cells are preconditioned with thapsigargin (59); however, calreticulin expression is not altered appreciably in pkASgrp78 cells (56). Overexpression of calreticulin protects LLC-PK\(_1\) cells against \(H_2O_2\)-induced cell injury and an increase in \([\text{Ca}^{2+}]_\text{i}\). Thus, the fact that increased ER \(\text{Ca}^{2+}\) buffering protects against oxidative injury supports the suggestions that the protective effect of ER stress is due, in part, to better \(\text{Ca}^{2+}\) buffering, decreased \(\text{Ca}^{2+}\) release, or an indirect mechanism involving cooperation between \(\text{Ca}^{2+}\) uptake by ER and net efflux across the plasma membrane. Each of these mechanisms could prevent a change in \([\text{Ca}^{2+}]_\text{i}\).

Renal epithelial LLC-PK\(_1\) cells have been used extensively to investigate cytotoxicity and stress gene activation (11, 12, 56, 60). However, the influence of cell signaling pathways on stress pathways and cell survival during oxidative stress is not well characterized. In comparison with native LLC-PK\(_1\) cells, cells
preconditioned with ER stress have greater ERK and less JNK phosphorylation following oxidative stress. The activation of the ERK pathway results from up-regulation of MEK1, the upstream kinase. GRP78 modulates these MAPK signaling pathways because prevention of GRP78 induction in pκASgrp78 cells alters the effects of H2O2 on ERK and JNK pathways. There is less ERK and more JNK activation upon exposure to H2O2 in pκASgrp78 cells, and these cells are more sensitive to oxidative injury. Constitutively active MEK1 rescues the pκASgrp78 cells from H2O2-induced cell injury. Thus, ERK1/2 phosphorylation is a downstream effector of the protection against oxidative injury afforded by GRP78. Our findings demonstrate for the first time that the ER stress response and specifically GRP78 expression act via ERK and JNK signaling pathways to modulate cell injury in response to oxidative stress.

Whether or not changes in [Ca2+]i, following H2O2 treatment are also involved in modulating JNK activation and cell death in a coordinate manner is not clear. The fact that [Ca2+]i can modulate MAPK signaling and cell injury may provide a link between these two events and ER stress. The ability of ER stress to suppress JNK activation in response to subsequent oxidative stress might be related to the prevention of an increase in [Ca2+]i, by GRP78 and other ER stress proteins. There is precedent for an interaction among proteins that regulate oxidative stress to suppress JNK activation in response to subsequent cell injury. Constitutively active MEK1 rescues the pκASgrp78 cells from H2O2-induced cell injury. Thus, ERK1/2 phosphorylation is a downstream effector of the protection afforded by GRP78. Our findings indicate that the MAPK signaling pathways are critical downstream effectors of the protection afforded by ER stress proteins against oxidative stress in renal epithelial cells.

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