Bax Inhibitor-1 Regulates Endoplasmic Reticulum Stress-associated Reactive Oxygen Species and Heme Oxygenase-1 Expression*\textsuperscript{S}

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The Bax inhibitor-1 (BI-1) is an anti-apoptotic protein that is located in endoplasmic reticulum (ER) membranes and protects cells from ER stress-induced apoptosis. The ER is associated with generation of reactive oxygen species (ROS) through oxidative protein folding. This study examined the role of BI-1 in the regulation of ER stress-induced accumulation of ROS and expression of unfolded protein response-associated proteins. BI-1 reduced the expression levels of glucose response protein 78, C/EBP homologous protein, phospho-eukaryotic initiation factor 2α, IRE1α, XBP-1, and phospho-JNK and inhibited the cleavage of ATF-6α p-90, leading to the inhibition of ROS. Although ROS scavengers offer some protection against ER stress-induced apoptosis, the expression of pro-apoptotic ER stress proteins was not affected. This study shows that the response of unfolded proteins is followed by ROS accumulation under ER stress, which is regulated in BI-1 cells. The mechanism for these BI-1-associated functions involves the expression of heme oxygenase-1 (HO-1) through nuclear factor erythroid 2-related factor 2. In BI-1 cells, the transfection of HO-1 small interfering RNA completely abolished the BI-1-induced protection. The endogenous expression of HO-1 through ER stress-initiated ROS is believed to be as a protection signal. In conclusion, these observations suggest that BI-1 can inhibit the ER stress proteins as well as the accumulation of ROS, thereby protecting the cells. Moreover, HO-1 plays an important role in the BI-1-associated protection against ER stress.

The endoplasmic reticulum (ER)\textsuperscript{3} is the key organelle in cells where the important steps in the folding and modification of proteins as well as the selection for transport to other compartments occur (1, 2). The ER can initiate apoptosis, when the accumulation of unfolded proteins or the inhibition of the ER-Golgi transport results in the so-called ER stress response (3). Many infectious agents, environmental toxins, and adverse metabolic conditions can interfere with protein folding, which can lead to ER stress.

Three ER-resident transmembrane proteins, endoribonuclease, IRE1, PKR-like kinase PERK, and the basic leucine-zipper transcription factor ATF-6 have been identified as the proximal sensors of ER stress. The ER has evolved highly specific signaling pathways to protect against ER stress, which is collectively known as the unfolded protein response (UPR) (4). The activation of the UPR causes the up-regulation of the genes that encode ER chaperone proteins such as GRP78, which increases the protein folding activity and prevents protein aggregation (5). However, it was reported that the accumulation of misfolded proteins within the lumen of the ER can lead to prolonged UPR activation, which in turn causes oxidative stress, resulting eventually in cell death (6, 7).

The Bax inhibitor-1 (BI-1) is an anti-apoptotic protein that contains several transmembrane domains that have a protective function on ER stress (8). BI-1 is conserved in both animal and plant species (9). The cytoprotective function of BI-1 was originally discovered during cDNA library screens for human proteins that suppress the death of yeast caused by the ectopic expression of the mammalian Bax protein (10). Although BI-1 has a regulatory effect on ER stress-related apoptosis, the roles of this protein during ER stress and the resulting signal pathways are only now beginning to emerge. This study examined...
whether or not BI-1 regulates ER stress-associated ROS accumulation and explored the mechanism by which BI-1 inhibits the accumulation of ROS and resultant cell death.

**EXPERIMENTAL PROCEDURES**

**Materials**

Thapsigargin and tunicamycin were supplied by Calbiochem. DiOC₆ (3,3’-dihexyloxacarbocyanine iodide) and DCF-DA were obtained from Molecular Probes (Eugene, OR). The antibodies against actin, HO-1, GRP78, JNK, IRE-1, XBP-1, CHOP, and Nrf-2 siRNA were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). ATF-6 antibody was obtained from Imgenex (San Diego, CA). The antibodies against phospho-JNK, eIF-2α, and phospho-eIF-2α were purchased from Cell Signaling Technologies (Beverly, MA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, trypsin, and other tissue culture reagents were supplied by Invitrogen. Bicinchoninic acid protein assay reagents were obtained from Pierce. All other chemicals were at least of analytical grade and were purchased from Sigma.

**Cell Culture and Viability**

The human HT1080 fibrosarcoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, 20 mM Heps, 100 μg/ml streptomycin, and 100 units/ml penicillin. HT1080 cells were stably transfected with the pcDNA3 (Neo), pcDNA3-BI-1-HA, or CA-BI-1-HA (C-terminal-deleted) plasmids using the superfect transfection reagent (Qiagen). The cells were then cultured for 3 weeks in 1 mg/ml G418 (Invitrogen). The tetracycline-inducible BI-1-transfected HeLa cells were cultured in complete DMEM containing 100 μg/ml hygromycin B, and 100 μg/ml G418 (hygromycin B from Sigma; G418 from Fisher). BI-1 was induced by adding doxycycline (1 μg/ml; Sigma) to the medium. Cell viability was assessed using the trypan blue exclusion technique and was calculated by dividing the number of nonstained (viable) cells by the total cell count.

**Apoptosis Assays**

**Annexin V and Propidium Iodide Double Staining Assay**—HT1080 cells (Neo, BI-1, and CA-BI-1) were plated in 100 × 20-mm plates at cell densities ranging from 1 × 10⁷ to 2 × 10⁷ cells/plate. After treatment, the cells were removed from the plate using trypsin and analyzed for the level of phosphatidylserine externalization using an annexin V and propidium iodide apoptosis kit (Oncogene Research Products, San Diego, CA) according to the manufacturer’s instructions, as well as a PAS Flow Cytometer equipped with Partec software (Partec, Germany).

**Morphological Detection of Apoptosis**—The cells were fixed for 5 min in phosphate-buffered saline containing 3% paraformaldehyde. After air drying, the cells were stained for 10 min in Hoechst 33258 (10 μg/liter), mounted in 50% glycerol containing 20 mmol/liter citric acid and 50 mmol/liter orthophosphate, and stored at −20 °C before analysis. Nuclear morphology was evaluated using a Zeiss IM 35 fluorescence microscope.

**Genomic DNA Fragmentation Assay**—The level of DNA fragmentation was measured using gel electrophoresis as described elsewhere (11). Briefly, the cells were plated at a density of 1 × 10⁷ cells/plate and treated with various agents. 1 × 10⁷ cells/ml were pelleted and resuspended in a lysis buffer (50 mM Tris–HCl, 10 mM EDTA, 0.5% SDS). The cell lysates were treated with 200 μg/ml proteinase K and incubated at 55 °C for 1 h. Subsequently, 0.5 mg/ml RNase A was added to the lysates, and the mixture was heated to 70 °C for 5 min. The DNA was precipitated with isopropanol, mixed with a loading dye (10 mM EDTA, pH 8.0, 40% sucrose, 0.25% bromphenol blue), and analyzed on a 2% agarose gel containing 0.5 μg/ml ethidium bromide. Gel images were captured using an Alpha Innotech digital camera equipped with a transilluminator and Alpha Ease 5.5 software (Alpha Innotech Corporation, San Leandro, CA).

**DCF-DA Assay**

The intracellular ROS level was measured as described elsewhere (12). The cells were incubated with 1 μM DCF-DA at 37 °C for 30 min and treated with various agents at 37 °C for an additional 30 min. After cooling on ice, the cells were washed with cold phosphate-buffered saline, scraped from the plate, and resuspended at 1 × 10⁶ cells/ml in phosphate-buffered saline containing 10 mM EDTA. The fluorescence intensity of the 2,7'-dichlorofluorescein formed by a reaction between DCF-DA and the intracellular ROS of more than 10,000 viable cells from each sample was analyzed by PAS flow cytometry (Partec, Münster, Germany) at excitation and emission wavelengths of 488 and 525 nm, respectively. The data were collected and analyzed using Partec software. The experiments were repeated at least three times with similar results. The data were expressed as representative histograms from three independent experiments.

**DiOC₆ Assay**

The stock solution of DiOC₆ (4 mmol/liter) was prepared in ethanol and stored in small lots at −20 °C; working solution (dilution 1:2000 for DiOC₆) was made up in experimental medium (DMEM) immediately before use. A total of 5 × 10⁵ cells were incubated in DMEM containing 100 nM DiOC₆ at 37 °C and analyzed using a PAS cytofluorometer (Partec) equipped with Partec software. Forward and side scatter were gated for the major population of normally sized cells, and a minimum of 10,000 cells was analyzed. The fluorescent probe DiOC₆ was excited using a 488-nm argon laser, and emissions were collected through an FL1 detector fitted with a 525 ± 5-nm band pass filter.

**Nrf-2 Promoter Assay**

HT1080 cells (1 × 10⁵) suspended in 1 ml of DMEM containing 10% (v/v) fetal bovine serum were seeded into each well of a 12-well plate. After incubating at 37 °C for 24 h, the cells were transiently transfected with 1 μg of the ARE-luciferase reporter plasmid and 1 μg of the β-galactosidase plasmid in DMEM without fetal bovine serum and antibiotics using the Lipofectamine reagent (Invitrogen). The luciferase activity was determined using a luciferase assay system with a reporter lysis buffer from Promega. Briefly, the cells were harvested by scrap-
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ing in 200 μl of a reporter lysis buffer into a 1.5-ml microcentrifuge tube, vortex-mixed for 15 s, and centrifuged at 12,000 × g for 30 s. The supernatant of the cell lysates was then collected. Luciferase activity was determined using 60 μl of cell lysate and 20 μl of substrate using a monolight luminometer. β-Galactosidase activity was determined as described previously (12).

**Microsomal Fractionation**

The cells were resuspended in buffer A (250 mM sucrose, 20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 X protease inhibitor complex (Roche Applied Science) on ice for 30 min as described (13). The cells were homogenized using a Dounce homogenizer, and the lysates were centrifuged at 750 × g for 10 min at 4 °C. The resulting supernatant was discarded, and the pellet was saved as a light membrane (LM; ER/microsome) fraction. The LM fraction was examined for HO-1 activity.

**Heme Oxygenase-1 Activity**

HO-1 activity was measured by determining the level of bilirubin generated in isolated microsomes (14). The cells were homogenized in a phosphate buffer (pH 7.4), sonicated on ice, and centrifuged at 1,000 × g for 10 min at 4 °C. The resulting supernatant was centrifuged at 100,000 × g for 60 min at 4 °C. The resulting supernatant was discarded, and the pellet was saved as a light membrane (LM; ER/microsome) fraction. The LM fraction was examined for its HO-1 activity.

**Western Blotting**

The cell lysates were prepared, and the level of protein expression was measured as described elsewhere (15). The total protein from the tissue lysates (four independent tissue lysates from the kidney, liver, and testis of BI-1 littermates) was resolved in precast 4–12% SDS-PAGE gradient gels. Immunoblotting was performed using the indicated antibodies. The ECL reagents (Amersham Biosciences) were used to visualize the signals.

**Transfection of siRNA**

The siRNAs were synthesized in duplex and purified forms using Bioneer technology (Daejon, South Korea). The sense and antisense strands of the human HO-1 siRNA and human BI-1 are as follows: HO-1, 5'-UUUGAGCAUGCAUUGCA-3' (sense) and 5'-UUUGACUUGCAUGCAUGCA-3' (antisense); BI-1, 5'-GAAGCGUUCUCCG-3' (sense) and 5'-UGAAGCCUUCG-3' (antisense). The sense and antisense strands of the non-specific siRNA duplex are as follows: CUG-AACAACCAUGCAAAU-3' (sense), and 5'-AUUUGCAUGCGUUGUUCAG-3' (antisense). The siRNAs were transfected into the HT-1080 cells using an Amaxa Nucleofector apparatus (Amaxa, Cologne, Germany). Five μg of the plasmid DNA was mixed with 0.1 ml of a cell suspension, transferred to a 2.0-mm electroporation cuvette, and nucleofected using an Amaxa Nucleofector apparatus (Amaxa, Cologne, Germany) according to the manufacturer’s protocol. The DNA quantity, cell concentration, and buffer volume were kept constant throughout the experiments. After electroporation, the cells were transferred immediately to 2.0 ml of complete medium and cultured in six-well plates at 37 °C until needed.

**Statistical Analysis**

The data were analyzed by an analysis of variance in the dose-response experiments as well as by two-tailed Student’s t tests. A p value <0.05 was considered significant. In each case, the statistical test used is indicated, and the number of experiments is stated individually in the legend of each figure.

**RESULTS**

**ROS Are Associated with ER Stress-induced Apoptosis, Which Is Regulated by Bi-1**—Recent studies have investigated ER stress-associated accumulation of endogenous ROS and their effect on the apoptotic process (7, 8). To examine association of ER stress with ROS generation, HT-1080 cells were transfected with pcDNA-3 vector and HA-BI-1. Neo resistant cells (Neo) or BI-1 stable overexpressing cells (BI-1) (Fig. 1A, inset) were confirmed by immunoblotting using anti-HA antibody. The cells were treated with thapsigargin, an ER-Ca-ATPase inhibitor, or tunicamycin, an N-glycosylation inhibitor, and cell death was evaluated. A thiol agent, N-acetylcysteine (NAC), and a precursor of L-cysteine, GSH, were also used to determine whether thapsigargin- or tunicamycin-induced cell death is the result of ROS generation. As shown Fig. 1A, treatment with thapsigargin or tunicamycin decreased cell viability of Neo cells significantly, whereas the BI-1 cells were significantly resistant to the cell death induced by these agents. Pretreatment with an antioxidant, NAC or GSH (1 mM), increased the number of viable cells by 64 ± 1.25 and 67 ± 1.21% for thapsigargin and by 65 ± 1.59 and 65.1 ± 2.2% for tunicamycin, respectively (Fig. 1A). In contrast, the antioxidant had no effect on the viability of BI-1 cells. These observations were confirmed by flow cytometric analysis of the cells stained with annexin V (Fig. 1B). In the thapsigargin-treated cells, 57.01 ± 3.01% of the cells (63.66 ± 1.85% of the tunicamycin-treated cells) were annexin V-positive. However, in the presence of either NAC or GSH, only 35.23 ± 3.46 or 35.4 ± 1.76% of the total cell population in the thapsigargin-treated cells were annexin V-positive (37.01 ± 1.45% or 35.24 ± 2.49% in the tunicamycin-treated cells). The number of annexin V-positive BI-1 cells was not changed by a pretreatment with antioxidants. Although the overall number of propidium iodide-positive stained cells was relatively low compared with the annexin V-stained cells, the protective effect of BI-1 and that of NAC and GSH in Neo cells were similar to the result of annexin V staining (supplemental Table S1). These results suggest that BI-1 overexpression protects the cells against ER stress through the regulation of ROS.
**BI-1 Inhibits Accumulation of ROS in ER Stress-exposed Cells**—

ROS levels in Neo and BI-1 cells were measured after treatment with an ER stress inducer, thapsigargin or tunicamycin. Basal levels of ROS in BI-1 cells were much lower than that in the Neo, control cells (Fig. 2). Moreover, thapsigargin treatment increased significantly the levels of ROS in Neo cells (∼40% increment), whereas the level of ROS in BI-1 cells was increased only slightly (∼12%). Tunicamycin also increased the level of ROS in the Neo cells more than in BI-1 cells. Pretreatment with NAC or GSH completely inhibited the accumulation of ROS in the Neo cells treated with the ER stress agent. In parallel with ROS analysis, we measured mitochondria membrane potential using DiO6. Fig. 2 shows that the mitochondrial membrane potential remained intact during the period of exposure to ER stress, which suggests that ER stress-induced accumulation of ROS precedes collapse of mitochondrial membrane potential and apoptosis. To confirm that DiOC6 dye had correctly stained the mitochondria, the cells were incubated with carbonyl cyanide p-chlorophenylhydrazone, an uncoupling agent that abolishes mitochondria membrane potential (16, 17). As expected, mitochondrial membrane potential collapsed in carbonyl cyanide p-chlorophenylhydrazone–treated cells (data not shown). Taken together, these results suggest that ER stress-induced generation of ROS is not a by-product of the apoptotic phenomena but is an upstream component of ER stress-associated cell death.

**Unfolded Protein Response Is Followed by the Release of ROS during ER Stress**—Because BI-1 expression reduced the level of ER stress-associated ROS accumulation and/or the generation (Fig. 2), we examined expression of several ER stress-inducible UPR proteins. ER stress-related proteins include GRP78, C/EBP homologous protein (CHOP), phospho-eIF-2α, eIF-2α, phospho-c-Jun N-terminal kinase (JNK), IRE-1, and CHOP expression-related transcription factors, XBP-1 and ATF-6.

During ER stress, increases occur in the expression of protective intra-ER molecular chaperone GRP78, which is intended to compensate for damage (4). To determine whether the protection afforded by BI-1 is upstream or downstream of these events, expression of GRP78 was monitored by immunoblotting in the presence of thapsigargin or tunicamycin. ER stress induced comparable increases in the GRP78 protein levels in Neo cells, which is indicative of ER stress. However, the
expression of GRP78 in BI-1 cells tended to be somewhat lower (Fig. 3, A and B).

CHOP is a transcription factor whose expression is induced during ER stress and that participates in ER-mediated apoptosis (18). Deregulated CHOP activity compromises cell viability, whereas cells lacking CHOP are significantly protected from the lethal consequences of ER stress (19). When assessed by immunoblot analysis in Neo and BI-1 cells, the CHOP protein became elevated in the Neo cells but was barely detectable in the BI-1 cells (Fig. 3, A and B).

Recently, inhibitors of tunicamycin-induced neuronal death were shown to suppress protein phosphatases responsible for the dephosphorylation of eIF-2α on serine 51, thereby increasing the accumulation of phospho-eIF-2α and providing protection from apoptosis induced by several inducers of ER stress (20). Interestingly, phospho-eIF-2α was strongly expressed in the Neo cells but was only slightly detectable in the BI-1 cells (Fig. 3, A and B).

In ER stress, JNK phosphorylates and enhances CHOP transcription and pro-apoptotic activity (21). Therefore, the status...
of JNK in Neo and BI-1 cells before and after ER stress was examined using the phospho-specific JNK antibody. After ER stress, the level of phospho-JNK increased significantly in Neo cells, whereas little phospho-JNK was detected in the BI-1 cells (Fig. 3, A and B). The Neo and BI-1 cells showed no change in the total levels of the JNK protein, as revealed by the phospho-independent antibody (Fig. 3, A and B). Additional ER stress-related proteins are IRE1 protein kinase and ATF-6 (21). Activated IRE1 protein kinase is essential for splicing the XBP-1 mRNA (22), and the spliced variant is a potent transcriptional activator. ATF-6 is also cleaved during ER stress, and its cytosolic domain (ATF-6(N)) translocates to the nucleus. Therefore, the changes in IRE1α, XBP-1, and ATF-6 in the Neo and BI-1 cells were examined before and after ER stress. After treating the cells with thapsigargin or tunicamycin, the expression of IRE1α and the resulting XBP-1 spliced form were increased in the Neo cells. The expression of IRE1α was also induced in BI-1-overexpressing cells, although not quite as much. However, expression of XBP-1 spliced isoform was markedly reduced in BI-1 cells compared with Neo cells (Fig. 3, A and B). The level of uncleaved ATF-6 (p90) was also lower in the Neo cells but levels of full-length ATF-6 remained unchanged in the BI-1 cells under ER stress (Fig. 3, A and B). Taken together, these results suggest that BI-1 modulates the activity of all three major pathways for ER stress signaling in HT-1080 cells, including PERK, IRE-1α, and ATF-6.

The above-mentioned UPR proteins were also analyzed in HT1080 cells that had been preincubated with either 1 mM NAC or GSH. These pretreatments had no effect on the expression of CHOP and the other UPR proteins (Fig. 3C), indicating that the release of ROS may be a product of ER stress-induced UPR but not an initiating messenger of UPR. The time course of ROS accumulation was examined. The ROS was delayed relating to UPR markers (supplemental Fig. S1). Thus, ROS appears to be a downstream consequence of UPR.

The BI-1 Increases the Expression of Heme Oxygenase-1 through Nrf-2—The level of ROS accumulation/generation is lower in the cells overexpressing BI-1 (Fig. 2). Therefore, we considered whether BI-1 could regulate the expression of the ROS scavenging protein. First, the activity of a transcription factor, Nrf-2 was determined. Nrf-2 is known to be a redox-sensitive transcription factor (23, 24). Nrf-2, which was determined using a direct marker of the Nrf-2, the ARE, was 15 times higher in the BI-1 cells than the Neo cells (Fig. 4A). The BI-1 cells also showed higher Nrf-2 expression in the nuclear fraction than the control (Fig. 4B), suggesting that BI-1 increased the activity of Nrf-2. Anti-PARP antibody was used as a marker of nuclear protein and anti-procaspase-3.
BI-1 Regulates ER Stress-associated ROS and HO-1 Expression

antibody as a marker of cytoplasmic protein (Fig. 4B, bottom panel).

It was reported that Nrf-2 mediates the induction of antioxidant enzyme expression such as heme oxygenase-1, glutathione S-transferase, glutamyl cysteine synthetase, and the phase II detoxifying enzymes (25–27). HO-1 functions as a part of cytoprotective mechanisms based upon a series of antioxidant activities (28). Therefore, HO-1 expression was evaluated in the BI-1 stable cells. The level of HO-1 expression was significantly higher in the BI-1 cells than in Neo cells (Fig. 5A). Nrf-2 siRNA was transfected, and the expression of HO-1 was measured to determine whether Nrf-2 is directly responsible for the HO-1. Fig. 5B shows that the HO-1 levels in BI-1 cells were reduced following knock-down of Nrf-2, suggesting that Nrf-2 plays an important role in the induction of HO-1 in BI-1 cells. The activity of HO-1 also was higher in the cells overexpressing BI-1 (Fig. 5C). In the Neo and BI-1 cells, transfection of BI-1 siRNA markedly decreased expression of HO-1 (Fig. 5D, inset) and caused increase of sensitivity to ER stress-induced cell death in Neo and BI-1 cells (Fig. 5D).

The correlation of BI-1 with HO-1 expression was further confirmed using tissues of BI-1−/− mice. Levels of HO-1 were significantly lower in the kidney, liver, and testis from BI-1−/− mice (Fig. 5E).

To confirm the correlation of HO-1 with BI-1 expression in another cell system, BI-1 was expressed using a tetracycline-inducible BI-1 promotion in HeLa cells. HO-1 was induced after the induction of BI-1 protein expression (supplemental Fig. S2A). Nrf-2 promoter activity was also measured in BI-1-tetracycline-inducible HeLa cells. Nrf-2 promoter activity increased in the presence of tetracycline (supplemental Fig. S2B). The amount of Nrf-2 protein was also higher in the nuclear fraction following incubation with tetracycline (supplemental Fig. S2C). PARP was used as a nuclear marker, and the pro-caspase-3 was used as a cytosolic marker in these immunoblot experiments. These results thus confirm the ability of BI-1 to modulate activity of Nrf-2 and expression of HO-1.

ER Stress Increases the Endogenous Expression of HO-1 through ROS Accumulation—We used antioxidants NAC and GSH to explain the relation of ROS to HO-1 expression. ER stress inducers thapsigargin and tunicamycin caused increase of expression of HO-1 in HT1080-Neo cells, whereas the higher basal expression of HO-1 was present in HT1080-BI-1 cells and unaffected by ER stress (Fig. 6A). Adding either NAC or GSH to the cell culture before ER stress completely blocked the induction of HO-1 expression (Fig. 6B) and HO-1 activity (Fig. 6C) in HT1080 cells. These data suggest that ROS is required for ER stress-initiated HO-1 expression and activation.

HO-1 Modulates the BI-1-cytoprotective Pathway—HO-1 siRNA was used to knock down endogenous HO-1 protein levels to determine the role of HO-1 in BI-1-induced protection against ER stress (Fig. 7A). The transfection of HO-1 siRNA into BI-1 cells effectively reduced the endogenous HO-1 protein levels (Fig. 7A, inset). Treatment with BI-1 siRNA decreased cell viability following treatment with thapsigargin or tunicamycin from the 75.33 ± 2.73% and 74.01 ± 2.89% to 54.01 ± 2.3% and 51.66 ± 2.3% compared with HT1080-BI-1 cells transfected with the nonspecific siRNA (Fig. 7A). In HT1080-Neo cells, knock-down of HO-1 increased sensitivity to ER stress, suggesting that the thapsigargin-initiated induction of HO-1 protects cells from the death stimuli (Fig. 7A). In addition to siRNA-mediated knock-down of HO-1, we explored the effects of HO-1 overexpression. When HO-1-overexpressing cells were treated with thapsigargin or tunicamycin, cell death was significantly reduced compared with the cells transfected with control vector (supplemental Fig. S3 and supplemental Table S2). Treatment of BI-1 overexpressing cells with HO-1 inhibitor zinc protoporphyrin c2 IX increased the level of ER stress-mediated cell death ~2-fold. In addition, the treatment with hemin (10 μM), a HO-1 activator, significantly prevented cell death caused by both ER stress agents (Fig. 7B). Hemin treatment did not have an additive effect on the BI-1-induced protection, suggesting that the BI-1-mediated protection includes or is modulated by HO-1 activation. Treatment of zinc protoporphyrin or hemin without ER stress agents did not affect the cell viability (Fig. 7B).

C-terminal Domain of BI-1 Is Required for HO-1 Induction and Cell Protection—Because the BI-1 C-terminal domain has been shown to be important for BI-1-mediated cytoprotection,
we explored the activity of the C-terminal deleted mutant on HO-1 expression. First, the BI-1 C-terminal deletion (CΔ-BI-1) exposed to the ER stress agents, 5 μM thapsigargin, or 5 μg/ml tunicamycin showed similar cell viability and a similar level of apoptosis compared with Neo cells (Fig. 8, A and B). Fig. 8B shows that the genomic DNA fragmentation, a hallmark of apoptosis, was similar in CΔ-BI-1 and Neo cells following exposure to ER stress.

The expression of HO-1 was examined in CΔ-BI-1 cells. As shown in Fig. 8C, CΔ-BI-1 cells had levels of HO-1 expression comparable with Neo control cells. This suggests that the C-terminal sequence, EKDKKKEKK, is essential for the induction of HO-1 and cell protection.

Because Bcl-2 and BclXL are well known anti-apoptotic proteins that are also localized to the ER (29), the expression of Bcl-2 and BclXL in CΔ-BI-1 cells was also examined by immunoblotting. Expression of the two anti-apoptotic proteins, Bcl-2 and BclXL, was not affected in the CΔ-BI-1 cells.

**DISCUSSION**

Previous studies have shown that BI-1 has a cytoprotective role in vivo in contexts where ER stress is known to occur, including ischemia/reperfusion injury in various tissues (brain, liver, and kidney) and pharmacological exposure to tunicamycin (9, 21). Absence of BI-1 in mice has also been associated with elevations in markers of ER stress, including sXBP1 production, cleavage of ATF-6, and increased phosphorylation of stress kinases (JNK and p38 MAPK) (21). These and other data indicated that BI-1 modulates the ER stress-associated pathway for cell death, but details of the mechanisms of BI-1 have been unclear.

In this study, we present evidence that BI-1 regulates ROS accumulation in the context of ER stress induced by thapsigargin and tunicamycin in cultured cells, with overexpression of BI-1 suppressing ROS accumulation. In seeking an explanation for the reduced ROS accumulation seen in BI-1 overexpressing cells, we evaluated HO-1 expression. HO-1 is the rate-limiting enzyme in the degradation of heme to biliverdin, CO, and free divalent iron (Fe²⁺) and thus is a candidate to explain reduced ROS accumulation in BI-1-overexpressing cells. Whereas BI-1 overexpression suppressed many markers of ER stress that become induced during exposure of cells to thapsigargin and tunicamycin (ATF-6 cleavage, eIF2α and JNK phosphorylation, CHOP and Grp78 expression, and sXBP1 production), expression of HO-1 protein and levels of HO-1 activity were elevated by BI-1. Conversely, siRNA-mediated reductions in BI-1 were associated with decreased HO-1 levels and increased sensitivity to cell death. The modulation of HO-1 expression by BI-1 appears not to be a downstream consequence of differences in ROS, in that antioxidants (NAC and GSH) did not affect the ability of BI-1 to regulate HO-1 expression.
HO-1 may be important for the cytoprotective activity of BI-1, considering siRNA-mediated knock-down and pharmacological inhibition of HO-1 (especially using zinc protoporphyrin) nullified BI-1-mediated protection from cell death induced by ER stress inducers (thapsigargin and tunicamycin). Also, a mutant of BI-1 lacking a C-terminal domain required for cytoprotective activity also failed to induce HO-1 protein expression. However, caution must be exercised in interpretation of these results, because HO-1 and BI-1 could regulate parallel pathways affecting cell death, such that each contributes independently to resistance to ER stress-induced killing in an additive manner.

The gene encoding HO-1 is a known target of Nrf-2, a transcription factor that regulates ARE-driven gene expression and that plays an important role in expression of phase 2 detoxifying and antioxidant enzymes as well as in the activation of other stress-inducible genes, including GSH S-transferase, quinine reductase (NQO1), and HO-1 (30). Nrf-2 undergoes translocation from cytosol to nucleus when activated. Nrf-2 transcriptional activity and nuclear levels of Nrf-2 were elevated in BI-1 overexpressing cells, whereas partial reduction in Nrf-2 achieved by siRNA resulted in a corresponding partial reduction in HO-1 expression. Moreover, using a conditional promoter system (tetracycline-inducible), a direct cause-and-effect relation was demonstrated between overexpression of BI-1 and induction of Nrf-2 activity and nuclear accumulation of Nrf-2 protein. Thus, Nrf-2 is a candidate upstream inducer of HO-1 in the BI-1 pathway.

This study suggests that the elevated HO-1, by reducing ROS, limits the oxidative dysregulation that causes misfolding of ER proteins, thereby decreasing the unfolded protein response and explaining why several markers of ER stress (CHOP and Grp78 expression, JNK and eIF2a phosphorylation, and sXBP-1 production) are reduced in BI-1-overexpressing cells.

Although BI-1 was shown to regulate basal levels of HO-1 expression in HT1080 and HeLa cells in culture, comparison of tissues from BI-1 mice showed that homozygous deficiency of BI-1 does not alter tissue levels of HO-1 protein in testis, whereas HO-1 was reduced in liver and kidney. Thus, additional factors in addition to BI-1 protein presumably contribute to the regulation of HO-1 expression in vivo in some tissues.

Although BI-1 overexpression induces HO-1 expression, exposure of cells to ER stress-inducing agents also induces increases in HO-1 protein levels and HO-1 activity. When taken together with our data showing that overexpression of HO-1 is protective while siRNA-mediated knock-down of HO-1 sensitizes to ER stress-induced apoptosis, this observation suggests that the induction of HO-1 expression following pharmacologically triggered ER stress is an adaptive response whereby cells attempt to reestablish homeostasis. We therefore speculate that this adaptive response is inadequate for protecting control cells from ER stress-induced killing, whereas BI-1-
overexpressing cells start with higher basal HO-1 and thus have a survival advantage. This interpretation is consistent with previously published work on the role of HO-1 in compensatory protection from ER stress (31).

The ability of BI-1-induced HO-1 to block ER stress-mediated apoptosis could be of clinical relevance. Cell death following ER stress is believed to contribute to the progression of neurodegenerative disorders such as Alzheimer and Parkinson disease, as well as to the loss of pancreatic β cells in diabetes (32–34). Furthermore, ER stress might underlie the cell death that occurs after ischemia/reperfusion. Therefore, approaches that increase BI-1 or HO-1 expression or activity might provide a promising route to new therapeutics strategies for ameliorating a variety of clinical disorders related to ER stress.

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