Critical Role of Endogenous Akt/IAPs and MEK1/ERK Pathways in Counteracting Endoplasmic Reticulum Stress-induced Cell Death*

Received for publication, July 8, 2004, and in revised form, August 31, 2004
Published, JBC Papers in Press, August 31, 2004, DOI 10.1074/jbc.M407700200

Ping Hu‡§, Zhang Han‡¶, Anthony D. Couvillon‡, and John H. Exton‡∥

From the ‡Howard Hughes Medical Institute, Department of Molecular Physiology and Biophysics, and the ¶Department of Urologic Surgery, Vanderbilt University Medical Center, Nashville, Tennessee 37232-0615

Endoplasmic reticulum (ER) stress has been implicated in the pathogenesis of many diseases and in cancer therapy. Although the unfolded protein response is known to alleviate ER stress by reducing the accumulation of misfolded proteins, the exact survival elements and their downstream signaling pathways that directly counteract ER stress-stimulated apoptotic signaling remain elusive. Here, we have shown that endogenous Akt and ERK are rapidly activated and act as downstream effectors of phosphatidylinositol 3-kinase (PI3K)-Akt signaling. We demonstrate that endogenous PI3K/Akt and MEK/ERK plays a critical role in controlling cell survival by resisting ER stress-induced cell death signaling.

The endoplasmic reticulum (ER) is a highly dynamic organelle that synthesizes and folds intraorganellar, secretory, and transmembrane proteins. Disruption of ER homeostasis interferes with protein folding and leads to the accumulation of unfolded and misfolded proteins in the ER lumen. This condition has been designated “ER stress” (1). ER stress can be triggered by a number of stimuli that perturb ER function, such as Ca²⁺ depletion from the ER lumen, inhibition of N-linked glycosylation, reduction of disulfide bonds, overexpression of certain proteins, and nutrient/glucose deprivation (2). ER stress has been implicated in the pathogenesis of a variety of human diseases, including neuronal degenerative diseases, such as Alzheimer’s disease (3), Parkinson’s disease (4), and diabetes (5); viral pathogenesis (6); and some genetic diseases (2). It is also related to cancer therapy (7). The balance between cell survival responses and cell death responses decides the occurrence and development of these diseases. Thus, a better understanding of cell survival responses to ER stress can aid the understanding and development of new therapies for these diseases.

The unfolded protein response (UPR) is an important adaptive response to ER stress, which includes transcriptional induction of UPR genes, translational attenuation of global protein synthesis, and ER-associated protein degradation (8). In mammals, three ER transmembrane proteins, IRE1, ATF6, and PERK, mediate the UPR. Activated IRE1 and ATF6 stimulate transcription of ER chaperone genes (9). On the other hand, PERK phosphorylates the translation initiation factor eIF2α, which halts translation and prevents the continual accumulation of newly synthesized proteins in the ER (10). ER-associated protein degradation stimulates the degradation and clearance of unfolded proteins in the ER lumen (11, 12). However, if these adaptive responses are not sufficient to relieve the ER stress, the cell dies through apoptosis.

Apoptosis is primarily mediated by a family of caspases. Two separable pathways leading to caspase activation have been characterized. The extrinsic pathway is initiated by ligation of transmembrane death receptors to activate caspase-8 and -10. The intrinsic pathway requires disruption of the mitochondrial membrane and release of mitochondrial proteins, including Smac/DIABLO, HtrA2, and cytochrome c. Cytochrome c functions with Apaf-1 to induce activation of caspase-9, thereby initiating the apoptotic caspase cascade, whereas Smac/DIABLO and HtrA2 bind to and antagonize IAPs (inhibitor of apoptosis protein) (13). It has been reported that activation of caspase-3, -4, -8, -9, and -12 is required for ER stress-induced cell death (14–18). The UPR rescues cells mainly through removing unfolded proteins. It has not been reported that the UPR has a direct inhibitory effect on the extrinsic and intrinsic apoptotic pathways. Here, we hypothesize that ER stress activates in parallel UPR and cell survival mechanisms that counteract apoptotic signals and facilitate UPR function.

In this study, we evaluate the hypothesis that certain survival elements directly counteract ER stress-induced apoptotic signaling. We demonstrate that endogenous PI3K/Akt and

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Both authors contributed equally to this work.
¶ To whom correspondence should be addressed: Dept. of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, 702 Light Hall, Nashville, TN 37232-0615. Tel.: 615-322-8494; Fax: 615-322-4381; E-mail: john.exton@vanderbilt.edu.
∥ The abbreviations used are: ER, endoplasmic reticulum; UPR, unfolded protein response; eIF, eukaryotic initiation factor; IAP, inhibitor of apoptosis protein; PI3K, phosphatidylinositol 3-kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; MTT, 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; pAb, polyclonal antibody; JNK, c-Jun N-terminal kinase; RT, reverse transcription; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; DN, dominant-negative; MAPK, mitogen-activated protein kinase; ASK1, apoptosis signal-regulating kinase-1; PAK, p21-activated kinase.
MEK1/ERK are acutely activated in ER stress and subsequently prevent the apoptosis response. We examine the possible interaction between the survival elements and anti- or pro-apoptotic genes. We demonstrate that endogenous Akt is required for transcriptional regulation of specific IAPs. Furthermore, we show that ablation of these IAPs sensitizes stressed cells to death and that this can be reversed by a caspase inhibitor. Smac/DIABLO are indeed released during ER stress. These findings reveal a previously unrecognized important response that controls cell survival during ER stress.

EXPERIMENTAL PROCEDURES

Materials—Tunicamycin, thapsigargin, Hoechst 33342, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. The broad-spectrum caspase inhibitor Z-VAD-fmk, the MEK1 inhibitor U0126, and the PI3K inhibitor LY294002 were obtained from Calbiochem. The mTOR inhibitor rapamycin was purchased from Cell Signaling Technology.

Cell Culture—The human breast cancer cell line MCF-7 and its stable transfectants, the human prostate cancer cell line PC-3, and the human lung cancer cell line SH-SY5Y were grown in Dulbecco’s modified Eagle’s medium. The human lung cancer cell line H1299 was cultured in RPMI 1640 medium. All media were supplemented with 10% (v/v) fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Before being challenged with tunicamycin or thapsigargin, the cells were serum-starved for 24 h.

Plasmids and Establishment of Stable Cell Lines—pcMV-HA-MEK1(K97M), encoding dominant-negative Akt (K179M) (a gift of Dr. Carlos L. Arteaga, Vanderbilt University), was digested with EcoRI and XbaI and cloned into the pcDNA3.1(+) plasmid (Invitrogen). pcDNA3.1(+) plasmid (Invitrogen) was digested with EcoRI and XbaI and cloned into the pcdNA3.1(+) (+) HA vector. The pMCL (+) HA-tagged MEK1(K97M) construct, containing dominant-negative Akt (K179M) (a gift of Dr. Natalie Ahn, University of Colorado, Boulder, CO), was digested with KpnI and HindIII and subcloned into the pcdNA3.1(+) plasmid (Invitrogen). pcDNA3.1(+) HA-Akt(K179M), pcDNA3.1(+) HA-Thapsigargin (Invitrogen), and pcDNA3.1(+) HA-MEK1(K97M) and pcDNA3.1(+) HA-XIAP (Invitrogen) were used to transfect MCF-7 and H1299 cells with each construct using LipofectAMINE Plus (Invitrogen). The stably expressing cells were selected in the presence of G418 (800 μg/ml) for 4 weeks, and the surviving clones were pooled (mass culture). The transfectants were identified by Western blotting by anti-hemagglutinin (HA) antibody. For each construct, three mass cultures were used for further experiments.

Western Blotting and Antibodies—After treatment as indicated, cells were washed once with phosphate-buffered saline and extracted with SDS sample buffer. Equal amounts of protein were subjected to electrophoresis on Novex Tris-glycine gels (Invitrogen) and transferred to polyvinylidine difluoride membranes (Millipore Corp.). After blocking, the membranes were incubated with each primary antibody, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. The protein bands were visualized using the ECL detection system (Amersham Biosciences). The following antibodies were used in our study: anti-ERF2a pAb, anti-phospho-Ser248 Tyr242 Akt pAb, anti-ERK1/2 pAb, anti-phospho-Thr202 Tyr204 ERK1/2 pAb, anti-phospho-Thr183 Tyr182 p38, anti-phospho-Thr183 Tyr182 JNK pAb, anti-phospho-Thr202 Tyr204 p38, anti-phospho-Thr202 Tyr204 Akt pAb, anti-Akt pAb, anti-phospho-Ser240 Tyr244 mTOR pAb, and anti-XIAP pAb (Cell Signaling Technology); anti-CAPI-2 monoclonal antibody (Chemicon International, Inc.); anti-β-actin monoclonal antibody (Sigma); and anti-HA monoclonal antibody (Covance).

RT-PCR—Cells were treated with the indicated reagents, and total RNA was extracted by the TRIzol method (Invitrogen). It was subjected to RT-PCR in a two-step protocol using SuperScriptTM II reverse transcriptase and Taq polymerase. The number of cycles and the annealing temperature were adjusted depending on the genes amplified.

Ablation of Endogenous cIAP-2 and XIAP by RNA Interference—Ablation of the two genes was achieved by transfection of specific siRNA duplexes using the RNAiMAX reagent (Invitrogen). The siRNA sequences were selected by褲anther et al. (2007). These sequences were used to transfect MCF-7 cells. The surviving clones were selected by G418 treatment (800 μg/ml) and cultured in mass culture. The transfectants were characterized by Western blotting of anti-HA (HA) and anti-XIAP pAb. For each construct, three mass cultures were used for further experiments.

RESULTS

Thapsigargin or Tunicamycin Induces the Unfolded Protein Response and Apoptosis—Incubation of human breast cancer MCF-7 cells with the ER stressors thapsigargin and tunicamycin rapidly induced mRNA expression of two known UPR target genes, viz. the ER resident molecular chaperone BiP and the transcription factor CHOP (Fig. 1A). Phosphorylation of the translation initiation factor eIF2α was also observed under the same conditions (Fig. 1B). These results are consistent with published reports that ER stress induces cell death in MCF-7 cells (Fig. 1D) and human lung cancer H1299 cells (data not shown) in a time-dependent manner. Furthermore, co-incubation of MCF-7 cells with the broad-spectrum caspase inhibitor Z-VAD-fmk significantly increased thapsigargin-induced cell death from 48 to 29%. Similar results were obtained in tunicamycin-treated cells (Fig. 1E). These data indicate that ER stress-induced cell death is caspase-dependent.

ER Stress-induced Activation of Endogenous PI3K/Akt Is a Cell Survival Response—In addition to the UPR, it remains

2 Primer sequences are available upon request.
Survival Responses to ER Stress

Thapsigargin and tunicamycin are two pharmacologically distinct ER stress inducers. Activation of the Akt pathway by both thapsigargin and tunicamycin in MCF-7 cells demonstrated that ER stress-induced Akt activation was not restricted to one ER stressor. To further explore whether ER stress-induced Akt activation is a general event among different cells, we investigated ER stress-induced Akt activation in cells originating from different human organs. Thapsigargin or tunicamycin also acutely induced phosphorylation of Akt in human lung cancer H1299 cells (Fig. 2B) and human prostate cancer PC-3 cells (data not shown). These results indicate that acute activation of the Akt pathway is a rather general cell response to ER stress.

To elucidate whether activation of the PI3K/Akt pathway is required to protect cells from ER stress-induced cell death, we first assessed the effect of the PI3K inhibitor LY294002. As shown by phase-contrast microscopy and a cell viability assay (MTT assay) in Fig. 2 (C and D), co-incubation with LY294002 significantly sensitized MCF-7 cells to thapsigargin- or tunicamycin-induced cell death. LY294002 treatment alone had no effect on cell viability. Similar results were obtained when the same experiments were performed with H1299 cells (Fig. 2D). To further confirm that Akt signaling is protective of cell survival during ER stress, we established MCF-7 cell pools stably expressing HA-tagged dominant-negative (DN) Akt in which a point mutation (K197M) was introduced at a site required for kinase activity. Control cells were transfected with empty vector. Stable transfectants were identified by Western blotting with an HA-specific monoclonal antibody. A cell viability assay showed that ectopic expression of DN-Akt significantly increased thapsigargin- and tunicamycin-induced cell death (Fig. 2E). Similar results were obtained in H1299 cells expressing DN-Akt (data not shown). These results indicate that activation of the PI3K/Akt pathway is a critical cell survival response to ER stress.

Akt-dependent Activation of mTOR Is Not Required for Akt-mediated Survival Signaling in ER Stress—Recent research has shown that mTOR, an established Akt effector, is essential for Akt-mediated survival signaling (24, 25). There is also much evidence that mitogens and growth factors stimulate phosphorylation of mTOR at Ser2448 (26, 27). It was therefore interesting to investigate the role of mTOR in regulation of ER stress considering its critical controlling role in protein synthesis. Acute phosphorylation of mTOR at Ser2448 was found in MCF-7 cells treated with thapsigargin or tunicamycin, which was blocked by either LY249002 or expression of DN-Akt (Fig. 3A). Next, we tested whether activated mTOR contributes to cell survival during ER stress. As shown in Fig. 3B, co-incubation relative to that of MeSO-treated controls. E, caspase-dependent cell death induced by ER stress. MCF-7 cells were exposed to thapsigargin (2 μM) or tunicamycin (2 μg/ml) with or without the broad-spectrum caspase inhibitor Z-VAD-fmk (zVAD; 100 μM) for 28 h. The percentage of cell death was measured by MTT assay as described for D.
tion of MCF-7 cells with rapamycin, an inhibitor of mTOR, did not increase ER stress-induced cell death. Our data indicate that ER stress induces phosphorylation of mTOR in an Akt-dependent manner; however, this is not involved in cell survival.

ER Stress-induced Endogenous ERK Activation Protects Cells from ER Stress-induced Cell Death—

In addition to the PI3K/Akt pathway, the MAPK family, comprising ERK, JNK, and p38 MAPK, also mediates many stress responses. Although the role of the ERK pathway in regulation of apoptosis in mammalian cells is controversial, it is well established in Drosophila that the activated Ras/Raf/MEK/ERK pathway promotes survival through phosphorylating head involution-defective protein (28). The exact role of ERK in ER stress has not been characterized. Activation of ERK occurs through phosphorylation of Thr and Tyr residues by an upstream MAPK kinase (MEK1). To investigate the role of the MAPK family in ER stress, we first tested whether MAPK is activated upon ER stress. Phosphorylation of ERK1/2 was induced in MCF-7 cells after exposure to thapsigargin or tunicamycin. JNK was also phosphorylated, consistent with previous reports (29, 30), but the phosphorylation of p38 was not induced (Fig. 4A).

Confirming the activation of the ERK pathway, the MEK1 inhibitor U0126 completely inhibited thapsigargin-induced ERK1/2 phosphorylation (see Fig. 5B). ER stress-induced activation of ERK was also observed in H1299 cells (Fig. 4B).
Next, we examined whether activation of the MEK1/ERK pathway could protect cells from ER stress-induced cell death. As shown by phase-contrast microscopy and MTT assay in Fig. 4 (C and D), the MEK1 inhibitor U0126 increased thapsigargin- and tunicamycin-induced cell death in MCF-7 and H1299 cells. Treatment with U0126 alone had no effect on cell viability. To further confirm the protective role of the MEK1/ERK pathway, we generated stable MCF-7 cell pools expressing an HA-tagged DN-MEK1 mutant. Control cells were transfected with empty vector. Stable transfectants were identified by Western blotting with anti-HA antibody. MTT assay showed that expression of DN-MEK1 increased thapsigargin- and tunicamycin-induced cell death (Fig. 4E). Similar results were obtained in H1299 cells expressing DN-MEK1 (data not shown). These results suggest that activation of the MEK1/ERK pathway is also a cell survival response to resist ER stress.

**Pi3K Mediates ER Stress-induced ERK Activation**—The aforementioned results demonstrated that ER stress activated both the Pi3K/Akt and MEK1/ERK pathways, so it was interesting to investigate whether there is cross-talk between the two pathways. MTT assay revealed that, in MCF-7 cells, inhibition of Pi3K with LY294002 resulted in more ER stress-induced cell death compared with treatment with U0126 (Fig. 5A). The effect of cotreatment with both LY294002 and U0126 on ER stress-induced cell death was similar to that with LY294002 alone (Fig. 5A). These results suggest that Pi3K may be an upstream signal that activates ERK during ER stress. To explore the relationship between Pi3K and ERK, MCF-7 cells were incubated with thapsigargin in the presence of LY294002, U0126, or vehicle (Me2SO). Western blotting showed that U0126 totally inhibited ERK activation, but had little or no effect on thapsigargin-induced phosphorylation of Akt (Fig. 5B). On the other hand, LY294002 completely blocked Akt activation, but also significantly decreased thapsigargin-induced phosphorylation of ERK1/2 (Fig. 5B). Similar results were obtained in tunicamycin-treated cells (data not shown). These results reveal that, in MCF-7 cells, ER stress-induced activation of ERK is Pi3K-dependent.

**ER Stress-induced Expression of cIAP-2 and XIAP Is Required for Sustaining the Survival of Cells under ER Stress**—Several lines of evidence indicate that caspase activation is an important component of ER stress-induced cell death (14–18). Our results showed that the broad-spectrum caspase inhibitor Z-VAD-fmk reduced ER stress-induced cell death compared with treatment with U0126 (Fig. 1E). Since Bel-2 and IAP family proteins play a critical role in regulation of caspase-dependent cell death, we investigated the expression pattern of genes for both the Bel-2 and IAP families during ER stress. As indicated in Fig. 6A, high level transcription of cIAP-2 and XIAP, two members of the IAP family, rapidly occurred in thapsigargin-treated MCF-7 cells, whereas the transcription of cIAP-1 and survivin was not significantly induced. However, RT-PCR could not detect altered transcription of Bel-2 family members under the same conditions, including the pro-apoptosis genes bax, bak, bik, bim, and bid and...
In the presence of thapsigargin (TG, 2 μM) or tunicamycin (TU, 2 μg/ml) with Me2SO (DMSO) as a control, LY294002 (LY, 20 μM), U0126 (20 μM), and LY294002 plus U0126 for 30 h. Cell viability was scored by MTT assay. Results are expressed as the means ± S.E. for three independent experiments. Cell survival is expressed as absorbance relative to that of the relevant controls, viz. Me2SO, Me2SO plus LY294002, Me2SO plus U0126, and Me2SO plus LY294002 and U0126. MCF-7 cells treated for 1 h with vehicle (Me2SO), LY294002 (20 μM), and U0126 (20 μM) were exposed to thapsigargin (2 μM) for the indicated times. The whole cell lysates were subjected to Western blotting with the antibodies indicated.

If induction of cIAP-2 and XIAP is a critical survival response to ER stress, decreasing the levels of endogenous cIAP-2 and/or XIAP should increase ER stress-induced cell death. To test whether ablation of IAPs increases the sensitivity to cell death through promoting activation of caspases, we co-incubated MCF-7 cells with the general caspase inhibitor Z-VAD-fmk. Phase-contrast microscopy and MTT assay showed that Z-VAD-fmk reversed the sensitization effect caused by knockdown of the IAPs (Fig. 6, D and E). Similar results were obtained with SH-SY5Y cells (data not shown). Our data indicate that cIAP-2 and XIAP are essential for cells to survive ER stress by inhibiting caspase activation.

One important function of IAPs in the prevention of apoptosis is to bind RHG motif-containing proteins, such as Smac/DIABLO and HtrA2/Omi, which are released from mitochondria to the cytosol to promote apoptosis (31, 32). The critical protective role of IAPs in ER stress led us to investigate whether Smac is released from mitochondria during ER stress. For this purpose, the subcellular location of Smac was examined by immunofluorescence. As shown in Fig. 7A, in MCF-7 cells, Smac staining showed a punctate perinuclear pattern that matched MitoTracker staining. Thapsigargin treatment for 12 h induced redistribution of Smac to a diffused cytosolic pattern. Subcellular fractionation analysis of the Smac protein further supported the immunostaining results. The Smac protein was detected in the soluble cytosolic fraction in response to thapsigargin treatment (Fig. 7B). Similar results were found with tunicamycin (data not shown). Our results suggest that Smac release from mitochondria may play a key role in ER stress-induced cell death and that IAPs protect cells through inhibiting caspase activation by neutralizing Smac. These data indicate that induction of IAPs is an important cell survival response to ER stress.

**Induction of cIAP-2 and XIAP Depends on Activation of PI3K/Akt**—The aforementioned results suggested that PI3K-dependent activation of Akt and the ERK pathway is a cell survival response. It was therefore interesting to determine a possible role for activated Akt and ERK in ER stress-stimulated expression of cIAP-2 and XIAP. Co-treatment with LY294002 or overexpression of DN-Akt significantly decreased thapsigargin-induced transcription of cIAP-2 and XIAP (Fig. 8A), and expression of cIAP-2 and XIAP proteins was also inhibited (Fig. 8B), as shown by RT-PCR and Western blotting, respectively. Similar results were obtained in tunicamycin-treated cells (data not shown). In contrast, we failed to detect a significant effect of DN-MEK1 on the expression levels of cIAP-2 and XIAP (Fig. 8B). The MEK inhibitor U0126 also failed to suppress cIAP-2 and XIAP expression (Fig. 8B). These results indicate that the PI3K/Akt survival pathway, not the ERK pathway, increases cIAP-2 and XIAP function by regulating their transcription in response to ER stress.

**DISCUSSION**

The fate of cells under ER stress is dependent on the balance between cell adaptive responses and cell death responses. Much progress has been made recently to gain insight into the mechanisms of ER stress-induced cell death. Caspase-3, -4, -9, and -12 and Bax, Bak, and PUMA from the Bcl-2 family have been demonstrated to mediate ER stress-induced apoptosis (14–18, 33). However, studies of cell adaptive responses have focused mainly on the UPR, which allows cells to survive through removal of misfolded proteins. In this study, we have presented evidence that, in addition to the UPR, ER stress activates in parallel some endogenous cell survival mechanisms that directly prevent apoptotic signals to provide time for the UPR to function. These mechanisms involve Akt, the ERK
pathway, and the anti-apoptotic proteins cIAP-2 and XIAP.

Akt is a serine/threonine protein kinase that is mainly regulated by PI3K. An important function of activated PI3K/Akt in cells is the inhibition of apoptosis. Activated Akt can protect cells from different kinds of apoptotic stimulation, including growth factor withdrawal, Fas ligand interaction, oxidative stress, N-methyl-D-aspartate, UV irradiation, matrix detachment, cell cycle discordance, DNA damage, transforming growth factor-β, and chemotherapeutic agents (23, 25). A number of pro-apoptotic proteins have been identified as direct Akt substrates, including BAD, caspase-9, Forkhead transcription factors, and ASK1, which are inactivated upon phosphorylation by Akt (23, 34). The role of Akt in regulation of ER stress-induced cell death has not been characterized. We found that Akt was activated early in several cell lines (MCF-7, PC-3, and H1299) during ER stress. The activation was shown to be mediated by PI3K since the PI3K inhibitor LY294002 completely blocked its phosphorylation in MCF-7 cells. More im-

![Fig. 6](http://www.jbc.org/Downloaded from)

**Fig. 6.** ER stress-stimulated induction of cIAP-2 and XIAP is required for cell survival during ER stress. A, ER stress-induced transcription of cIAP-2 and XIAP. MCF-7 cells were incubated with thapsigargin (TG; 2 μM) for the indicated times. Total RNA was prepared. The mRNA levels of the indicated genes were analyzed by semiquantitative RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control. B, protein expression of cIAP-2 and XIAP induced by ER stress. MCF-7 and SH-SY5Y cells were treated with 2 μM thapsigargin, 2 μg/ml tunicamycin (TU), or vehicle (Me₂SO). At the indicated time points, cells were extracted. 10 μg of total protein was separated on Novex Tris/glycine gels and probed using the indicated antibodies. β-Actin served as a loading control. C, ablation of cIAP-2 and/or XIAP protein by RNA interference. MCF-7 and SH-SY5Y cells were transfected with a 21-nucleotide siRNA duplex to cIAP-2 (cIAP-2 siRNA) and/or XIAP (XIAP siRNA) or control (Contr) siRNA (100 nM) using Oligofectamine. After two consecutive transfections with 24-h intervals, cells were treated with thapsigargin (2 μM) for the indicated times. Cells were harvested for Western blot analysis. β-Actin was used as a loading control. D, knockdown of cIAP-2 and/or XIAP by RNA interference sensitizes cells to ER stress-induced cell death. MCF-7 cells were transfected with the indicated siRNAs as described for C. Cells were incubated with thapsigargin (2 μM) or tunicamycin (2 μg/ml) in the presence or absence of Z-VAD-fmk (zVAD; 100 μM) and assessed by phase-contrast microscopy. DMSO, Me₂SO; Cont, control, Bar, 50 μM. E, quantification of cell viability after 13 h by MTT assay. Cell survival is expressed as absorbance relative to that of Me₂SO- or Me₂SO/Z-VAD-fmk-treated controls.
Importantly, inhibition of the PI3K/Akt pathway by either LY294002 or overexpression of kinase-dead mutant Akt significantly sensitized cells to ER stress-induced cell death. We therefore speculate that ER stress-induced activation of the endogenous PI3K/Akt pathway is a cell survival response.

How do the cells sense ER stress to activate PI3K? PERK and IRE1 are two ER resident kinases involved in regulation of ER stress. However, previous studies indicate that PI3K activation requires the binding of the p85 regulatory subunit to tyrosine-phosphorylated molecules (35). Considering that both PERK and IRE1 are Ser/Thr kinases, it seems that they are probably not directly responsible for ER stress-induced PI3K activation. Ca\(^{2+}\) is an important second messenger that is required for numerous cell functions. ER is the main intracellular storage compartment for Ca\(^{2+}\), and disruption of Ca\(^{2+}\) homeostasis is one of the most important characteristics of ER stress (33, 36–38). A recent study shows that Ca\(^{2+}\) and calmodulin play important roles in activation of PI3K (39), and activation of calmodulin and calcineurin is necessary for long-term survival of cells undergoing ER stress (40). Therefore, we speculate that activation of PI3K is a cell response to ER stress-induced increases in intracellular Ca\(^{2+}\) and that Ca\(^{2+}\) is a potential linker between ER stress and PI3K activation.

mTOR, an established target of Akt, regulates translation in response to nutrients and growth factors by phosphorylating key components of the protein synthesis machinery, including the ribosomal protein S6 kinase, p70\(^{S6K}\) and the eIF4E-binding protein, 4E-BP1 (41). It has been suggested that mTOR is involved in regulation of cell survival (24, 25). However, another study has shown that the negative regulator of mTOR, TSC2, protects cells from glucose depletion-induced apoptosis (26). This discrepancy is likely due to differences in stress and cell types. Our results show that, although Akt-dependent phosphorylation of mTOR was induced in ER stress, it was not involved in regulation of cell death because the mTOR inhibitor rapamycin had no effect on thapsigargin- or tunicamycin-induced cell death. We therefore propose that mTOR is not required for Akt-mediated survival signaling in ER stress.

The MAPK family, comprising JNK, p38 MAPK, and ERK, mediates stress responses. Activation of JNK during ER stress is mediated by IRE1 (29), and primary neurons from ASK1-knockout mice are defective in ER stress-induced JNK activation and cell death (30). Unlike in Drosophila, the role of ERK in regulation of cell death in mammalian cells is controversial. In some cases, ERK activation exerts a pro-apoptotic influence (42, 43), but in most cases, it provides anti-apoptotic signals (44, 45). It is probable that the kinetics and duration of ERK activation determine whether it acts in a pro-apoptotic or anti-apoptotic manner. In our study, we found that ER stress activated the ERK pathway early in different cell types. We have also provided evidence that ER stress-induced activation of...
ERK provides survival signals. For example, either the MEK1 inhibitor U0126 or expression of DN-MEK1 made cells vulnerable to ER stress-induced cell death. We therefore propose that activation of the endogenous MEK1/ERK signaling pathway in ER stress is a cell survival response.

Cross-talk between the PI3K and Raf/MEK1/ERK pathways has been reported to occur at multiple levels. Some research indicates that PI3K activity is essential for activation of the Raf/MEK1/ERK cascade (46). Other studies suggest that the PI3K pathway enhances and/or synergizes with the Raf/MEK1/ERK pathway to provide a more robust signal (47). In our studies, we demonstrated that, in MCF-7 cells, U0126 had no effect on thapsigargin-induced phosphorylation of Akt, but LY294002 significantly inhibited phosphorylation of ERK1/2. We therefore speculate that PI3K is an upstream signaling pathway mediating activation of ERK in ER stress. It has been reported that members of the PAK family may play a role in regulation of PI3K-mediated activation of the Raf/MEK1/ERK cascade (48, 49). MEK1 has recently been proved to be directly phosphorylated by PAK1 to regulate ERK activation (50). However, PI3K can also attenuate Raf activity through Akt (51). In our experiments, we found that, in contrast to LY294002, expression of kinase-dead Akt had no effect on ER stress-induced ERK activation. According to these results, we propose that signals from activated PI3K diverge to activate both Akt and the Raf/MEK1/ERK cascade in ER stress. Whether Rac1 and PAK are involved in PI3K-dependent activation of ERK in ER stress needs further investigation.

Caspases are the primary mediators of apoptosis. It has been reported that activation of caspase-3, -4, -8, -9, and -12 plays a central role in ER stress-induced cell death (14–18). The members of the IAP family are the only known cellular caspase inhibitors. The IAP family includes XIAP, cIAP-1, cIAP-2, survivin, neuronal apoptosis inhibitory protein, melanoma IAP, and Bruce, all of which contain one or more repeats of the characteristic baculovirus IAP repeat BIR domain. IAPs block apoptosis in many cells exposed to different kinds of apoptotic stimulus (52, 53). Our data show that the expression of cIAP-2 and XIAP was significantly induced at the mRNA and protein levels in different human cells under ER stress. It is interesting to note that the expression of IAPs is induced by ER stress because the synthesis of XIAP is controlled by a unique mechanism. The 5′-untranslated region of XIAP mRNA contains an internal ribosome initiation site or internal ribosome entry site element (54). Internal ribosome entry site-containing transcripts can continue to direct protein synthesis under a number of cellular stress conditions in which cap-dependent translation is shut down. A recent study shows that an internal ribosome entry site element also exists in the transcript of cIAP-1 (55). The internal ribosome entry site element seems to be a general feature in IAPs.

In our experiments, reduction of the level of cIAP-2 or XIAP in MCF-7 and SH-SY5Y cells by RNA interference sensitized the cells to ER stress-induced cell death. The IAPs can regulate caspase activity by binding directly to activated caspases and inhibiting their function (52, 56). On the other hand, some IAPs, such as XIAP, have anti-apoptotic activities unrelated to their ability to inhibit caspases (57). Our data show that the general caspase inhibitor Z-VAD-fmk reversed the effect of knockdown of the IAPs on ER stress-induced cell death. This indicates that ablation of these IAPs increases ER stress-induced cell death by promoting caspase activation. The fact that ablation of IAPs induces only a partial decrease in cell viability suggests that some other signaling pathways may exist. Both cIAP-2 and XIAP can bind to mammalian RING motif-containing proteins, such as Smac/DIABLO and HtrA2/Omi, which promote apoptosis (23, 24). The ubiquitin-protein isopeptide ligase activity of IAPs can also down-regulate Smac through protein degradation (58). A proposed model of IAP inhibition of the amplification of caspase activation is through binding and neutralizing released Smac. The critical protective role of IAPs in ER stress may indicate the important role of Smac release from mitochondria in ER stress-induced cell death.

We have further shown that enhanced transcription and translation of cIAP-2 and XIAP were mediated by activated Akt in MCF-7 cells. The transcription factor that is responsible for Akt-dependent induction of cIAP-2 and XIAP needs further investigation. Inhibition of the PI3K/Akt pathway does not completely block ER stress-induced expression of IAPs. It is therefore possible that other mechanisms might operate. It has been reported that NF-κB and the cAMP/cAMP-responsive element-binding protein pathway activate induction of cIAP-2 and XIAP (59, 60). Whether these pathways are involved in ER stress-induced expression of IAPs needs further study. Except for regulation at the level of transcription, modulation of the abundance of IAPs also occurs at the translational and post-translational levels. Fibroblast growth factor-2 increases expression of XIAP and cIAP-1 principally through translational regulation in an ERK-dependent manner (61). A recent study shows that Akt phosphorylates XIAP at Ser87 and that phosphorylated XIAP is more resistant to ubiquitination-induced degradation (62). Post-translational modification might play an important role in the induction of IAPs during ER stress.

In summary, we have provided evidence that acute activation of endogenous Akt/IAPs and MEK/ERK governs cell survival during ER stress by directly counteracting ER stress-induced cell death (Fig. 9). We propose that this is an important physiological response to ER stress. We have presented evidence that the IAP induction required for suppressing cell death is regulated by Akt. Akt/IAPs can protect against cell death by directly interfering with the post-mitochondrial apoptotic pathway. The activation of Akt/IAPs and ERK was observed in different cell types, suggesting that this is a common response during ER stress in mammals. The critical protective role of the IAPs in ER stress relates to the fact that they bind or stimulate degradation of Smac, which is released from mi-
tochondria to the cytosol to promote caspase activation. These results provide significant novel insights into the molecular mechanisms for anti-apoptotic behavior in ER stress. This study appears to be the first identification of endogenous Akt/ IAPs and ERK signaling pathways in regulation of cell fate in ER stress.

Acknowledgments—We thank Dr. Carlos L Arteaga for the dominant-negative MEK1 plasmid and Dr. Natalie Ahn for the dominantly-negative MEK1 plasmid.

REFERENCES

1. Kaufman, R. J. (1999) Genes Dev. 13, 1211–1233
2. Kaufman, R. J. (2002) J. Clin. Investig. 110, 1389–1398
3. Katayama, T., Imai, Y., Soda, M., Inoue, H., Hattori, N., Mizuno, Y., and Takahashi, R. (2001) Cell 105, 891–902
4. Burstein, E., Ganesh, L., Dick, R. D., Van De Sluis, B., Wilkinson, J. C., Klomp, L. W., Wijmenga, C., Brewer, G. J., Nabel, G. J., and Duckett, C. S. (2004) J. Cell Biol. 165, 286–297
5. Imai, Y., Soda, M., Inoue, H., Hattori, N., Mizuno, Y., and Takahashi, R.(2001) Cell 106, 158–166
6. Hitomi, J., Katayama, T., Eguchi, Y., Kudo, T., Taniguchi, M., Koyama, Y., and Yuan, J. (2000) Nature 403, 891–895
7. Xia, G. H., Jeffers, M., Bellacona, M., Mitsuishi, Y., Vande Woude, G. F., and Testa, J. R. (2003) Proc. Natl. Acad. Sci. U. S. A. 99, 247–252
8. Baranano, D. E., Rao, M., Ferris, C. D., and Snyder, S. H. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16993–16998
9. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) Genes Dev. 13, 2905–2927
10. Chung, J., Bachelor, R. E., Lipscomb, E. A., Shaw, L. M., and Mercurio, A. M. (2000) J. Cell Biol. 150, 165–174
11. Wendel, H. G., De Stanchina, E., Fridman, S. A., Malina, A., Ray, S., Kogan, M., Cardon-Cordero, C., Pelletier, J., and Lowe, S. W. (2004) Nature 428, 332–337
12. Inoki, K., Zhu, T., and Guan, K. L. (2003) Mol. Cell. Biol. 23, 7696–7706
13. Kam, Y., and Exton, J. H. (2004) FASEB J. 18, 311–319
14. Bergmann, A., Agapite, J., McCall, K., and Steller, H. (1998) Cell 95, 331–341
15. Imai, Y., Soda, M., Inoue, H., Hattori, N., Mizuno, Y., and Takahashi, R.(2001) Cell 106, 158–166
16. Plemper, R. K., Bohmler, S., Bordallo, J., Sommer, T., and Wolf, D. H. (1997) J. Cell Biol. 138, 153–164
17. Jimbo, A., Fujita, E., Kouroku, Y., Ohnishi, J., Inohara, N., Kuida, K., Sabatini, D. D., and Ron, D. (2001) Genes Dev. 15, 138–149
18. Hitomi, J., Katayama, T., Eguchi, Y., Kudo, T., Taniguchi, M., Koyama, Y., and Yuan, J. (2000) Nature 403, 891–895
19. Xia, G. H., Jeffers, M., Bellacona, M., Mitsuishi, Y., Vande Woude, G. F., and Testa, J. R. (2003) Proc. Natl. Acad. Sci. U. S. A. 99, 247–252
20. Baranano, D. E., Rao, M., Ferris, C. D., and Snyder, S. H. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16993–16998
21. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) Genes Dev. 13, 2905–2927
22. Chung, J., Bachelor, R. E., Lipscomb, E. A., Shaw, L. M., and Mercurio, A. M. (2000) J. Cell Biol. 150, 165–174
23. Wendel, H. G., De Stanchina, E., Fridman, S. A., Malina, A., Ray, S., Kogan, M., Cardon-Cordero, C., Pelletier, J., and Lowe, S. W. (2004) Nature 428, 332–337
24. Inoki, K., Zhu, T., and Guan, K. L. (2003) Mol. Cell. Biol. 23, 7696–7706
25. Kam, Y., and Exton, J. H. (2004) FASEB J. 18, 311–319
26. Bergmann, A., Agapite, J., McCall, K., and Steller, H. (1998) Cell 95, 331–341
27. Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, Y., Chong, H., and Harding, H. P., and Ron, D. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 44701–44708
28. Chae, H. J., Kim, H. R., Xu, C., Baily-Maitre, B., Krajewska, M., Krajewski, S., Yamamoto, A., Kuida, K., and Tanaka, S. (2000) J. Cell Biol. 148, 333–342
29. Whitlock, B. B., Gardali, S., Fadok, V., Bratton, D., and Henson, P. M. (2000) J. Cell Biol. 151, 1305–1320
30. Bergmann, A., Agapite, J., McCall, K., and Steller, H. (1998) Cell 95, 331–341
Critical Role of Endogenous Akt/IAPs and MEK1/ERK Pathways in Counteracting Endoplasmic Reticulum Stress-induced Cell Death
Ping Hu, Zhang Han, Anthony D. Couvillon and John H. Exton

J. Biol. Chem. 2004, 279:49420-49429.
doi: 10.1074/jbc.M407700200 originally published online August 31, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M407700200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 62 references, 36 of which can be accessed free at http://www.jbc.org/content/279/47/49420.full.html#ref-list-1