Endoplasmic Reticulum Stress-induced Death of Mouse Embryonic Fibroblasts Requires the Intrinsic Pathway of Apoptosis*§

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Members of the caspase family are essential for many apoptotic programs. We studied mouse embryonic fibroblasts (MEFs) deficient in caspases 3 and 7 and in caspase 9 to determine the role of these proteases in endoplasmic reticulum (ER) stress-induced apoptosis. Both caspase 3−/− and caspase 9−/− MEFs were resistant to cytotoxicity induced via ER stress and failed to exhibit apoptotic morphology. Specifically, apoptosis induced by increased intracellular calcium was shown to depend only on caspases 3 and 9, whereas apoptosis induced by disruption of ER function depended additionally on caspase 7. Caspase 3−/− and caspase 9−/− MEFs also exhibited decreased loss of mitochondrial membrane potential, which correlated with altered caspase 9 processing, increased induction of procaspase 11, and decreased processing of caspase 12 in caspase 3−/− cells. Furthermore, disruption of ER function was sufficient to induce accumulation of cleaved caspase 3 and 7 in a heavy membrane compartment, suggesting a potential mechanism for caspase 12 processing and its role as an amplifier in the death pathway. Caspase 8−/− MEFs were not resistant to ER stress-induced cytotoxicity, and processing of caspase 8 was not observed upon induction of ER stress. This study thus demonstrates a requirement for caspases 3 and 9 and a key role for the intrinsic pathway in ER stress-induced apoptosis.

The endoplasmic reticulum (ER)3 is a critical organelle responsible both for the proper synthesis, folding, and modification of secretory and transmembrane proteins and lipids and for intracellular Ca2+ storage (1, 2). Thus, a significant number of ER resident proteins either sequester Ca2+ or function as molecular chaperones as part of the quality control machinery designed to monitor folding and prevent production of nonfunctional proteins (3). Unfolded and misfolded proteins are normally exported to cytosolic proteasomes, but the accumulation of such proteins or perturbation of the ER Ca2+ equilibrium leads to a condition known as ER stress and triggers unfolded protein response (UPR) signaling (1). This pathway results in attenuation of protein synthesis, via PERK and IRE1α, and ATF6-induced up-regulation of XBP-1 and ER chaperones BiP/GRP78 and CHOP/GADD153. Prolonged UPR signaling and ER stress lead to apoptosis. This latter case is pathological and has been implicated in several neurological diseases involving protein accumulation, including Alzheimer and Parkinson (2).

Caspases are a family of cysteinyl aspartate proteases that mediate apoptosis induced by many stimuli (4). The family can further be divided into inflammatory (caspases 1, 2, 4, 5, 10, 11, 12), initiator (caspases 2, 8, 9), and effector (caspases 3, 6, 7) subfamilies. Several groups have attempted to elucidate the specific caspase activation pathway responsible for the ER stress-induced apoptotic program. To date, it is known that depletion or efflux of ER luminal Ca2+ is a critical component of the pathway, and this is modulated by Bcl-2 family members, including Bax and Bak, on both the mitochondrial and ER membranes (5–7). Further, many groups have reported the involvement of initiator caspases 2, 8, and 9 and effector caspases 3 and 7 in ER stress-induced apoptosis. Current models suggest that caspase 12 acts as the initiator caspase in ER stress. Caspase 12 can be cleaved via IRE1α/TRAF2 (8), calpain (9), or caspase 7 (10) or induced by ATF6 (2), and cleaved caspase 12 can either directly process caspase 9 or cleave Bap31 (1, 3), resulting in mitochondrial permeabilization, cytochrome c release, and Apaf-1-mediated caspase 9 processing, leading to caspase 3 cleavage (4). Alternatively, caspase 12-mediated processing of caspase 9 has been proposed via Apaf-1- (11) and cytochrome c-independent (12) pathways. There is also debate about the role of caspase 12 because the knock-out is not protected from apoptosis (13–16), ER stress-inducers have been shown to activate caspase 11 (17), and inflammatory stimuli leading to IL-1β processing have been shown to induce ER stress (18). Recently, it has been suggested that caspase 12 is...
Cleaved downstream of the mitochondria in an Apaf-1-dependent manner (19) and that it co-translocates with AIF to the nucleus (20). Some groups have also postulated the existence of an extrinsic pathway of ER stress-induced apoptosis involving activation of caspase 8 (21) and subsequent cleavage of Bap31 (22, 23), whereas others have implicated caspase 2 (24, 25) as a potential initiator caspase. It is still not known, however, which of these caspases are required in vivo for ER stress-induced apoptosis under physiological conditions.

We have previously reported the creation of caspase 3−/−/caspase 7−/− mice and embryonic fibroblasts (MEFs) (26). We immortalized these cells, along with caspase 9−/− MEFs, to create stable cell lines to study the physiological roles of these effector caspases in ER stress-induced apoptosis. Our findings indicate that caspase 3−/−/caspase 7−/− and caspase 9−/− MEFs are consistently resistant to apoptosis by canonical ER stress-inducing stimuli, which correlates with upstream effects on caspase 9 and caspase 12 processing by caspase 3 in wild-type cells. These effector caspases were also shown to accumulate in a heavy membrane compartment, and their absence preserved mitochondrial membrane potential during stress. Thus, we have demonstrated that ER stress-induced apoptosis is primarily dependent on the mitochondrion and the intrinsic pathway.

**EXPERIMENTAL PROCEDURES**

*Cell Lines—*The creation of caspase 3−/−/caspase 7−/− MEFs (26) and caspase 9−/− MEFs (27) was previously described. Immortalization of MEFs cultured from various intercrossed progeny was performed via SV40 large T antigen-mediated transformation, as described previously (28). Multiple transformed lines were pooled by genotype for experimentation. Caspase 8−/− MEFs were derived from caspase 8-deficient embryos and subsequently immortalized (29).

*MEF Cytotoxicity—*Cells were plated in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum, penicillin/streptomycin, and β-mercaptoethanol. Prior to induction of apoptosis, MEFs were placed in low serum (0.5% fetal calf serum) medium for 6 h. Apoptosis was induced by adding indicated concentrations of thapsigargin (Sigma), tunicamycin (Sigma), calcium ionophore A23187 (Sigma), and brefeldin A (Sigma). At 36 h, attached cells were trypsinized and pooled with floating cells. Viability was assessed using the Live/Dead Viability/Cytotoxicity kit (Molecular Probes) and FACS analysis. Live cells were determined by gating on the population that was FL-1 high and FL-2 low.

*Light Microscopy—*After indicated treatments, cells were visualized on a Leica DMIL light microscope and then directly imaged in culture with a Kodak DC290 digital camera.

*Whole Cell Lysates, Cell Fractionation, and Western Blotting—*After indicated treatments, attached MEFs were trypsinized, pooled with floating MEFs, and washed with phosphate-buffered saline. Some cells were treated with UV irradiation (20 J/m²) and 1 μg/ml Jo2 antibody (BD Biosciences) as positive controls for caspase activation. Cyclohexamide (Sigma) was added at 10 μg/ml to Jo2 treatments. For whole cell lysates, cells were resuspended in cell lysis buffer (Cell Signaling) supplemented with phenylmethylsulfonyl fluoride and protease inhibitor mixture (Roche Applied Science), incubated on ice for 10 min, and then centrifuged at 10000 rpm for 10 min. Supernatant was saved as whole cell lysate. Heavy membrane/cytosolic fractionation was performed using the ProteoExtract Subcellular Proteome Extraction kit (Calbiochem), according to the manufacturer's instructions.

Proteins from each sample were run on SDS-PAGE gels and transferred to Immobilon P membrane (Millipore). Western blotting was performed using antibodies at a dilution of 1:1000 (unless otherwise noted) to caspase 3, caspase 6, caspase 7, caspase 12 (Cell Signaling), caspase 9 (Stressgen), caspase 11 (Sigma), protein disulfide isomerase and BiP (1:10,000) (Abcam), cytochrome c (BD Biosciences), caspase 8 (Alexis), and caspase 2, CHOP, XBP-1, and actin (1:2000) (Santa Cruz Biotechnology).

*Mitochondrial Membrane Potential—*After indicated treatments, attached MEFs were trypsinized, pooled with floating MEFs, and washed with phosphate-buffered saline. Membrane potential was assessed using the JC-1 Mitochondrial Membrane Potential Detection kit (Cell Technology) according to the manufacturer's instructions. Cells were analyzed by FACS in the FL-1 and FL-2 channels.

**RESULTS**

Caspases 3, 7, and 9 are important in both the intrinsic and extrinsic pathways of apoptosis (4). To determine the physiological roles of these proteases in ER stress-induced apoptosis, caspase 3, 7, and 9 knock-out MEFs were studied. Fibroblasts from heterozygous and homozygous knock-out embryos were immortalized to create eight genetically distinct cell lines (cells from multiple transformations were pooled to form lines) and treated with 3 μM thapsigargin (TG) (Fig. 1A), 10 μg/ml tunicamycin (TN) (Fig. 1B), 1 μM calcium ionophore A23187 (CA) (Fig. 1C), or 40 μM brefeldin A (BFA) (Fig. 1D) for 36 h. Harvested MEFs were assayed for viability using the Live/Dead Viability/Cytotoxicity kit. As shown in Fig. 1, caspase 3−/−/caspase 7−/− and caspase 9−/− MEFs were generally protected against apoptosis induced by all four ER stress inducers, with at least a 70% increase in survival compared with wild-type controls in all treatments. Dose response experiments were performed to assess the extent of this protection for each ER stress inducer, and representative ones are shown in Fig. 1, E and F. The survival of heterozygous cell lines, however, depended on the ER stress pathway engaged. The survival of caspase 3−/−/caspase 7−/− MEFs resembled that of caspase 3−/−/caspase 7−/− MEFs following treatment with either TG or CA but was intermediate between that of wild-type and caspase 3−/−/caspase 7−/− MEFs after treatment with either TN or BFA. Conversely, caspase 3+/−/caspase 7−/− MEFs resembled wild-type MEFs upon TG or CA treatment and exhibited intermediate survival with TN or BFA treatment. Both TG and CA induce an increase in intracellular [Ca²⁺], whereas TN and BFA impair ER function by, respectively, inhibiting N-linked glycosylation of ER luminal proteins and ER/Golgi transport. Thus, the survival pattern in the heterozygous MEFs suggests that ER stress induced via Ca²⁺ efflux is dependent upon caspases 3 and 9 only, whereas ER stress pathways affecting protein transport activate caspases 3, 7, and 9.
To determine whether the ER stress-induced apoptotic program was solely dependent on the intrinsic pathway of apoptosis, immortalized MEFs deficient in caspase 8 were studied; caspase 8−/− MEFs retained susceptibility to ER stress-induced cytotoxicity (supplemental Fig. S1). Because caspase 8 is known to initiate the extrinsic apoptotic pathway (4), this finding indicates that the extrinsic pathway is not required for ER stress-induced death. Taken together with the general protection against ER stress-induced apoptosis in caspase 3−/−/caspase 7−/− and caspase 9−/− MEFs, these findings suggest the presence of caspase-7-dependent and -independent ER stress apoptotic programs that converge on caspases 3 and 9.

At both the genetic and protein levels, caspases 3 and 7 exhibit a high degree of homology, and this has been correlated with observations of overlapping function in many systems (4). As shown in Fig. 1, the genotypically variant MEF cell lines used in this experiment allow for functional analysis of the caspase 3 and 7 alleles. In all treatments, the survival of wild-type MEFs was similar to that of caspase 3−/−/caspase 7−/− MEFs, suggesting that heterozygote MEFs are able to undergo apoptosis normally so long as at least one copy of each caspase gene is functional. In addition, caspase 3−/−/caspase 7+/- and caspase 3+/-/caspase 7−/− MEFs generally exhibited similar survival to each other and to wild-type MEFs in all treatments. This finding implies that two functional alleles of caspase 3 or 7 can almost completely compensate for loss of both alleles at the other locus. Thus, having any combination of two alleles of caspase 3 and 7 appears to be sufficient for the ER stress-induced apoptotic program. The effect of allelic dosage, however, was observed in some MEF lines at the caspase 7 locus. Caspase 3−/−/caspase 7+/- MEFs and caspase 3+/-/caspase 7−/− MEFs generally exhibited similar survival, whereas a 2-fold increase in survival compared with caspase 3−/−/caspase 7−/− MEFs with all treatments, indicating that loss of a caspase 7 allele significantly impaired ER stress-induced apoptosis. Thus, with respect to ER stress, the data suggest that the caspase 3 locus is haplo-sufficient, whereas normal caspase 7 function requires expression of both allelic copies.

To confirm the protection against ER stress-induced cytotoxicity observed in knock-out MEFs, live cell light microscopy

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**FIGURE 1.** Improved survival following ER stress in caspase 3−/−/caspase 7−/− and caspase 9−/− MEFs. Immortalized MEFs with the indicated genotypes for caspases 3, 7, and 9 were treated with thapsigargin (3 μM) (A), tunicamycin (10 μg/ml) (B), calcium ionophore A23187 (1 μM) (C), or brefeldin A (40 μM) (D). After 36 h, cells were harvested and survival was determined using the Live/Dead Cytotoxicity/Viability assay (Molecular Probes). The data are represented as means ± S.D. from a single experiment in triplicate, representative of at least three independent experiments. The * denotes a statistically significant difference (p < 0.05) in the survival of wild-type and knock-out MEFs. E and F, thapsigargin dose response for wild-type, caspase 3−/−/caspase 7−/−, and caspase 9−/− MEFs. Cells were analyzed as above.
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was performed as shown in Fig. 2. Cells of all eight genotypes were plated onto slides and treated with 10 μg/ml tunicamycin and 1 μM CA for 20 h before observation. Untreated control cells of all genotypes exhibited normal fibroblast morphology, including adhesion to the slide surface and extension of cytoplasmic projections. Conversely, many treated cells exhibited hallmarks of late stage apoptosis, as exemplified by the arrows in Fig. 2B, including cell shrinkage, membrane blebbing, and loss of adhesion. Moreover, the presence of apoptotic cells correlated with caspase 3, 7, or 9 deficiency in MEFs. Cells were treated with either tunicamycin or A23187 and then directly imaged in culture after 20 h using a light microscope and a Kodak DC290 digital camera. Loss of cellular morphology is indicated by arrows (B). The data are from a single experiment, representative of three independent experiments.

FIGURE 2. Resistance to ER stress-induced death in caspase 3-, 7-, or 9-deficient MEFs. Cells were treated with either tunicamycin or A23187 and then directly imaged in culture after 20 h using a light microscope and a Kodak DC290 digital camera. Loss of cellular morphology is indicated by arrows (B). The data are from a single experiment, representative of three independent experiments.

FIGURE 3. Preservation of Δψm in knock-out MEFs during ER stress. Cells with the indicated genotypes were treated with 10 μg/ml tunicamycin for 24 h. Loss of membrane potential was determined by incubating harvested cells with JC-1 reagent and performing FACS analysis. The JC-1 enters cells and accumulates in aggregates in mitochondria with intact Δψm to produce a red fluorescent signal (FL-2, y-axis) but remains monomeric and produces a green fluorescent signal (FL-1, x-axis) otherwise. Thus, cells with preservation of Δψm are both FL-1 high and FL-2 high (top right in each plot), whereas cells with loss of Δψm are FL-1 high, FL-2 low (bottom right in each plot). Plots are shown with the percentage of total events in each gate indicated. The data are from a single experiment, representative of three independent experiments.
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To explain the resistance of caspase 3−/−/caspase 7−/− and caspase 9−/− MEFs to ER stress-induced apoptosis and the preservation of ΔΨm, we characterized the activation of caspases 3, 7, and 9 in wild-type (+/+ ) MEFs through Western blotting of whole cell lysates after treatment with various stimuli (Fig. 4B). In wild-type cells, extensive cleavage of caspases 3 and 9 was observed with TN and CA treatment compared with untreated controls (UT) and UV-irradiated cells. Bands corresponding to the full-lengthzymogens were also reduced in treated versus untreated cells. In contrast, levels of full-length caspase 7 appeared equivalent between treated and untreated wild-type cells, and little cleaved caspase 7 was detected in response to treatment with TN or CA compared with that observed in UV-irradiated cells. Given the finding of many groups that caspase 8 is involved in ER stress- and calcium flux-induced apoptosis (22, 23, 30), we also studied caspase 8 activation in wild-type MEFs. Very little cleaved caspase 8, however, was detected following TN or CA treatment compared with that observed in Jo2-treated cells, in which the band corresponding to full-length caspase 8 was barely observed. Taken together, the data from whole cell lysates indicate that cleaved caspases 3 and 9 play an important role in TN- and CA-induced apoptosis, whereas cleaved caspase 7 is much less physiologically relevant. When compared with actin levels, it also appears that CA consistently induces less activation of caspases 3, 7, and 9 than does TN. This effect was observed as early as 12 h following treatment (data not shown).

Characterization of caspase 3 and 7 activation in wild-type MEFs did not sufficiently explain the significant susceptibility of caspase 3+/−/caspase 7−/− MEFs to CA-induced apoptosis and the mechanism by which this occurs. Based on evidence from other systems that activated caspases can migrate to specific cellular compartments (4, 31), we sought to determine whether differential localization of cleaved caspase 3 and 7 to the ER might explain the difference in survival of knock-out MEFs. Wild-type MEFs were treated with either 10 or 20 μg/ml TN or 1 or 2 μM CA for 16 h, and cytosolic and heavy membrane lysates were isolated and probed on a Western blot (Fig. 4E). The composition of the heavy membrane fraction was analyzed by probing for protein disulfide isomerase, an ER-resident enzyme, which was undetectable in cytosolic lysates (data not shown) and present in excess in the heavy membrane fraction, suggesting the latter was enriched with ER. Both procaspase 3 and cleaved caspase 3 were detected in the heavy membrane fraction, and treatment with TN was observed to induce a significantly greater amount of cleaved caspase 3 than did treatment with CA. The greater amount of procaspase 3 in the lane corresponding to untreated MEFs might be due to a larger amount of total protein, as is indicated by the larger amount of protein disulfide isomerase in the untreated lane, explaining the presence of cleaved caspase 3 in the untreated heavy membrane lysate. In contrast, cleaved caspase 7 was only observed in the heavy membrane fraction of wild-type MEFs treated with TN. Procaspases are not known to be natively expressed in the ER (4), but the data suggest that caspase activation can and does occur at the ER in the presence of ER stress inducers. Moreover, the fact that procaspase 7 is cleaved in the heavy membrane

FIGURE 4. A, UPR signaling remains intact in knock-out MEFs. Lysates from heterozygous, caspase 3−/−/caspase 7−/−, and caspase 9−/− MEFs treated for 0, 4, and 8 h with 10 μg/ml tunicamycin were probed with the indicated antibodies. B, cleavage of caspases 3 and 9 in ER stress-induced apoptosis of wild-type MEFs. Lysates from untreated (UT) wild-type cells and cells treated for 24 h with either 10 μg/ml tunicamycin (TN), 1 μM calcium ionophore A23187 (CA), 20 J/m2 UV, or 1 μg/ml Jo2 antibody + 10 μg/ml cyclohexamide were probed with the indicated antibodies. C, differential localization of cleaved caspase 3 and 7 during ER stress. Heavy membrane and cytosolic lysates were obtained, using the ProteoExtract Subcellular kit, from untreated (UT) wild-type MEFs and MEFs treated with either 10 or 20 μg/ml tunicamycin (TN) or 1 μM calcium ionophore A23187 (CA) for 16 h. Western blots were probed with the indicated antibodies. D, altered caspase 11 and 12 activity in caspase 3−/−, 7−/−, or 9-deficient MEFs. Cells with the indicated genotypes were treated as in panel C, and Western blots were probed with the indicated antibodies.

cytotoxicity assay of TN-treated MEFs (Fig. 1), although it does suggest that even a small loss in ΔΨm can indicate enough degradation of the outer membrane to allow apoptotic molecules, such as cytochrome c, to enter the cytosol and induce death.

The current model of ER stress proposes a threshold level of initial UPR signaling that, once crossed, additionally stimulates cleavage and activation of these inflammatory caspases (1–3). To verify that the observed resistance to ER stress-induced apoptosis in caspase 3+/−/caspase 7−/− and caspase 9−/− MEFs was not a consequence of abrogated early signaling events, heterozygous, caspase 3−/−/caspase 7−/−, and caspase 9−/− MEFs were assayed for levels of BiP and CHOP via Western blotting of lysates 0, 4, and 8 h following treatment with TN (Fig. 4A). Increases in both BiP and CHOP were observed in a time-dependent manner, regardless of MEF genotype. Levels of XBP-1 were similarly observed to increase following TN treatment in knock-out MEFs (data not shown), suggesting that UPR signaling remains intact in the absence of caspase 3, 7, or 9.
fraction in TN-induced, but not CA-induced, apoptosis suggests an explanation for the increased loss of TN-induced apoptosis on caspase 7.

To determine the functional consequences of loss of caspases 3, 7, and 9, processing of canonical initiator caspases 2 and 9, effector caspase 6, and inflammatory caspases 11 and 12 was assayed via Western blotting of whole cell lysates in wild-type and knock-out MEFs following TN or CA treatment for 24 h (Fig. 4D). Levels of full-length caspase 2 were unchanged by treatment compared with controls in all genotypes, and no cleavage bands were detected (data not shown). This was similarly observed with levels of full-length caspase 6, except in the case of caspase 3+/−/caspase 7+/− MEFs, in which the decrease in procaspase 6 was reproducibly observed. In this situation, caspase 6 might have been cleaved in the absence of caspase 7, explaining the lack of caspase 6 cleavage in caspase 3−/−/caspase 7+/− MEFs. Processing of caspase 9, however, was increased in caspase 3−/−/caspase 7+/− MEFs compared with wild-type cells. Effector caspases 3 and 7 can cleave caspase 9 (4), so loss of these caspases in caspase 3−/−/caspase 7+/− and caspase 3+/−/caspase 7−/− MEFs may prevent extensive cleavage of procaspase 9. In contrast, the absence of effector caspases in caspase 3−/−/caspase 7−/− MEFs might have created a futile feedback cycle in which cleaved caspase 9 processed remaining procaspase 9. Caspase 11 induction, which precedes its role in inflammation (18) and has been proposed to work via the ER stress-related CHOP transcription factor (17), was also affected by the genotype of treated cells. In all MEFs, an increase in the level of full-length caspase 11 was observed following CA treatment, compared with untreated and TN-treated cells. When compared with actin, however, the amount of caspase 11 observed was much greater in single knock-out, caspase 3−/−/caspase 7−/−, and caspase 9−/− cells, both treated and untreated, than that observed in wild-type cells, arguing against a significant role for caspase 11 in ER stress-induced apoptosis.

The specificity of the caspase 11 induction to CA treatment is supported by the lack of induction after UV treatment of wild-type MEFs. The general increase in protein levels might result from a stable compensatory adjustment due to permanent loss of caspase 3, 7, or 9 and might result in an increased inflammatory response due to high cytosolic calcium, although this was not further investigated. Caspase 11 is known to be lipopolysaccharide-inducible and is thought to be transcriptionally or translationally up-regulated in response to many inflammatory stimuli (18). Conversely, caspase 12 processing was observed to be decreased in caspase 3−/−/caspase 7−/−, caspase 9−/−, and caspase 3−/−/caspase 7+/− MEFs. Caspase 12 processing seemed unchanged in caspase 3+/−/caspase 7−/− cells relative to that in wild-type MEFs, suggesting that caspase 3 has a more significant physiological role in caspase 12 processing than does caspase 7, contrary to other findings (2, 10). Thus, it seems that caspase 12 processing is either downstream of effector caspase activation or a tertiary event not directly related to the apoptotic cascade.

**DISCUSSION**

In recent years, ER stress has garnered attention both as a model example of cellular quality control and for its potential role in several disease processes. Further, many groups have concluded that ER stress-induced apoptosis involves a novel pathway distinct from the well characterized intrinsic and extrinsic ones. We studied MEFs deficient in caspase 3, 7, or 9 in order to determine the roles of these proteases in the ER stress apoptotic program. Our study established a role for caspases 3 and 9 in ER stress-induced apoptosis. Specifically, caspase 3−/−/caspase 7−/− and caspase 9−/− MEFs exhibited (1) resistance to cytotoxicity induced via ER stress, (2) preservation of Δψm, and (3) significantly abrogated caspase 12 processing. These observations correlated with accumulation of cleaved caspase 3 and 7 in the ER compartment, providing a potential mechanism for caspase 12 processing.

Caspases 3 and 9 are a central component of most models of ER stress-induced apoptosis (1–3), but their roles have never been definitively established in a knock-out model. Moreover, several groups have shown that the ER and mitochondria, physiologically juxtaposed organelles, jointly maintain intracellular calcium stores (6, 7). Rapid efflux of calcium from the ER is believed to result in uptake by the mitochondria and subsequent release of pro-apoptotic molecules, such as cytochrome c. Many have also reported the existence of mitochondria-independent pathways of ER stress-induced apoptosis (11, 12). We have previously shown that caspase 3−/−/caspase 7−/− MEFs exhibit delayed loss of Δψm, Bax translocation, and cytochrome c release in response to UV irradiation (26), suggesting that these caspases contribute to upstream mitochondrial effects. Release of cytochrome c from mitochondria and loss of Δψm were previously shown to be mutually exclusive events (32, 33), indicating that caspases 3 and 7 specifically regulate Δψm.

In this study, we similarly observed that a significantly higher percentage of caspase 3−/−/caspase 7−/− and caspase 9−/− MEFs maintain Δψm during ER stress than do wild-type MEFs, suggesting a central role for the mitochondria and providing an impetus for further study of caspases 3, 7, and 9.

The finding that caspase 3+/−/caspase 7−/− MEFs exhibit reduced cytotoxicity, compared with wild-type MEFs, following direct disruption of ER/Golgi function (via TN or BFA), but not after an increase in cytosolic calcium levels (via CA or TG), suggests an interesting physiological difference between the two classes of stimuli. Several groups have established the critical role of calcium efflux from the ER, primarily through Bap31 (1, 2, 23) and Bcl-2 family members (5, 7), as part of the stress response. Many groups have observed that efflux-induced calcium and calcineurin activity can lead to mitochondrial permeabilization and activation of caspases 9 and 3 (1, 2, 9). In general, ER stress induced by TN and BFA probably engages multiple different UPR signaling pathways, in addition to calcium release, to either increase calcium-induced mitochondrial outer membrane permeabilization or stimulate effector caspase activation independent of the mitochondrion, as has been suggested by some groups (Fig. 5) (11, 12). Such a model would explain not only the differential dependence of caspase 7 but also the greater amount of cleaved caspase 3 induced by TN and BFA compared with that induced by CA and TG. We initially hypothesized a role for caspase 8 might exist in mediating CA- and TG-dependent differences in caspase activation, based on previously published findings, but cleaved caspase 8 was unde-
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![Diagram of ER stress-induced apoptosis]

- ER stress leads to efflux of calcium from the ER and into the mitochondria, due to UPR signaling, and may also result in activation of additional, non-mitochondrial signaling pathways (dashed line).
- Increased calcium levels in the mitochondria stimulate release of pro-apoptotic molecules like cytochrome c. Cytochrome c and Apaf-1 form the apoptosome to process procaspase 9, resulting in downstream cleavage of caspase 3 cleavage in both the cytosol and ER lumen. We have previously shown that activated caspase 3 can induce loss of ΔΨm, potentiating further cytochrome c release. One potential function of active caspase 3 in the ER might be processing of procaspase 12 bound to the membrane to amplify processing of caspases 9 and 3 in the cytosol.

Our data also question the roles of murine caspases 11 and 12 as potential initiators of the ER stress apoptotic program. The current model of ER stress proposes a threshold level of initial UPR signaling that, once crossed, additionally stimulates cleavage and activation of these inflammatory caspases (1–3). Following treatment with TN, however, UPR signal transduction appeared intact, as determined by levels of BiP, CHOP, and XBP-1, despite significant differences in the expression and activation of caspases 11 and 12 between wild-type and knock-out MEFs. The increased expression of procaspase 11 in untreated knock-out cells, compared with wild-type cells, was not observed to correlate with increased susceptibility to apoptosis. In general, levels of procaspase 11 were not detectable by Western blotting of whole cell lysates from untreated and treated wild-type MEFs, providing strong evidence against a causative role in apoptosis. Similarly, caspase 3- and caspase 9-deficient MEFs were shown to exhibit significant abrogation of caspase 12 processing 24 h after treatment with either TN or CA. This finding implies that caspase 12 cleavage is actually contingent upon the activity of caspases 9 and 3, placing it downstream of the mitochondria. Combining this observation with the fact that cleaved caspase 3 was shown to accumulate in the ER at 16 h, we propose a new model in which ER stress-induced activation of caspase 9 leads to processing of procaspase 3 and accumulation of the cleaved form at the ER, where it can catalyze processing of ER membrane-bound caspase 12, resulting in amplification of the apoptotic cascade through increased processing of caspases 9 and 3. This model is attractive because it supports the observation of several groups that caspase 12 processing is associated with ER stress-induced apoptosis (8, 12, 13, 19, 20) and the finding by Saleh et al. (16) that caspase 12-deficient and wild-type MEFs exhibit similar amounts of cytotoxicity following treatment with ER stress inducers. In general, murine caspases 11 and 12 are better characterized as inflammatory mediators (16–18, 34), so it is likely that they become physiologically active as a result of ongoing ER stress and apoptosis rather than as a cause of these events.

Thus, we conclude that ER stress-induced apoptosis is significantly blocked in caspase 3−/−/caspase 7−/− and caspase 9−/− MEFs due to abrogated processing of caspase 12, decreased permeation of the mitochondrial outer membrane, and reduced cleavage of caspase 3 substrates. We therefore propose that the ER stress program is analogous to the well-characterized intrinsic pathway. This model (Fig. 5) explains both the fact that neither caspase 11 nor caspase 12 functions as an initiator and our observations that ER stress-induced apoptosis physiologically requires caspases 3 and 9 but not caspase 8. Future study of the UPR signaling events that potentiate caspase 3 and 9 activation and of the roles of cleaved caspase 3 at the ER should shed light on ways to block ER stress-related pathologies.

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