Inhibition of the ER Ca\(^{2+}\) pump forces multidrug-resistant cells deficient in Bak and Bax into necrosis

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Summary

Tumor cells deficient in the proapoptotic proteins Bak and Bax are resistant to chemotherapeutic drugs. Here, we demonstrate that murine embryonic fibroblasts deficient for both Bak and Bax are, however, efficiently killed by thapsigargin, a specific inhibitor of ER Ca\(^{2+}\) pumps that induces ER stress by depleting ER Ca\(^{2+}\) stores. In the presence of Bak and Bax, thapsigargin eliminates cells by release of mitochondrial cytochrome c and subsequent caspase activation, which leads to the proteolytic inactivation of the molecular necrosis switch PARP-1 and results in apoptosis. By contrast, in the absence of Bak and Bax, a failure to activate caspases results in PARP-1-mediated ATP depletion. The subsequent necrosis is not prevented by autophagy as an alternative energy source. Moreover, in cells deficient for both Bak and Bax, thapsigargin induces permanent mitochondrial damage by Ca\(^{2+}\) overload, permeability transition and membrane rupture. Thus, even though deficiency in Bak and Bax protects these cells against apoptosis, it does not compromise necrosis induced by SERCA inhibitors. Importantly, thapsigargin induces caspase-independent cell death also in colon and prostate carcinoma cells deficient in Bak and Bax expression. Therefore, targeted application of ER stressors such as thapsigargin might be a promising approach for the treatment of Bak- and Bax-deficient, drug-resistant tumors.

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Key words: ER stress, PARP, SERCA, Bak, Bax, Necrosis, Multidrug resistance, UPR, Autophagy, Ire1

Introduction

Although the role of mitochondria in apoptosis is well established, the endoplasmic reticulum (ER) is becoming increasingly recognized as an important cell death regulator (Boyce and Yuan, 2006; Xu et al., 2005; Szegedi et al., 2006). In the ER, secretory and membrane proteins are synthesized and folded into their native conformation. Therefore, the ER has to provide a proper environment that can be perturbed, for instance, when post-translational protein modification fails or Ca\(^{2+}\) concentrations are altered (Schroder and Kaufman, 2005). Normally, a Ca\(^{2+}\) gradient exists across the ER membrane with millimolar levels in the ER lumen and about four orders of magnitude lower concentrations in the cytoplasm. This gradient is maintained by the sarco-endoplasmic reticulum calcium ATPase (SERCA) pump that transports Ca\(^{2+}\) into the ER. Application of the specific SERCA inhibitor thapsigargin depletes ER Ca\(^{2+}\) stores and causes ER stress (Dong et al., 2006).

ER stress activates signaling pathways, collectively termed the unfolded protein response (UPR). ER stress is sensed by three integral stress receptors including the pancreatic ER kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1α (IRE1α; also known as ERN1), which are kept inactive through their association with the chaperone BiP (also known as Grp78 or Hspsa5) (Schroder and Kaufman, 2005). Misfolded proteins in the ER associate with BiP/Grp78, thereby releasing and activating PERK, ATF6 and IRE1α that trigger induction of UPR target genes. The UPR then drives a coordinated program to restore ER homeostasis after mild insults by increasing biosynthesis of factors involved in protein folding, secretion and ER-associated protein degradation. Severe stress, however, can trigger necrotic and apoptotic cell death through mechanisms largely unknown. Multiple mediators have been implicated in ER stress-induced death including caspases, Bel-2 proteins, phosphatases, kinases and transcription factors (Boyce and Yuan, 2006; Szegedi et al., 2006). ER stress also induces autophagy (Hoyer-Hansen and Jaattela, 2007), in which parts of the cytoplasm are sequestered within double-membrane vesicles. Normally, autophagy enables cells to endure low nutrient supply and assists the UPR in removing misfolded proteins. Enhanced autophagic vacuolization has been implicated in cell death, but whether autophagy contributes to cell death during ER stress remains unclear (Levine and Yuan, 2005; Maituri et al., 2007).

Characteristic features of cell death are mitochondrial outer membrane permeabilization (MOMP) and the loss of the mitochondrial transmembrane potential ΔΨ\(_{m}\) (Kroemer et al., 2007). Stress such as mitochondrial Ca\(^{2+}\) overload can cause the breakdown of ΔΨ\(_{m}\) by activation of the mitochondrial permeability transition (MPT) pore, a large channel spanning the outer and inner mitochondrial membrane. MPT pore opening results in the swelling of mitochondria and subsequent outer membrane rupture that causes the leakage of proteins including cytochrome c (Green and Kroemer, 2004). Recent evidence suggests that MPT pore opening and subsequent loss of ΔΨ\(_{m}\) plays a role in necrotic but not apoptotic cell death. For example, animals deficient in cyclophilin D, an MPT component, are resistant to necrosis, but not apoptosis, induced...
during ischemia-reperfusion or elevation of cytosolic Ca\textsuperscript{2+} (Baines et al., 2005; Nakagawa et al., 2005).

During apoptosis, loss of ΔΨ\textsubscript{m} seems to be a secondary event not required for cytochrome c release (Chipuk and Green, 2008). MOMP and the release of cytochrome c are instead controlled by Bcl-2 proteins acting on the outer mitochondrial membrane. Bcl-2 proteins are classified into two major groups: antiapoptotic (e.g. Bcl-2) and proapoptotic members, which are further subdivided into multidomain proteins (e.g. Bak, Bax) and BH3-only proteins (e.g. Bim, Bid). In response to apoptotic stimuli the multidomain proteins Bak and Bax undergo a conformational change, leading to their assembly into multimers with channel-forming properties in the outer mitochondrial membrane, which mediate cytochrome c release (Youle and Strasser, 2008). Conformational change of Bak or Bax is inducible by BH3-only proteins acting as allosteric sensors of apoptotic signaling. Once released into the cytosol, cytochrome c and cytosolic factors form the apoptosome, an activating platform for caspases that execute apoptosis by the cleavage of target proteins.

Knockout models provided evidence for the essential involvement of Bax and Bak in apoptotic cell death (Lindsten and Thompson, 2006). Deficiency of Bax and Bak therefore confers resistance to most conventional tumor therapies that employ apoptosis to achieve cell death (Wei et al., 2001; Meijerink et al., 1998). Single inactivation or downregulation of Bak (Kondo et al., 2000; Wang et al., 2001) or Bax (Brimmell et al., 1998; Gutierrez et al., 1999), frequently found in various solid and hematological neoplasias, can be sufficient to induce therapy resistance and poor prognosis (Friess et al., 1998; Sturm et al., 1999; Ionov et al., 2000; Wang et al., 2001; LeBlanc et al., 2002).

Lack of therapies for drug resistant tumors, as exemplified by cells lacking Bax or Bak, represents a severe problem. Since most current anticancer drugs signal through mitochondria, we speculated that the ER might be an alternative target for the induction of tumor cell death. In this report, we demonstrate that, even though deficiency in Bak and Bax (hereafter referred to as Bak/Bax deficiency) protects against apoptosis, it does not compromise necrosis induction by SERCA inhibitors. Our results suggest that interfering with ER function might be a therapeutic approach for certain multidrug-resistant tumor subsets.

**Results**

**Multidrug-resistant Bak/Bax-deficient MEFs are efficiently eliminated by the SERCA inhibitor thapsigargin**

Tumor cells deficient in both Bak and Bax are protected against mitochondria-mediated apoptosis that is exploited by most chemotherapeutic drugs to achieve tumor cell death (Fischer et al., 2007). Employing murine embryonic fibroblasts (MEFs) as a model system, our experiments confirmed that MEFs derived from Bak and Bax double knockout mice resist the cytotoxic effects of a wide variety of chemotherapeutic and apoptosis-inducing drugs (Fig. 1A,B and supplementary material Fig. S1). In a screen for new, alternative anti-tumor drugs that could overcome this resistance, we identified the SERCA inhibitor thapsigargin, as a potent inducer of death in Bak/Bax-deficient cells (Fig. 1B). Intriguingly, bak/bax\textsuperscript{−/−} (note that genes encoding Bak and Bax are referred to as Bak and Bax in the MGI database) cells were less sensitive to tunicamycin, an ER stress inducer that exerts a different mode of action by inhibiting protein N-glycosylation.

Cell death studies of up to 3 days (Fig. 1C) as well as dose-response studies (supplementary material Fig. S2) revealed that, although bak/bax\textsuperscript{−/−} MEFs were less sensitive to tunicamycin, they were killed by thapsigargin with a time and dose dependency similar to wild-type (WT) cells. To investigate effects on long-term survival, we performed clonogenicity assays (Fig. 1D). Unlike WT cells, Bak/Bax-deficient MEFs survived and retained proliferative capacity after treatment with low doses of tunicamycin (0.1 µg/ml, 48 hours). However, at higher doses (1 µg/ml) bak/bax\textsuperscript{−/−} cells also succumbed to tunicamycin-induced toxicity. By contrast, after treatment with even low doses of thapsigargin (0.1 µM) neither WT nor Bak/Bax-deficient MEFs retained clonogenicity. Compared with WT cells, even fewer bak/bax\textsuperscript{−/−} colonies grew, indicating that SERCA inhibitors could potentially circumvent therapy resistance of certain tumor subsets.
Caspase inhibition protects WT but not Bak/Bax-deficient MEFs

To ascertain whether WT and bak/bax−/− cells died by apoptosis, we investigated activation of caspases (Fig. 2A,B). Cleavage of caspase-3 in response to ER stress was readily observed in WT MEFs, but almost absent in bak/bax−/− MEFs. These results were confirmed by analyses of caspase substrate cleavage showing that ER stress induced high caspase activity in WT, but not bak/bax−/− cells. The weak residual caspase activity observed in bak/bax−/− MEFs might be attributed to caspases functioning upstream of mitochondria, such as caspase-2 or caspase-12, whose functional relevance for ER stress is controversial. Pharmacological caspase inhibition by q-VD-oPh (QVD) resulted in marked reduction of death in WT MEFs, but not bak/bax−/− cells after 48 hours of treatment with thapsigargin (Fig. 2C). Therefore, SERCA inhibition triggers caspase-dependent death in Bak/Bax-expressing cells, but caspase-independent death in Bak/Bax-deficient cells.

Autophagy is associated but not functionally involved in cell death during ER stress

Autophagy can exert both survival- and death-promoting effects (Hoyer-Hansen and Jaattela, 2007). Electron microscopy demonstrated that thapsigargin and tunicamycin induced formation of autophagic vacuoles in both WT and bak/bax−/− MEFs (Fig. 3A). Moreover, treatment with both drugs resulted in conversion of the microtubule-associated protein light chain (LC3) from its cytosolic form to the lipidated form LC3-II, a marker for autophagic activity (Fig. 3A). Thus, in both cell types ER stress is associated with autophagic alterations.

Fig. 2. Thapsigargin-induced death of wild-type, but not bak/bax−/− MEFs is caspase dependent. (A) ER stress induces caspase-3 cleavage in WT cells, but not Bak/Bax-deficient MEFs. 48 hours after treatment with thapsigargin (TG; 1.5 μM) or tunicamycin (TU; 1 μg/ml) cell lysates were analyzed by immunoblotting. Protein bands specific for full-length caspase-3 (Casp-3) and a β-actin control are indicated. (B) ER stress induces caspase activity in WT (left panel), but not Bak/Bax-deficient MEFs (right panel). Cells were treated with tunicamycin or thapsigargin or left untreated. After 48 hours caspase activity was monitored employing the substrate DEVD in a luminescence assay. (C) ER stress-induced cell death is caspase dependent in WT, but not bak/bax−/− MEFs. Cells were treated for 48 hours with thapsigargin or tunicamycin in the presence or absence of the caspase inhibitor QVD. Cell death was measured by PI uptake (values are mean ± s.d.; *significant inhibition of drug-induced death by QVD; P<0.05). w/o, no treatment.

To analyze the functional role of autophagy, we first used Bak/Bax-expressing atg-5−/− (Atg5−/−) MEFs that are deficient in autophagosome formation. Treatment with ER stress agents revealed no difference in cell death between WT and atg-5−/− cells, suggesting that in Bak/Bax-proficient cells autophagy plays no critical role (Fig. 3B). Furthermore, treatment of Bak/Bax-deficient MEFs with the autophagy inhibitor 3-methyladenine (3-MA) resulted in only a minor reduction of cell death in response to the ER stressors (Fig. 3C). Moreover, siRNA-mediated knockdown of Beclin-1, a protein essentially involved in autophagy, did not provide protection against ER stress in either WT or bak/bax−/− cells (data not shown). Thus, despite autophagic alterations, neither the death of Bak/Bax-proficient nor of Bak/Bax-deficient cells seems to be caused by autophagy.

Abrogation of UPR components does not protect against ER stress-induced cell death

ER stress triggers the UPR that has been implicated in some forms of cell death (Schroder and Kaufman, 2005). Therefore, we investigated a contribution of critical UPR components. Even in the absence of Bak/Bax, thapsigargin and tunicamycin induced a time-dependent increase of the ER chaperone BiP, enhanced phosphorylation of the PERK substrate eukaryotic initiation factor 2α (eIF2α) and the IRE1α substrates JNK1/2, indicating a functional ER stress response also in bak/bax−/− cells (Fig. 3D). In addition, we observed induction of the transcription factor CHOP (Fig. 3E) that has been implicated in ER stress-induced cell death (McCullough et al., 2001). Concurrently, increased levels of the BH3-only protein Bim, a target gene of CHOP (Puthalakath et al., 2007) were detected. Although CHOP has also been reported to activate apoptosis by transcriptional repression of Bcl-2, we found no reduction in Bcl-2. Instead, cleavage and hence activation of the BH3-only protein Bid were evident (Fig. 3E). Similarly to a previous study (Cheng et al., 2001), we detected slightly elevated levels of Bim in bak/bax−/− cells as compared to WT MEFs, which might be due to a missing survival constraint in the absence of Bax and Bak or other unknown mechanisms.

To clarify the impact of the UPR on cell death, we employed knockout MEFs of the major UPR mediators. Knockout of perk did not decrease cell death in response to thapsigargin or tunicamycin (supplementary material Fig. S3). The kinase IRE1α has also been implicated in ER stress-triggered death pathways and was proposed to form a complex with Bak and Bax that is essential for IRE1α activation (Hetz et al., 2006). Other studies implicated a ternary complex composed of IRE1α, TRAF-2 and the kinase ASK-1 in ER stress-mediated cell death and JNK activation (Nishitoh et al., 1998). However, the impact of such pathways is controversial and several lines of evidence argue against an essential role of IRE1α and associated components. First, we found no impairment of JNK activation in Bak/Bax-deficient cells upon ER stress (Fig. 3D and data not shown), arguing against a non-functional IRE1α pathway in the absence of Bak/Bax. Second, although bak/bax−/− cells were protected against tunicamycin, cell death still occurred in ire1α−/− cells to a similar extent as in WT cells (Fig. 3F). Moreover, the caspase inhibitor QVD prevented thapsigargin-induced death in WT and ire1α−/−, but not in bak/bax−/− cells. These results indicate that distinct mechanisms are involved in death signaling of ire1α−/− and bak/bax−/− cells. In the presence of Bak/Bax, cells die by a caspase-dependent pathway that is not impaired by lack of IRE1α, whereas in bak/bax−/− cells, death does not require caspases.
To substantiate these results, we analyzed MEFs from jnk-1−/−, jnk-2−/− and jnk-1−/−/jnk-2−/− (also known as Mapk8) mice. JNK-deficient cells were even more sensitive to ER stress than their WT counterparts (Fig. 3G, supplementary material Fig. 4S). Moreover, no protection was found in ask-1−/− MEFs (supplementary material Fig. S5). Finally, the JNK inhibitor SP-600125 did not affect cell death induced by ER stress at all, in either WT or in bak/bax−/− MEFs (Fig. 3H). In conclusion, neither genetic ablation of the ER stress sensors PERK and Ire1α nor disturbance of JNK signaling diminished cell death in response to thapsigargin.

Thapsigargin induces rupture of mitochondria in bak/bax−/− MEFs

To elucidate how bak/bax−/− MEFs died, we analyzed their morphology by electron microscopy. After 36 hours of treatment with thapsigargin WT cells showed typical signs of apoptosis including remodeling of mitochondrial cristae (Fig. 4A). By contrast, bak/bax−/− mitochondria displayed massive swelling indicative of MPT and rupture of the outer membrane. A permanent MPT can lead to a loss of ΔΨm and contribute to cytochrome c release in a Bak/Bax-independent manner. To explore whether the morphological alterations of bak/bax−/− mitochondria were associated with a loss of ΔΨm, we stained cells with the potentiometric dye DiOC6 and analyzed by immunoblotting (Fig. 4B,C). WT MEFs responded with a loss of ΔΨm (Ricci et al., 2004). Provided that caspases are blocked, mitochondria have been shown to restore mitochondrial cytochrome c and ΔΨm (Morita et al., 1999). Our results show that caspases are not activated in bak/bax−/− cells. Nevertheless, these cells are unable to regain respiratory function, further indicating an MPT-mediated loss of ΔΨm.

In WT cells, caspases cleave the p75 subunit of complex I of the respiratory chain resulting in permanent loss of ΔΨm (Martinou et al., 2004). Provided that caspases are blocked, mitochondria have been shown to restore mitochondrial cytochrome c and ΔΨm and remain structurally and functionally intact after MOMP (Martinou et al., 1999). Our results show that caspases are not activated in bak/bax−/− cells. Nevertheless, these cells are unable to regain respiratory function, further indicating an MPT-mediated loss of ΔΨm.
Bak/Bax-deficient, but not WT MEFs are rescued from thapsigargin-induced death by Ca^{2+} chelation and inhibition of mitochondrial respiration. MPT pore opening can be induced by elevated mitochondrial calcium concentration \([\text{Ca}^{2+}]_{\text{Mi}}\). Thapsigargin-mediated \([\text{Ca}^{2+}]_{\text{ER}}\) depletion has been reported to increase mitochondrial \(\text{Ca}^{2+}\) (Dong et al., 2006). As we found that Bak/Bax-deficient cells were not protected against death induced by a \(\text{Ca}^{2+}\) ionophore (Fig. 5A), we analyzed whether elevated \([\text{Ca}^{2+}]\) was involved in thapsigargin-mediated cell death. Indeed, \(\text{Ca}^{2+}\) chelation by BAPTA-AM protected Bak/Bax-deficient but not WT cells from death and reduced DNA fragmentation in a dose-dependent manner (Fig. 5B,C). Similar results were obtained with cyclopiazonic acid (CPA), another SERCA inhibitor. That BAPTA-AM did not completely prevent cell death is presumably due to close contact sites between the ER and mitochondria, which allow fast \(\text{Ca}^{2+}\) transfer (Szalai et al., 2000).

Mitochondrial \(\text{Ca}^{2+}\) uptake is driven through \(\Delta\Psi_{\text{m}}\), generated by the respiratory chain. Consequently, inhibition of respiration should protect against thapsigargin. When we applied SERCA inhibitors
in combination with rotenone, an inhibitor of complex I, bak/bax<sup>−/−</sup> MEFs were rescued and even clonogenicity was preserved (Fig. 5D,E). By contrast, WT cells were not rescued by Ca<sup>2+</sup> chelation or inhibition of respiration, either in short- or in long-term assays (Fig. 5B,D,E). This indicates that the death of apoptosis-competent MEFs does not require mitochondrial Ca<sup>2+</sup> accumulation and MPT.

ER stress induces necrosis in Bak/Bax-deficient cells. Our results demonstrate that ER stress eliminates bak/bax<sup>−/−</sup> MEFs by a calcium-dependent, non-apoptotic mechanism. When we analyzed the morphology of bak/bax<sup>−/−</sup> MEFs, we found that thapsigargin triggered signs of necrosis, including swelling of mitochondria, cell volume increase and rupture of the plasma membrane, alterations that were not observed in WT cells (Fig. 6A). Recently, the release of high-mobility group 1 protein (HMGB1) was shown to distinguish apoptotic from necrotic cell death (Scaffidi et al., 2002). This strict specificity has recently been questioned, because HMGB1 release was also detected in secondary necrotic, apoptotic cells (Bell et al., 2006). Therefore, to exclude secondary necrosis, we analyzed the release of HMGB1 in the presence of the caspase inhibitor QVD. We found that WT MEFs released only small amounts of HMGB1, and this was completely inhibitable by QVD (Fig. 6B). By contrast, bak/bax<sup>−/−</sup> MEFs released substantial amounts of HMGB1 upon stimulation with thapsigargin. This release could not be blocked by QVD and was therefore not caused by secondary postapoptotic necrosis. Thus, these results indicate that the death of bak/bax<sup>−/−</sup> cells was indeed due to necrosis.

Recently, it has been demonstrated that activation of poly(ADP-ribose)polymerase-1 (PARP-1) causes translocation of HMGB1 from the nucleus into the cytoplasm of necrotic cells, from where it is released after plasma membrane rupture (Ditsworth et al., 2007). Strong activation of PARP consumes large amounts of NAD<sup>+</sup> (Herceg and Wang, 2001). Strong activation of PARP consumes large amounts of NAD<sup>+</sup> and, in efforts to resynthesize NAD<sup>+</sup>, causes massive ATP depletion (Ha and Snyder, 1999). Depletion of cellular energy may switch apoptotic cell death that is energy-dependent to necrosis (Leist et al., 1997; Ferrari et al., 1998; Los et al., 2002). By contrast, during apoptosis, caspases cleave PARP-1 and thereby maintain ATP levels. Our results show that PARP-1 is cleaved in WT, but not in Bak/Bax-deficient cells upon treatment with thapsigargin (Fig. 6C). This finding indicates that ER stress might trigger PARP-dependent energy depletion in Bak/Bax-deficient MEFs.

Therefore, we analyzed cellular ATP content, which revealed that apoptosis induction by staurosporine led to complete ATP loss after 48 hours in WT, but not bak/bax<sup>−/−</sup> MEFs. By contrast, ER stress caused complete ATP consumption in both WT and bak/bax<sup>−/−</sup> cells (Fig. 6D). To confirm the functional relevance of PARP-1 in thapsigargin-induced death of bak/bax<sup>−/−</sup> MEFs, we analyzed cell death, ATP content and PARP-1 cleavage in the presence or absence of the PARP-1 inhibitor 3-aminobenzamide (3-AB). These experiments revealed that bak/bax<sup>−/−</sup> cells treated with 3-AB in addition to thapsigargin had significantly increased ATP levels (Fig. 6E), showed reduced poly(ADP-ribosylation) of PARP-1 (Fig. 6F) and were significantly rescued from necrotic death (Fig. 6E).

To test whether ER stress-induced necrosis of Bak/Bax-deficient cells is a general finding not limited to MEFs, we analyzed thapsigargin-induced cell death in different tumor cells (Fig. 7B,C). Similar to several other cell lines tested, HCT116 colon carcinoma cells were more sensitive to thapsigargin than to equimolar concentrations of etoposide (Fig. 7A). In response to thapsigargin, HCT116 cells expressing both Bak and Bax died at least partially by apoptosis, as assessed by the protective effect of the caspase

![Fig. 6. Thapsigargin induces necrotic death in Bak/Bax-deficient MEFs.](Image)

(A) WT and bak/bax<sup>−/−</sup> MEFs were treated and analyzed as in Fig. 4A. Low magnification electron micrographs of whole WT MEFs (left panels) and bak/bax<sup>−/−</sup> MEFs (middle panels). Boxed regions are shown at higher magnification (right panels). (a,b) Arrowheads mark disrupted plasma membrane sections (scale bars: 1 μm). (B) Bak/bax<sup>−/−</sup> and WT MEFs were treated with thapsigargin in the presence or absence of QVD for 24 hours. Release of HMGB1 into the extracellular culture supernatant was assessed by immunoblotting. Loading was controlled by Coomassie Blue staining of the gel. Remaining intracellular HMGB1 content was assessed by immunoblot of the corresponding cell lysates. (C) Thapsigargin induces PARP activation in Bak/Bax-deficient cells. WT and bak/bax<sup>−/−</sup> MEFs were treated with thapsigargin for 48 hours and PARP-1 expression was investigated by immunoblot analysis. Loss of full-length PARP-1 in WT cells is indicative of PARP-1 cleavage and inactivation. Bak/Bax-deficient cells retain expression of PARP-1. (D) Upper panel: thapsigargin causes ATP depletion in Bak/Bax-proficient and Bak/Bax-deficient MEFs. Cells were treated for 48 hours with thapsigargin or staurosporine (STS) or left untreated. ATP levels were measured by a luminescence assay (mean of light values ± s.d.). Asterisks denote significant ATP loss, P<0.05. Lower panel: death induced by thapsigargin and staurosporine in Bak/Bax-proficient and Bak/Bax-deficient cells after 48 hours. Death was measured by flow cytometry (values are mean ± s.d.; *significantly elevated cell death of treated compared to untreated samples; P<0.05). (E) Inhibition of PARP-1 reduces death and ATP loss. Bak/bax<sup>−/−</sup> MEFs were treated with thapsigargin in the presence or absence of PARP-1 inhibitor 3-amino-benzamide (3-AB; 10 mM). Cell death and ATP content were analyzed after 48 hours. Asterisks indicate significant suppression of cell death and ATP loss in samples treated with thapsigargin and 3-AB as compared with thapsigargin-treated controls, P<0.05. (F) Immunoblot analysis of PARP-1 in bak/bax<sup>−/−</sup> MEFs treated as described in E. 3-AB reduced the occurrence of slower migrating ADP-ribosylated PARP-1 in the knockout cells.
inhibitor QVD (Fig. 7B). The shRNA-mediated knockdown of Bak alone did not abrogate apoptosis. Strikingly, however, HCT116 cells deficient in Bax or Bax and Bak were protected from caspase-dependent apoptosis, but not from necrotic death. Similarly, thapsigargin killed Bax-deficient DU145 prostate carcinoma cells by a caspase-independent mechanism (Fig. 7C), whereas re-expression of Bax switched the mode of cell death to apoptosis. Thus, by employing different cellular systems we demonstrate that thapsigargin can efficiently trigger non-apoptotic death of Bak/Bax-deficient cells.

**Discussion**

Chemotherapeutic agents induce cell death mostly via the mitochondrial apoptotic pathway that is frequently compromised in tumors (Fischer et al., 2007). In this study, we demonstrate that multidrug-resistant Bak/Bax-deficient MEFs can still be efficiently eliminated by ER stress-inducing drugs, such as SERCA inhibitors, which trigger an MPT-dependent necrotic program (Fig. 8). We show that thapsigargin not only eliminates Bak/Bax-deficient MEFs, but also severely impairs their clonogenicity after short-term treatment. Similarly, tumor cells deficient in Bak/Bax expression succumb to a caspase-independent, non-apoptotic death after treatment with thapsigargin. Our results thus suggest that SERCA inhibition might be an attractive strategy for the treatment of multidrug-resistant tumors.

SERCA inhibitors and other ER stress inducers trigger the UPR that is launched primarily to maintain ER homeostasis and cell survival. Classical UPR components were, however, not critical in determining the cell death decision in response to thapsigargin. Distinct regulators of the UPR, such as PERK and ATF6, have been implicated in apoptosis upon ER stress (Szegedi et al., 2006). However, our results show that thapsigargin-mediated cell death is not compromised in PERK-deficient cells. Similarly, genetic targeting of ATF6 was reported not to protect against ER stress-mediated cell death (Wu et al., 2007; Yamamoto et al., 2007). However, since both PERK and ATF6 independently regulate survival. Classical UPR components were, however, not critical in determining the cell death decision in response to thapsigargin. Distinct regulators of the UPR, such as PERK and ATF6, have been implicated in apoptosis upon ER stress (Szegedi et al., 2006). However, our results show that thapsigargin-mediated cell death is not compromised in PERK-deficient cells. Similarly, genetic targeting of ATF6 was reported not to protect against ER stress-mediated cell death (Wu et al., 2007; Yamamoto et al., 2007). However, since both PERK and ATF6 independently regulate transcription of the UPR mediator CHOP (Szegedi et al., 2006), these findings do not exclude a possible role of CHOP in

**Fig. 7.** Thapsigargin-induced cell death in different human tumor cell lines. (A) Thapsigargin efficiently eradicates a wide variety of tumor cell lines of different tissue origin. T98 glioblastoma, U2OS osteosarcoma, HT1080 fibrosarcoma, HeLa cervix carcinoma, and HCT116 colorectal carcinoma cells were treated for 48 hours with thapsigargin or etoposide (both 1 μM) or left untreated. Cell death was assessed by flow cytometric measurement of PI uptake (mean ± s.d.; *significantly more drug-induced cell death compared with etoposide treated cells; P<0.05). (B) Death of Bax-deficient HCT116 cells is caspase independent. Right panel: immunoblot analysis of Bak and Bax in HCT116 and isogenic BAX−/− (bax−/−) cells stably transfected with Bax-specific shRNA or a corresponding empty vector. Left panel: HCT116 WT, bax−/− and bax−/−/bax−/− cells were treated with thapsigargin for 5 days in the presence or absence of QVD. Cell death was determined by PI uptake (values are mean ± s.d.; solid asterisks: significant induction of death in thapsigargin-treated compared with untreated cells, P<0.05; open asterisk: significant inhibition of thapsigargin-mediated death by caspase inhibition, P<0.05). (C) Upper left: Bax-deficient prostate carcinoma DU145 cells are more efficiently eliminated by thapsigargin compared with equimolar concentrations of etoposide (both 1 μM). Cells were treated for 72 hours and death was analyzed by flow cytometry. Upper right: Bax-deficient DU145 cells die by a caspase-independent mechanism in response to thapsigargin, whereas reconstitution with GFP-tagged Bax leads to caspase-dependent cell death. Cells were treated for 48 hours with thapsigargin in the presence or absence of QVD. Cell death was analyzed as described above. Lower left: Bak and Bax expression of DU145 cells was analyzed by immunoblotting. Hey ovarian carcinoma cells expressing both Bak and Bax served as a control. Lower right: flow cytometry confirmed that >90% of the reconstituted DU145 cells expressed GFP-Bax.

**Fig. 8.** Proposed model of ER stress-induced cell death in wild-type and Bak/Bax-defective cells. Left panel: in WT cells ER stress triggers a BH3-only protein-mediated conformational change of Bak/Bax and subsequent cytochrome c release. The subsequent loss of ΔΨm prevents further Ca2+-uptake and damage of mitochondria. Caspase activation then shuts off respiration permanently by amplification of MOMP and proteolytic destruction of components of the respiratory chain. Activation of PARP-1 presumably by ROS-mediated DNA damage is counteracted by caspase-mediated cleavage. As a result cells die by apoptosis. Right panel: when cells lack Bak and Bax BH3-only proteins cannot induce cytochrome c release. Initial mitochondrial Ca2+-uptake further enhances ΔΨm, which in turn drives further Ca2+ uptake, creating a vicious circle that results in MPT, rupture of the outer mitochondrial membrane and passive leakage of intermembrane proteins such as cytochrome c or AIF. Respiration cannot be restored. In the absence of caspase activation PARP-1 depletes residual ATP and cells eventually die by necrosis.

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thapsigargin-induced cell death. In both WT and bak/bax−/− MEFs we observed induction of CHOP and its downstream target Bim that has been implicated in apoptosis (Puthalakath et al., 2007), but not necrosis during ER stress.

Another mechanism by which the UPR could impact on cell death is the IRE1α pathway. Bak/Bax were suggested to directly interact with IRE1α resulting in activation of JNK (Hetz et al., 2006). However, neither the knockout of IRE1α, JNK nor other components of this pathway or JNK inhibition conferred protection from ER stress. Our observation that death of ire1α−/− cells was caspase dependent, whereas it was caspase independent in bak/bax−/− cells, rather suggests that Bak/Bax and IRE1α are acting on separate pathways.

In WT and Bak/Bax-deficient MEFs ER stress rapidly induced autophagic alterations. This indicates that autophagic alterations per se are independent of proapoptotic Bcl-2 proteins. Autophagy has been implied as a death mechanism substituting for deficient apoptosis under certain conditions (Levine and Yuan, 2005; Maiuri et al., 2007). Interestingly, autophagy was more pronounced in apoptosis under certain conditions (Levine and Yuan, 2005; Maiuri et al., 2007). Nevertheless, we found no protection against ER stress in Bak/Bax-deficient MEFs (data not shown). ER Ca²⁺ release was also shown to activate calcineurin that enhances the proapoptotic potential of Bad (Wang et al., 1999) and loss of Puma protected motoneurons from ER-stress-induced apoptosis but not necrosis (Kieran et al., 2007). It is also possible that mitochondria of Bak/Bax-deficient MEFs could not activate cytochrome c is not quickly released via Bak/Bax pores and caspasas are not activated. This probably enhances ΔΨm and Ca²⁺ uptake until MPT induces swelling and final rupture of bak/bax−/− mitochondria.

It is also possible that mitochondria of Bak/Bax-deficient MEFs have a higher sensitivity to MPT-induced membrane rupture. Unlike elongated mitochondria of WT cells, mitochondria from bak/bax−/− MEFs are small and spherical, probably because of deficient mitochondrial fusion (Karbowska et al., 2006). Therefore, Bak/Bax-deficient mitochondria can be assumed to have a maximal volume to surface ratio, resulting in higher susceptibility to swelling-induced outer membrane rupture. By contrast, elongated WT mitochondria could presumably withstand a substantial volume increase without loosing outer membrane integrity.

Apart from mitochondrial disruption, we find that PARP-1 was activated in bak/bax−/− cells, whereas inhibition of PARP-1 prevented ER stress-induced death. PARP-1 is a major regulator of necrotic cell death and persistent PARP-1 activation is known to cause deleterious ATP depletion in necrotic cells (Ha and Snyder, 1999). As we show here, PARP-1 is cleaved and inactivated by caspasas in WT cells, but remained active in Bak/Bax-deficient MEFs under ER stress. ATP consumption triggered by PARP-1 under ER stress is probably crucial for Bak/Bax-deficient cells, as
mitochondrial disruption compromises their main source of ATP synthesis. It is tempting to speculate that the pronounced activation of autophagy in bak/bax−/− MEFs treated with ER stress drugs might be a mechanism to compensate for the loss of ATP to enable survival. PARP-1 activation is readily triggered by DNA damage and oxidative stress. Since calcium overload, PT pore opening and mitochondrial rupture are accompanied by increased oxidative stress (Green and Reed, 1998), similar mechanisms are presumably responsible for PARP-1 activation in bak/bax−/− MEFs.

In conclusion, our study demonstrates that induction of necrosis by ER stress might be an attractive approach to overcome the resistance of Bax- or Bak/Bax-deficient tumor cells. Compared with agents that affect other ER functions, SERCA inhibitors might be particularly promising candidate drugs. Experiments performed within the National Cancer Institute’s anticancer screens already demonstrated that thapsigargin possesses a broad growth-inhibitory activity in hematopoietic and non-hematopoietic tumor cells with a 10- to 100-fold greater efficiency as compared with traditional antitumor agents. Some of the drugs that affect other ER functions, SERCA inhibitors might be particularly promising candidates. Experiments performed within the National Cancer Institute’s anticancer screens already demonstrated that thapsigargin possesses a broad growth-inhibitory activity in hematopoietic and non-hematopoietic tumor cells with a 10- to 100-fold greater efficiency as compared with traditional antitumor agents.

Materials and Methods

Cell culture and treatments

Knockout and respective wild-type (WT) MEFS were kindly provided by Randal J. Kaufman (University of Michigan Medical Center, Ann Arbor, MI), Hans-Uwe Simon (University of Bern, Bern, Switzerland), Andreas Strasser (Walter and Eliza Institute, Melbourne, Australia), Hidenori Ichijo (Tokyo Medical University, Tokyo, Japan), Erwin F. Wagner (Institute of Molecular Pathology, Vienna, Austria) and Stanley J. Korsmeyer (Harvard Medical School, Boston, MA) and maintained in DMEM supplemented with 10% FCS and antibiotics. U2OS, Hey and DU145 cells were grown in the same medium. T98, HT1080 and HeLa cells were kept in complete RPMI-1640. HT116 WT and Bax-deficient cells were provided by Bert Vogelstein (Johns Hopkins Cancer Center, Baltimore, MD) and cultured in McCoy’s 5A medium. Stable knockdown of Bak was performed in HT116 WT and HT116 Bax knockout cells as described previously (Hemmati et al., 2006).

Cells were seeded at 5×104 cells per well in six-well plates and treated for 24-96 hours with thapsigargin (1.5 μM, or as indicated), tunicamycin (1 μg/ml, or as indicated) or staurosporine (1 μM, all from Calbiochem, Darmstadt, Germany) in the presence or absence of QVD (20 μM; MP-Biomedicals, Eschwege, Germany), BAPTA-AM (Alexis Biochemicals, Lörach, Germany), rotenone, 3-amino-benzoamide, 3-methyladenine (Sigma-Aldrich, Steinheim, Germany) or SP-600125, cyclopiazonic acid and A23187 (Calbiochem). Taxol, etoposide, cisplatin and 3-methyladenine (Sigma-Aldrich, Steinheim, Germany) or SP-600125, cyclopiazonic acid and A23187 (Calbiochem).

Immunoblotting

Cells were lysed in buffer containing 1% NP-40, 50 mM Tris, pH 7.5, 350 mM NaCl, 0.5 mM EDTA, 2 mM DTT (dithiothreitol), 10 mM NaF, 0.5 mM sodium vanadate and protease inhibitor cocktail. Proteins were separated on 8-15% polyacrylamide gels, transferred to polyvinylidene fluoride membranes and detected by chemiluminescence.

The following primary antibodies were used: β-actin (clone AC-74) and α-tubulin (clone DM1A) from Sigma-Aldrich, Bim (clone 3C5) from Alexis Biochemicals, active Bak and Bax (rabbit polyclonal) from Upstate (Lake Placid, NY), Bel-2 (clone C-2), CHOP/GADD153 (clone B-3), GAPDH (clone 0411) from Santa Cruz Biotechnology (Santa Cruz, CA), LC3 (clone 2G6) from Nanotools (Teningen, Germany), BIP/GPR78 (clone 40), caspase-3 (clone 46), cytochrome c (clone 6H1, B4), PARP-1 (clone 7D6) from BD Biosciences, Bid (goat polyclonal) from R&D Systems (Wiesbaden, Germany), and pELF2-Ser51, JNK, pJNK-Thr183/Tyr185, pJNK-Thr221/Tyr223 (all rabbit polyclonal) from Cell Signaling Technology (Danvers, MA).

Measurement of cellular ATP content

ATP content was measured by bioluminescent detection (ApoSENSOR, Biovision, Mountain View, CA) according to the manufacturer’s instructions. Triplicates of 104 cells per condition were transferred into white 96-well plates and incubated for 5 minutes in nucleotide-releasing buffer. After addition of luciferase, light emission was measured in a luminometer (Berthold Technologies, Bad Wildbad, Germany).

HMGB1 release

HMGB1 release was assessed by immunoblot analysis. Cells were seeded at 105 per well into six-well plates. After 24 hours the supernatant was replaced with serum-free medium containing the indicated drugs. After a further 24 hours the medium was collected and cleared by centrifugation. A 1/100 volume of 2% sodium deoxycholate was added and samples were incubated at 4°C for 30 minutes. Proteins were precipitated with trichloroacetic acid at 4°C overnight. Immunoblot analysis was performed using a rabbit anti-HMGB1 antibody (BD Biosciences).

Caspase activity assays

Caspase activity was measured using a luminescence assay (Caspase-Glo 3/7, Promega) according to the manufacturer’s protocol. Cell lysates (25 μg protein) were added to a prominescent caspase substrate containing the tetrapeptide sequence DEVD. Light production indicative of substrate cleavage was monitored for up to 180 minutes.

Transmission electron microscopy

For electron microscopy, MEFS were plated at a density of 175 cell/μm2 cell culture flasks. After 24 hours the cells were stimulated with either thapsigargin or tunicamycin or left untreated and harvested 36 hours later. Sections (50 nm) were prepared following a standard protocol (Viemann et al., 2007) and examined on an electron microscope (Zeiss EM 902A, Zeiss, Jena, Germany).

Flow cytometric analyses

Cell death was determined by the uptake of propidium iodide (PI, 2 μg/ml) and analyses of DNA fragmentation were performed as described previously (Nicoletti et al., 1991). Loss of ΔΨm was analyzed using the cationic dye DIOCl (Fischer et al., 2003). Cytochrome c release was analyzed as previously described (Waterhouse and Trapani, 2003). GFP-Bax expression was controlled by measuring fluorescence in FI-1. Each experiment was performed independently at least three times. An individual experiment analyzing 10,000 cells on a FACS Calibur flow cytometer (Becton Dickinson) was carried out in duplicate.

Clonogenicity assays

The colony-forming potential of adherent cells was measured as described previously (Janssen et al., 2007). In brief, 3×103 cells per well were seeded onto six-well plates and treated with the indicated drug concentrations on the following day. After 24 or 48 hours cells were washed and maintained in normal medium for another 24 hours until the untreated controls reached 90% confluency. Cells were split 1:10 and reseeded onto new plates. After 6 days viable cells were stained with 0.5% Crystal Violet and solubilized in 33% acetic acid. Absorbance was measured at 590 nm (4590). Relative proliferation was defined as A590 of test well × 100 divided by the A590 of untreated well. Experiments were performed independently three times in duplicate.

Statistical analysis

Statistical analyses were performed using two-tailed t-tests for equality of means (SPSS Statistics 17.0 software). A P-value of less than 0.05 was considered significant.

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References


Figure S1: Loss of Bak/Bax expression promotes clonogenic survival after treatment with chemotherapeutic reagents. WT and bak/bax−/− MEFs were treated with taxol (1 µM), cisplatin (5 µM), camptothecin (0.1 µM) or actinomycin D (0.1 µg/ml). After 48 hours cells were washed and kept in normal medium. 24 hours later cells were split, cultured for further 7 days and stained with crystal violet.
Figure S2: ER stress induced by thapsigargin eradicates Bak/Bax-deficient cells more efficiently than tunicamycin. Bak/bax<sup>−/−</sup> and WT MEFs were treated for 48 hours with the indicated concentrations of tunicamycin (left panel) and thapsigargin (right panel), respectively. Cell death was assessed by flow cytometric measurement of PI uptake. Mean values ± s.d. are shown. *Significant inhibition by genetic knockout of bak and bax (P<0.05).
Figure S3: Genetic ablation of PERK does not protect against ER stress-induced cell death. WT and perk−/− MEFs were treated with thapsigargin (1.5 µM) or tunicamycin (1 µg/ml) and analyzed for cell death after 48 hours as described in Figure S2.
Figure S4: Deficiency of JNK1 or JNK2 confers no protection against ER stress-mediated death. Jnk-1 and jnk-2 knockout MEFs and respective WT cells were treated with thapsigargin (1.5 µM) or tunicamycin (1 µg/ml) for 48 hours. Cell death was measured as described in Figure S2.
Figure S5: Genetic ablation of ASK1 does not protect against ER stress-induced cell death. WT and ask1−/− MEFs were treated with thapsigargin (1.5 μM) or tunicamycin (1 μg/ml). Cell death was measured after 48 hours as described in Figure S2.