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Regulation of endoplasmic reticulum Ca^{2+} dynamics by proapoptotic BCL-2 family members

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Abstract

Uncontrolled cytosolic Ca^{2+} overload is a common cause of cell death in several pathological conditions. Recent evidences reveal a more regulated role for intracellular Ca^{2+} stores in controlling cell death. Proteins of the BCL-2 family include anti- and proapoptotic members that control the mitochondrial amplification loop of apoptosis. The antiapoptotic protein BCL-2 prevents this mitochondrial loop, while the ''multidomain'' proapoptotic proteins BAX and BAK are crucial to initiate it. BCL-2, BAX and BAK localize also to the endoplasmic reticulum (ER), the main intracellular Ca^{2+} store. Overexpression of BCL-2 reduces resting ER Ca^{2+} and death in response to apoptotic stimuli that mobilize Ca^{2+}. Our recent data indicate that multidomain proapoptotics also influence Ca^{2+} metabolism. Cells deficient for *Bax*, *Bak* (DKO) display lowered steady state ER Ca^{2+} concentrations ($[Ca^{2+}]_{er}$) and secondarily decreased mitochondrial Ca^{2+} uptake. Genetic and pharmacologic correction of $[Ca^{2+}]_{er}$ indicates that it controls death in response to Ca^{2+}-dependent, mitochondria utilizing signals such as oxidative stress and lipid mediators; and that it participates in the regulation of the apoptotic response to most intrinsic stimuli, such as staurosporine. Thus, BAX and BAK control apoptosis not only at the mitochondria, but also at the ER, an obligate checkpoint for Ca^{2+}-dependent apoptotic stimuli.

Keywords: Apoptosis; BCL-2; BAX; BAK; Endoplasmic reticulum; Ca^{2+}; Oxidative stress

1. Introduction

Programmed cell death is a well-conserved pathway whose basic tenets appear common to all metazoans. Key components identified by genetic and biochemical approaches regulate the commitment step and/or participate in effecting cell demise [1]. Evidence indicates that mitochondria are pivotal to amplify apoptotic signals [2], but other organelles, including the ER, have also been implicated [3]. The ER can be an initiator of apoptosis when accumulation of unfolded proteins or inhibition of the ER–Golgi transport results in the so-called ER stress response [4]. It is also potentially a regulator of the progression of apoptosis, at least for two reasons: it is the main intracellular store of Ca^{2+}, one of the most versatile and ubiquitous second messengers, and it is physically and physiologically interconnected with mitochondria. This spatial and functional organization impacts on the regulation of mitochondrial function and on complex cellular processes [5,6]. On one hand, mitochondria modulate and synchronize Ca^{2+} signalling [7]. Stimuli that generate inositol 1,4,5-trisphosphate (IP3) cause the release of Ca^{2+} from the ER, which is rapidly taken up by closely juxtaposed mitochondria [8]. Mitochondrial Ca^{2+} uptake can shape the spatio-temporal distribution of cytosolic Ca^{2+} waves during signalling [9]. After the discharge of intracellular Ca^{2+} stores, clearance of cytosolic Ca^{2+} by strategically located mitochondria also modulates the opening of channels in the plasma

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Abbreviations: DKO, Bax, Bak double knockouts; ER, endoplasmic reticulum; IP3, inositol 1,4,5-trisphosphate; mtBAX, mitochondrially targeted BAX; PT, mitochondrial permeability transition; PTP, permeability transition pore; SERCA, sarcoplasmic–endoplasmic reticulum Ca^{2+} ATPase; wt, wild-type.

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membrane that are responsible for the capacitative entry of Ca^{2+} from the extracellular space [10]. On the other hand, Ca^{2+} modulates mitochondrial function. An increase in mitochondrial matrix Ca^{2+} ($[Ca^{2+}]_m$) regulates metabolism, including the activation of enzymes of the tricarboxylic acid cycle, resulting in a net increase in ATP production [5,11]. Ca^{2+} is also a prominent modulator of the PT, controlled by the PTP, a high conductance channel that in its fully open conformation allows permeabilization of the inner membrane to solutes up to 1500 Da [12]. PT has been implicated in both apoptotic and necrotic cell death following selected stimuli [13–15]. In addition to its role in modulating mitochondrial function, Ca^{2+} has long been recognized as a participant in different apoptotic pathways [16,17]. Ca^{2+}-dependent endonucleases mediate DNA fragmentation late in the course of apoptosis [18]. Several apoptotic stimuli, including the pan-kinase inhibitor staurosporine [19], the lipid mediators arachidonic acid and ceramide [20,21], and growth factor deprivation [22] are reported to increase cytosolic Ca^{2+} early in the course of apoptosis.

The BCL-2 family proteins serve as critical death regulators that reside immediately upstream of mitochondria and consist of both anti- and proapoptotic members. The proapoptotic BCL-2 members can be subdivided into ''multidomain'' and ''BH3-only'' proteins. Multidomain proapoptotic members such as BAX and BAK display sequence conservation in the BCL-2 homology (BH) domains 1–3. Cells lacking BAX, BAK indicate they are required for apoptosis following a diverse array of intrinsic death signals and extrinsic death receptor signals in Type II cells that require a mitochondrial amplification loop [23,24]. BH3-only members display sequence conservation only in the amphipathic α-helical BH3 region, which constitutes the critical death domain [25]. BH3-only proteins reside upstream in the pathway and their activation by transcriptional regulation or post-translational modification interconnects proximal death signals to the core apoptotic pathway [26]. The BH3-only proteins BID, BAD, BIM and NOXA require BAX, BAK to mediate cell death [27]. BH3-only proteins either directly or indirectly activate BAX, BAK, inducing their intramembranous homo-oligomerization which results in permeabilization of the outer mitochondrial membrane (OMM) and release of intermembrane space proteins, including cytochrome *c* and SMAC/DIABLO [28,29]. Other programs of mitochondrial remodeling insure the complete release of these proteins and mitochondrial dysfunction [30]. BAK resides at mitochondria and can be activated by the BH3 domain of tBID to release cytochrome *c* [28]. tBID can also activate BAX which is largely in the cytosol of cells *in situ* [23,31], but can also be loosely attached to intracellular membranes including the OMM of cultured cell lines [32]. Once released, cytochrome *c* complexes with Apaf-1 and caspase-9 to form a postmitochondrial apoptosome that amplifies effector caspase activation [33].

Evidence exists that BCL-2 also localize at the endoplasmic reticulum and that it can modulate Ca^{2+} fluxes during the course of cell death. Interestingly, several groups showed that overexpressed BCL-2 reduces resting ER Ca^{2+} concentration and the extent of capacitative Ca^{2+} entry, pointing to a specific role of BCL-2 at the ER in the control of cell death [34,35]. BCL-2 overexpression confers at least partial resistance to the stimuli that are reported to increase cytosolic Ca^{2+}, such as ceramide, arachidonic acid, growth factor deprivation and staurosporine. BCL-2 overexpression also protects from death induced by thapsigargin, an irreversible inhibitor of the sarcoplasmic–endoplasmic reticulum Ca^{2+} ATPase (SERCA) responsible for the reuptake of Ca^{2+} from the cytosol into the ER lumen [36]. Spatially restricted, ER located Bcl-2 inhibits apoptosis by ceramide, ionizing radiation, serum withdrawal, c-*myc* expression and the ER stressors tunicamycin and brefeldin A [37–40]. Andrews and co-workers suggested that ER restricted BCL-2 can inhibit forms of apoptosis in which the mitochondrial dysfunction precedes cytochrome *c* release, whereas it has no effect on those deaths in which the mitochondrial dysfunction is a consequence of the loss of cytochrome *c* [40].

Here we will review our recent data that support a novel role for BAX and BAK as regulators of Ca^{2+} concentration in the ER. In this way, BAX and BAK control the apoptotic response to Ca^{2+}-dependent stimuli, upstream of mitochondria.

2. A novel role for BAX and BAK in controlling steady state ER Ca^{2+} levels

Bax, *Bak* deficient cells are resistant to death induced by the irreversible SERCA inhibitor thapsigargin [23]. When we analyzed the subcellular distribution of both BAX and BAK, we found them associated not only with mitochondria, but also with the endoplasmic reticulum. These observations prompted us to investigate whether BAX, BAK influence the dynamics of Ca^{2+} signaling at the ER and mitochondria and the impact of such regulation on apoptosis [41].

Ca^{2+} metabolism presents striking differences in DKO cells compared to their wild-type (wt) counterparts. Inhibition of the SERCA pump by thapsigargin causes the passive release of ER Ca^{2+} stores and a rise in cytosolic Ca^{2+} ($[Ca^{2+}]_i$). In the absence of extracellular Ca^{2+}, the amount of cytosolic Ca^{2+} increase induced by thapsigargin is proportional to the ER Ca^{2+} content. The extent of capacitative entry of extracellular Ca^{2+} can be then assessed by re-addition of Ca^{2+}. DKO cells displayed similar kinetics, but a substantially lower peak $[Ca^{2+}]_i$ than wt cells as measured using the ratiometric probe Fura-2, while the capacitative Ca^{2+} entry proved similar in wt and DKO cells (Fig. 1A). A decreased $[Ca^{2+}]_i$ rise in response to the discharge of ER stores can be a consequence either of a

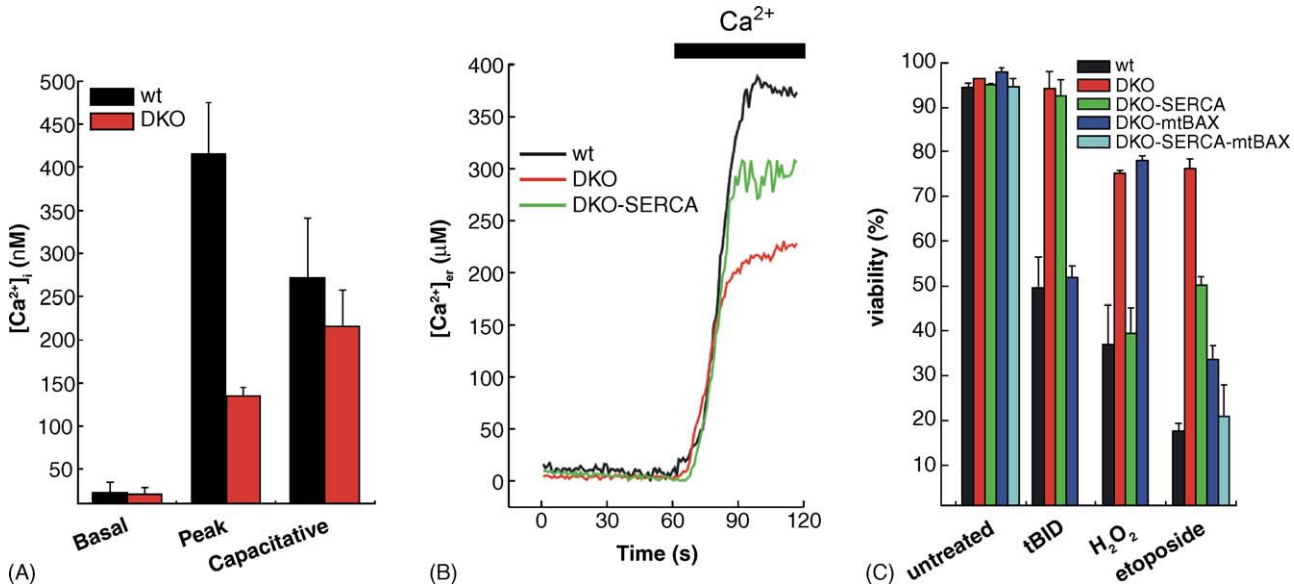

Fig. 1. Bax, Bak maintain steady state ER Ca^{2+} concentration: an ER gateway to control cell death. (A) Basal, peak after thapsigargin (200 nM) and capacitative (800 μM free extracellular Ca^{2+} final concentration) cytosolic $[Ca^{2+}]$ determined by Fura-2 in wt and DKO mouse embryonic fibroblasts (MEFs) incubated in Ca^{2+}-free medium as described in [41]. Means ± SE from nine independent experiments are showed. (B) Representative recordings of $[Ca^{2+}]_{er}$ measured with ER targeted AEQ in wt, DKO and SERCA corrected DKO cells. ER Ca^{2+} depleted cells were perfused where indicated with high-Ca^{2+} buffer (2 mM Ca^{2+}). See [41] for details. (C) Viability (mean ± SE of three experiments) of wt, DKO, DKO–mtBAX, DKO–SERCA and dual-corrected DKO–mtBAX–SERCA MEFs in response to stimuli that belong to three different classes: tBID, H_2O_2 (1 mM) and staurosporine (1 μM). For further details, see [41].

reduction in the ER Ca^{2+} content or of an increased mitochondrial Ca^{2+} uptake. We assessed Ca^{2+} metabolism both at mitochondria and ER in DKO cells. Using fluorescent probes specific for mitochondria and ER, such as rhod-2 and magfura-2, and aequorins, genetically encoded Ca^{2+}-sensors targeted to these organelles, we showed that DKO mitochondria do not display any intrinsic defect in Ca^{2+} uptake, nevertheless they respond with greatly reduced Ca^{2+} transients to stimuli that release Ca^{2+} from the ER. This is secondary to the diminished steady state ER Ca^{2+} levels in DKO cells [41] (Fig. 1B). This lowered steady state ER Ca^{2+} explains why cytosolic Ca^{2+} transients are blunted in DKO cells.

3. Do BAX and BAK control apoptosis from the ER?

The reduced ER Ca^{2+} level observed in DKO cells prompted us to question its relative impact on the regulation of apoptosis. We genetically and pharmacologically corrected the multiple defects of *Bax*, *Bak* deficient cells: the mitochondrial defect by targeting BAX exclusively to mitochondria, the ER defect by overexpressing SERCA, both the mitochondrial and the ER defects by generating doubly corrected cells that co-expressed SERCA and mitochondrially targeted BAX (mtBAX) (Fig. 1B and [41]). Of note, expression of mtBAX in the DKO cells did not correct $[Ca^{2+}]_{er}$, allowing us to selectively correct the multiple defects of the DKO cells. DKO cells selectively reconstituted with SERCA or mtBAX enabled us to test whether the outcome to different apoptotic stimuli is primarily controlled by ER Ca^{2+} levels or by mitochondria-based BAX, BAK. This analysis revealed that apoptotic stimuli can be divided in three categories. Certain stimuli require mitochondria-based multidomain proapoptotics and can proceed despite depleted intracellular Ca^{2+} stores. They include BH3-only proteins such as tBID, whose expression kills wt but not DKO cells [28] (Fig. 1C). While mtBAX restored apoptosis of DKO cells to essentially wt levels, DKO–SERCA cells remained resistant to tBID (Fig. 1C) [41]. The second class of stimuli includes molecules that rapidly mobilize Ca^{2+} as a critical component of their cell deaths: lipid mediators such as ceramide and arachidonic acid, or oxidative stress [14,21,36,41,42] The representative oxidant H_2O_2 induced death of wt but not DKO cells, which was restored in DKO–SERCA but not in DKO–mtBAX cells (Fig. 1C) [41]. This suggests that ER Ca^{2+} levels controlled by BAX, BAK determine the ability of hydrogen peroxide to kill. ''Intrinsic'' proapoptotic stimuli such as etoposide, staurosporine, and brefeldin A induce death by different mechanisms but all require BAX, BAK [23]. DKO cells reconstituted with mtBAX displayed considerable death in response to them (Fig. 1C) [41]. This confirmed the mitochondrial dependence of these agents and supports a model in which they activate BH3-only molecules. Of note, DKO–SERCA reconstituted cells displayed some death in response to these stimuli, but complete

correction was achieved only in the dual-corrected DKO–mtBAX–SERCA cells (Fig. 1C) [41].

4. Regulation of ER Ca^{2+} by BCL-2 family members: a control point for Ca^{2+}-dependent apoptotic signals positioned upstream of mitochondria

Ca^{2+} plays a prominent role in controlling several cellular functions and it has been implicated as a second messenger early in the course of cell death [16]. Several studies now reveal a genetic control over this second messenger, exerted by both pro- and antiapoptotic BCL-2 family members. Their regulation of steady state $[Ca^{2+}]_{er}$ appears to be a crucial checkpoint for Ca^{2+}-dependent apoptotic stimuli [21,34,35,41]. A common theme emerges as ablation of proapoptotic multidomain or overexpression of antiapoptotic multidomain members results in reduced steady state $[Ca^{2+}]_{er}$ and diminished mitochondrial Ca^{2+} uptake. This is consistent with a rheostat model in which the ratio of pro- to antiapoptotic BCL-2 members dictates the susceptibility to death signals. While loss of BAX, BAK and gain of BCL-2 share the reduction in $[Ca^{2+}]_{er}$, differences also exist. BCL-2 overexpression reduces the extent of the capacitative Ca^{2+} entry [34,35] and the levels of calreticulin and SERCA2b [43]. Cells expressing BCL-X_L display a significant reduction in the levels of the IP3 receptor Type I and a reduction of ER Ca^{2+} release elicited by IP3 coupled stimuli [44]. Conversely, capacitative Ca^{2+} entry and the levels of the aforementioned proteins are unaffected in DKO cells [41]. These differences argue for some distinct actions of proapoptotic vs. antiapoptotic BCL-2 members in controlling aspects of Ca^{2+} dynamics.

ER Ca^{2+} level is crucial to control the fate of the cell in response to certain stimuli such as ceramide, arachidonic acid and oxidative stress. Genetic [45] or pharmacologic [21] reduction of steady state ER Ca^{2+} levels is associated with resistance to apoptosis induced by ceramide. On the other hand, when BAX, BAK are overexpressed, they induce ER Ca^{2+} release with subsequent increase in mitochondrial Ca^{2+} and augmented cytochrome *c* release [46]. Altogether, it is tempting to speculate the existence of a dual control exerted by BAX, BAK over Ca^{2+} fluxes. Prior to apoptosis they are required for homeostatic, steady state $[Ca^{2+}]_{er}$ that secondarily dictates mitochondrial Ca^{2+} uptake [41]. In Bax-null cells the release of Ca^{2+} from the ER in response to Ca^{2+}-dependent apoptotic stimuli is reduced and can be restored only by re-expression of BAX [47]. Following apoptotic signals, conformational activation of BAX, BAK might modulate the release of Ca^{2+}, conceivably reflecting their capacity to form ion conductive pores [48,49] or regulate resident ER channels. Such an effect is suggested by models in which overexpression of BAX, known to cause its oligomerization [50], induces Ca^{2+} efflux from the ER [51] to enhance the release of cytochrome *c* during apoptosis [52]. The homeostatic effect of BAX, BAK on ER Ca^{2+} concentration serves as a gateway for Ca^{2+}-dependent apoptotic stimuli, while the release of Ca^{2+} induced by these multidomain BCL-2 family members during apoptosis would reflect an amplification loop between ER and mitochondria.

Ca^{2+}-dependent stimuli that require BAX, BAK at the endoplasmic reticulum kill through a mitochondrial pathway. Their death is characterized by mitochondrial dysfunction, cytochrome *c* release and caspase activation [41]. The lowered $[Ca^{2+}]_{er}$ in cells overexpressing BCL-2 or lacking BAX, BAK results in reduced mitochondrial Ca^{2+} uptake and in the abolishment of the program of mitochondrial dysfunction elicited by these stimuli. Restoration of adequate ER Ca^{2+} levels by SERCA not only corrects apoptosis to the Ca^{2+}-dependent stimuli, but also restores all the parameters of mitochondrial dysfunction normally induced by them [41]. A candidate mechanism for their mitochondrial effect is the PTP, which displays a striking Ca^{2+} dependency [53]. For example, subliminal levels of ceramide mediate the conversion of normal IP3-induced mitochondrial Ca^{2+} uptake into an apoptotic signal *via* the opening of the PTP [54], and death by oxidative stress and arachidonic acid, two PTP inducers, is potentiated by mitochondrial Ca^{2+} uptake [41,55]. Thus, the ER checkpoint controlled by BCL-2 family members appears to reside upstream of the mitochondrion. It regulates apoptosis by modulating mitochondrial Ca^{2+} uptake and Ca^{2+}-controlled mitochondrial parameters.

In summary, converging genetic and pharmacologic data indicate that pro- and antiapoptotic BCL-2 family members use the ER as a checkpoint, positioned upstream of mitochondria, to modulate the responsiveness of the cell to Ca^{2+}-dependent, mitochondria utilizing apoptotic stimuli.

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