Minireview

Participation of endoplasmic reticulum and mitochondrial calcium handling in apoptosis: more than just neighborhood? ☆

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Abstract Over the past few years, extensive progress has been made in elucidating the role of calcium in the signaling of apoptosis. This has led to the characterization of calcium's role in the induction of apoptosis and in the regulation of effector proteases. In this review, we attempt to summarize the current knowledge regarding a segment of these studies, the interaction between the endoplasmic reticulum (ER) and mitochondria. This interface has been shown to play a crucial role in transferring agonist induced Ca^{2+} signals to mitochondria during physiological processes. Recent evidence, however, extended the role of this Ca^{2+} transfer to apoptotic pathways, showing that modulation of mitochondrial Ca^{2+} uptake from the ER side has a prominent role in modulating cellular fate.

Keywords: Endoplasmic reticulum; Mitochondria; Apoptosis; Ca^{2+} release; Ca^{2+} overload; Voltage dependent anion channel (VDAC)

1. Introduction

Since the term "apoptosis" was first introduced in 1972 by John Kerr [1] to designate the common morphologic features of programmed cell death occurring in tissue remodeling [2], the process has gained enormous importance in normal and pathological cell conditions. The significance of this process is underscored by the fact that the process and its molecular components are conserved through virtually all metazoan species studied so far [3]. As the extensive investigations of the last three decades disclosed, apoptosis lies in the center of three fundamental aspects of the life of multicellular organisms. First, it is essential for maintaining the cellular balance during tissue development and homeostasis. In parallel, however, it can also contribute to many forms of pathological cell loss including Alzheimer's and Parkinson's disease and heart failure. Furthermore, inhibition of apoptosis is a strategy operative at the basis of the abnormal cell growth in tumors.

The apoptotic pathways running in mammalian cells (having close homology to the well studied mechanisms in *Caenorhabditis elegans* and *Drosophila*) can have an intrinsic and extrinsic origin leading equally to the activation of death effector proteases, the caspases [4]. The activation of these effector caspases by upstream regulatory members of the protease family is responsible for the morphological and functional changes observed during apoptosis, and the versatility of the activation process makes it capable of participating in a wide range of death patterns. The most important protein family, which evolved to either up or down-regulate the caspase cascade, is the BCL-2 family of proteins [5], having a variable number of BCL-2 homology domains (BH 1–4). These interactions represent an important switch between apoptosis induction vs. protection. The single domain, pro-apoptotic BH3-only proteins (like BAD, BID, BIM, NOXA and PUMA) serve as upstream sensors to specific cell stresses, like proteolytic activation of BID by caspase-8 after activation of cell surface death receptors (extrinsic pathway); BAD phosphorylation downstream of growth factor receptors; or transcriptional regulation of NOXA and PUMA in p53 induced apoptosis. Whether their activation leads to amplification of the death process is dictated by the action and interaction of multi-domain pro-apoptotic (BAX and BAK) and antiapoptotic (BCL-2, BCL-X_L, MCL-1) proteins of the BCL-2 family. The presence of BAX and BAK is inevitable for virtually all apoptotic models involving the intrinsic pathway, and together with their antiapoptotic counterparts, they represent the link to the next terrain of apoptosis regulation, i.e., the endoplasmic reticulum (ER) and mitochondria. The final step in apoptosis execution, i.e., the formation of the apoptosomes and activation of caspase-3 initiates from the interplay of these organelles. Our review will outline this scene with particular attention to Ca^{2+} movement between these organelles and its role in regulating the final outcome of cell fate (see Fig. 1).

2. Ca^{2+} enters the contest

The involvement of Ca^{2+} in cell death has been recognized almost as early as the apoptotic form of programmed cell

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Abbreviations: AIF, apoptosis inducing factor; $[Ca^{2+}]_c$, cytosolic Ca^{2+} concentration; $[Ca^{2+}]_m$, mitochondrial Ca^{2+} concentration; $[Ca^{2+}]_{er}$, Ca^{2+} concentration in the endoplasmic reticulum; Drp-1, dynamin-like protein type 1; ER, endoplasmic reticulum; IMM, inner mitochondrial membrane; $InsP_3$, inositol 1,4,5 trisphosphate; $InsP_3R$, inositol 1,4,5 trisphosphate receptor; MCU, mitochondrial calcium uniporter; OMM, outer mitochondrial membrane; PTP, permeability transition pore; ROS, reactive oxygen species; SERCA, sarcoplasmic-endoplasmic reticulum Ca^{2+} adenosine triphosphatase

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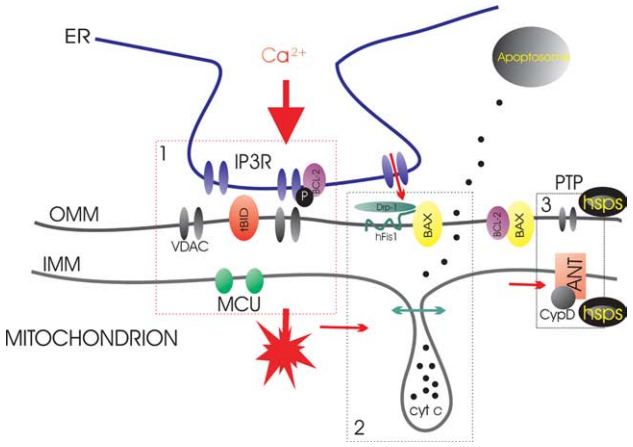

Fig. 1. Schematic representation of the components involved in ER-mitochondria interaction during Ca^{2+} mediated apoptotic processes. Boxes outlined with dashed lines represent different units drawn in different functions. (1) Ca^{2+} transfer from the ER to the mitochondria through $InsP_3$ receptors, VDAC, tBID and the MCU. (2) Unit of mitochondrial division, driven by hFis1, Drp-1, comprising cristae of the IMM, in which cytochrome *c* is compartmentalized. (3) The permeability transition pore (PTP) complex.

death, by A. Fleckenstein et al. in 1974, showing that excess Ca^{2+} entry into cardiac myofibers causes cell demise, playing a pathological role in cardiac myopathies [6]. Along these lines, soon it was demonstrated in several cellular models that Ca^{2+} ionophores are highly toxic to cells [7]. The ultimate permit, however, that let Ca^{2+} to enter into the field of cell death came from the findings that pathophysiological overstimulation of muscle and neural cells at their postsynaptic cholinergic of glutamate receptors, and the following Ca^{2+} influx is capable of inducing cell death [8,9]. Indeed, over the last few years, several studies have shown that increases of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) occur both at early and late stages of the apoptotic pathway [10–14]. Ca^{2+} overload has even been suggested to be the final common pathway of all types of cell death, with the distinction that very high intracellular Ca^{2+} levels can promote cell death through necrosis, whereas lower intracellular Ca^{2+} increases induced by milder insults promote cell death through apoptosis [15,16]. After these initial observations, a more specific question which arose is to define the participation of different Ca^{2+} sources in specific types of cell death. Along these lines, it has been well established that Ca^{2+} influx through NMDA receptor channels is chiefly responsible for glutamate induced neurotoxicity [17], but it has also been suggested that both Ca^{2+} release from the ER (Ca^{2+} induced Ca^{2+} release through ryanodine receptors in neurons) and the following capacitative Ca^{2+} influx are apoptogenic [18–20]. These studies also shed light on the inevitable, but rather unspecified role of mitochondria and mitochondrial Ca^{2+} uptake in cell death induction, representing a link between Ca^{2+} deregulation and apoptotic pathways outlined in the first caption.

3. The ER–mitochondrial interplay in the Ca^{2+} scene

Mitochondria take up Ca^{2+} avidly from the cytoplasm during agonist induced Ca^{2+} signals due to the strong driving force ensured by their membrane potential (−180 mV, negative inside) built by the respiratory chain of the inner mitochondrial membrane (IMM) [21,22]. The molecular nature of the channel responsible for Ca^{2+} uptake [mitochondrial Ca^{2+} uniporter (MCU)] is still not known, but Ca^{2+} uptake and electrophysiology measurements on isolated mitochondria showed that it is a highly selective Ca^{2+} channel and requires high external $[Ca^{2+}]$ for its opening [23,24]. In fact, efficient mitochondrial Ca^{2+} uptake in intact cells was shown to be dependent on the close apposition of mitochondria to Ca^{2+} release (ER) and entry sites (plasma membrane), where microdomains with high $[Ca^{2+}]$ are formed [25,26]. Ca^{2+} dependent stimulation of NAD(P)H and consequent ATP production, through activation of Ca^{2+} sensitive dehydrogenases and metabolite carriers, serves to adapt energy and substrate production to increased cellular needs [27]. In addition, mitochondria serve also as an intracellular Ca^{2+} buffer shaping global cellular Ca^{2+} signals [28,29].

On the other hand, mitochondrial Ca^{2+} overload has been shown to play a crucial role in apoptosis induction caused by certain pro-apoptotic stimuli, acting both on mitochondrial and ER, such as C_2 ceramide [20,30]. What is the Ca^{2+} source from which such a sustained $[Ca^{2+}]_m$ increase has its origin? The above depicted high $[Ca^{2+}]$ microdomains are obvious candidates in the case where an apoptotic stimulus is sufficient to maintain $[Ca^{2+}]$ for prolonged periods. Indeed, C_2 ceramide was shown to directly induce Ca^{2+} release from the ER Ca^{2+} store [31] and also to sensitize mitochondria to Ca^{2+} impulses from inositol 1,4,5 trisphosphate receptor ($InsP_3R$) or ryanodine receptor (RyR) mediated Ca^{2+} release, leading to mitochondrial permeability transition (MPT) and depolarization. This is followed by the release of pro-apoptotic factors activating the effector caspases and finally triggering apoptotic cell death [32]. On the other hand, mitochondrial Ca^{2+} uptake directly from the cytoplasm for prolonged periods may also lead to significant Ca^{2+} accumulation, even if the mean elevation does not exceed the micromolar level [33,34]. While mitochondrial Ca^{2+} uptake following Ca^{2+} influx in muscle and neuronal cells may represent examples for the latter mechanism, a recent line of evidence suggests that the ER–mitochondrial connection may have a prominent role also in cell death induction.

4. The key role of ER Ca^{2+} content and release in mitochondrial Ca^{2+} overload and induction of cell death

The first observations pointing to a major role of the releasable Ca^{2+} pool of the ER came from the exploration of the mechanism of antiapoptotic action of BCL-2 [35–39]. BCL-2 has been detected in association with the outer mitochondrial membrane (OMM), with the ER and with the nucleus and a cytoplasmic form of BCL-2 is also known to exist [40,41]. It has been suggested that the distribution of the membrane bound form p26-BCL-2-α in membranes renders it capable of participating in the formation of multi-protein complexes [42] and, accordingly, inhibition of apoptosis by BCL-2 has been suggested to be due to its capacity to interact with other anti- or pro-apoptotic proteins such as BCL-X_L, BAX procasp-8 and p28-BAP31 [43–46]. On the other hand, an effect on cellular ion homeostasis is suggested by the observation that BCL-2 forms ion channels of limited cation selectivity when added to lipid bilayers, thus potentially interfering with ion fluxes in organelles [47,48].

Thus, since the precise mechanism of the anti-apoptotic action of the BCL-2 protein was still debated, the indications that it is localized in key organelles of cellular Ca^{2+} handling (the mitochondria and the ER) and that it may act as ion channel led many groups to investigate whether the overexpression of this oncogene was affecting the cellular and organelle Ca^{2+} homeostasis. The first evidence of an effect of BCL-2 on ER Ca^{2+} homeostasis was obtained by Distelhorst and coworkers [49], who showed a reduction in the thapsigargin-induced efflux of Ca^{2+} from the ER in stably expressing clones of WEHI7.2 lymphoma cells. In turn, the use of targeted aequorins and GFP-based Ca^{2+} indicators allowed the direct measurement of Ca^{2+} concentration in the ER of cells transiently expressing BCL-2. It was shown that BCL-2 overexpression in HeLa [31] or HEK-293 cells [50] causes a reduction of the steady state Ca^{2+} concentration in the endoplasmic reticulum ($[Ca^{2+}]_{er}$), and thus of the amount of agonist-releasable Ca^{2+} pool, due to an increase in the Ca^{2+} leak across the ER membrane [31]. Importantly, the observed reduction of steady state $[Ca^{2+}]_{er}$ was modest (approx. 30%), but sufficient to reduce agonist induced $[Ca^{2+}]$ increase in the cytoplasm and Ca^{2+} uptake into mitochondria. In this model, apoptotic stimuli, known to act through Ca^{2+} release from the ER, such as C_2 ceramide, induced a prolonged increase in $[Ca^{2+}]_m$ followed by dramatic alterations in the morphology of mitochondria, i.e., swelling of the organelle and rupture of the mitochondrial network. If, however, steady state $[Ca^{2+}]_{er}$ was reduced not only by BCL-2 overexpression, but by a variety of experimental approaches (incubation at lower extracellular $[Ca^{2+}]$, partial inhibition of the ER Ca^{2+} ATPase or overexpression of the plasma membrane Ca^{2+} ATPase), there was a marked inhibition in apoptotic death and no mitochondrial modifications occurred [20]. Similarly, following overexpression of calreticulin (an abundant luminal ER Ca^{2+} buffer) which does not raise $[Ca^{2+}]_{er}$, but does increase the releasable Ca^{2+} pool, cell survival is drastically reduced upon C_2-ceramide treatment [20]. Furthermore, cell lines derived from calreticulin knock-out mice are more resistant to apoptosis, indicating that the crucial requirement is the amount of released Ca^{2+} and not $[Ca^{2+}]_{er}$ [51].

Scorrano et al. [52] provided further evidence in favor of the hypothesis that Ca^{2+} movement from the ER to mitochondria is a key process in the activation of apoptosis by a number of stimuli. They showed that mouse embryonic fibroblasts deficient of the two pro-apoptotic proteins BAX and BAK (double knock out, DKO, cells) are markedly resistant to a variety of apoptotic stimuli and have a much reduced Ca^{2+}concentration in the ER. If the ER Ca^{2+} levels are restored by recombinantly overexpressing the sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase (SERCA), not only mitochondrial Ca^{2+} uptake in response to stimulation is re-established, but the cells regain sensitivity to apoptotic stimuli such as arachidonic acid, C_2-ceramide and oxidative stress. These results are in keeping with previous work by Ma et al. [53], demonstrating that SERCA overexpression in Cos cells causes ER Ca^{2+} overload and increases spontaneous apoptosis. The work of Scorrano and colleagues further demonstrated that another group of apoptotic stimuli (staurosporine and etoposide) are partially insensitive to these alterations in the levels of ER Ca^{2+}, while they require the presence of pro-apoptotic proteins on the mitochondrial membrane. Thus, BAX specifically targeted to the OMM did not induce any change in $[Ca^{2+}]_{er}$ but it made cells sensitive to apoptosis induced by the BH3-only protein tBID.

The overall picture emerging from these studies is that an important mechanism underlying the sensitivity of cells to apoptosis is the up- and down-regulation of steady state $[Ca^{2+}]_{er}$ by pro- and anti-apoptotic factors, respectively. The resulting increase or decrease of Ca^{2+} release from the ER and Ca^{2+} uptake into mitochondria in turn is pivotal in triggering apoptotic signals.

5. ER and mitochondria: what is between?

The works discussed above settled Ca^{2+} release from the ER as a key component of cell death induction, but also promptly evoke the question of the mechanism of Ca^{2+} transfer to the mitochondria. Regarding this question up to now only few data are available. The elegant work of Darios et al. put forward a mechanism which presumes a very intimate connection between these organelles without even the contribution of cytoplasmic Ca^{2+} increase [54]. In PC12 cells, following C_2-ceramide treatment, inhibition of Ca^{2+} release through ryanodine receptors was able to inhibit $[Ca^{2+}]_c$ increase but not the prolonged $[Ca^{2+}]_m$ elevation and cell death induction. Furthermore, they proposed that Ca^{2+} transfer implicates caspase-8 activation, cleavage of BID, and tBID translocation to mitochondria. Along these lines, proteins of the OMM lying most likely in the route of Ca^{2+} transfer between the organelles may have particularly important role in apoptotic Ca^{2+} signaling. Indeed, overexpression of VDAC, the voltage-dependent anion channel of the OMM [55], and tBID [56] enhances mitochondrial Ca^{2+} uptake and in turn sensitizes the cells to apoptotic stimuli. Thus, the expression level of VDAC (that is upregulated in apoptosis-sensitive cells [57]) as well as mitochondrial translocation of the pro-apoptotic BCL-2 family member may serve to regulate tunnelling Ca^{2+} in a restricted space between ER and mitochondria.

In contrast to RyRs, very recently, $InsP_3$ receptors have been identified as an important component of ER-mitochondria interaction by S.H Snyder's group. They have shown, that cytochrome *c*, after its initial Ca^{2+} dependent release from the mitochondria translocates to the ER and interacts with $InsP_3$ receptors, further augmenting Ca^{2+} release. This mechanism serves in turn as a positive feed-back loop leading to a generalized form of cytochrome *c* release and cell death. DT40 cells lacking all three $InsP_3R$ isoforms thus are less sensitive to apoptotic stimuli [58].

6. Mitochondrial contribution to the effector phase of apoptosis

One of the key events in mitochondrial apoptotic signaling is the release of normal constituents [such as cytochrome *c* and apoptosis inducing factor (AIF)] into the cytosol. Released cytochrome *c* then binds to apoptotic protease activating factor-1 (APAF-1), which recruits and activates caspase-9 to form the apoptosome and thus activates the effector caspase-3. The release of the content of the intermembrane space requires the permeabilization of the OMM. Its mechanism and relation to mitochondrial Ca^{2+} signaling, however, are still controversial. The main components of the presumed mechanisms are the

translocation and activation of extramitochondrial factors (such as BAX, BAK and tBID) to the OM, and the opening of the PTP, with ensuing organelle swelling. Furthermore, reorganization of the IMM structure, in parallel with changes of the overall morphology of the mitochondrial reticulum, has been proposed to play an important role in this process. The potential mechanisms of OM permeabilization by extramitochondrial factors has been recently reviewed [59,60]. Here, we discuss only their links to Ca^{2+} mediated pathways potentially leading to pro-apoptotic factor release.

The first obvious candidate proposed to link Ca^{2+} to cytochrome *c* release is the process of MPT. This process leads to massive swelling of the organelle (mostly described in isolated mitochondria, [61], but see also [62]) and, importantly, the rupture of the OMM [63]. The presumed basis for this process is a multiprotein complex that transverses the intermembrane space; the putative essential components of this complex are VDAC in the OMM, the adenine nucleotide translocase (ANT) in the IMM, and cyclophilin-D in the mitochondrial matrix. The partial and transient PTP opening has been suggested to drain the proton gradient in order to protect mitochondria from hyperpolarization and increased reactive oxygen species (ROS) production [64]. In contrast, complete and irreversible PTP opening (induced, for example, by Ca^{2+} or ROS) renders the mitochondria permeable to larger molecules, leading to a complete loss of mitochondrial membrane potential and respiratory function (for review see [61]). However, the active role of PTP opening in inducing apoptosis has been questioned by the fact that Ca^{2+}-induced permeability transition can occur in BAX/BAK deficient, non-apoptotic cells [65]. An alternative, interesting possibility is that PTP serves as an anchor to target these molecules to the OMM, close to contact sites where OMM permeabilization occurs [62].

As an alternative, the phenomenon of mitochondrial fragmentation, which is prominent in several apoptotic models, has been directly implicated in the transduction of apoptotic signals involving OMM permeabilization [66–68]. These works identified dynamin-like protein-1 (Drp-1, member of the large GTPase mechanoenzyme family, mediating mitochondrial fission) in complexes with pro-apoptotic members of the BCL-2 family at mitochondrial fission sites, such as BAX, which mediate OMM permeabilization, release of cytochrome *c* and induction of apoptosis [69]. Moreover, Drp-1 overexpression amplified BAX-mediated release of cytochrome *c* induced by the pro-apoptotic agent staurosporine [59,70]. Conversely, expression of the GTPase mutant Drp-1^{K38A} prevented mitochondrial scission together with cytochrome *c* release and cell death without inhibiting BAX translocation to the OMM. A simple model suggested recently is that morphological alteration induced by Drp-1 is responsible for the OMM permeabilization [65,71]. Indeed, even if mild fragmentation of mitochondria by Drp-1 overexpression has not been shown to pro-apoptotic in itself, expression of its OM anchoring protein hFis1 renders cells sensitive to the mitochondrial apoptotic pathway [72]. Further studies are needed to clarify the role of additional components (possibly present in complex with BAX/Drp-1/hFis1) in OMM permeabilization (for reviews see [73,74]). Regarding the role of Ca^{2+} signals, Shore and coworkers showed that Ca^{2+} release from the ER by the BAP31 cleavage product p20 and subsequent mitochondrial Ca^{2+} accumulation precedes translocation of Drp1 to mitochondria, i.e., the induction of mitochondrial fragmentation is strictly Ca^{2+} dependent [68]. Interestingly, in the model proposed, this mitochondrial pathway serves as an amplification loop to augment caspase-8 mediated apoptosis.

In conclusion, recent developments in exploring Ca^{2+} dependent processes in apoptotic pathways disclosed that several aspects of mitochondrial participation in induction of apoptosis rely upon Ca^{2+} regulated mechanisms. Among them the release of pro- and anti-apoptotic factors from the intermembrane space has been related to mitochondrial Ca^{2+} overload and probably Ca^{2+} mediated morphological changes in mitochondrial network. Even if the exact mechanism and site of Ca^{2+} action still awaits investigation, an important characteristic of mitochondrial Ca^{2+} uptake/overload is its strict dependency on changes of ER Ca^{2+} handling. In our review, we summarized recent data strengthening this view. Discussion of further aspects of Ca^{2+} regulated processes in apoptosis has been recently reviewed [32,75,76], including cytoplasmic sites of Ca^{2+} action and changes in global Ca^{2+} signaling during apoptosis.

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