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Review

Calcium and mitochondria in the regulation of cell death

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ABSTRACT

The calcium ion has long been known to play an important role in cell death regulation. Hence, necrotic cell death was early associated with intracellular Ca^{2+} overload, leading to mitochondrial permeability transition and functional collapse. Subsequent characterization of the signaling pathways in apoptosis revealed that Ca^{2+}/calpain was critically involved in the processing of the mitochondrially localized, Apoptosis Inducing Factor. More recently, the calcium ion has been demonstrated to play important regulatory roles also in other cell death modalities, notably autophagic cell death and anoikis. In this review, we summarize current knowledge about the mechanisms involved in Ca^{2+} regulation of these various modes of cell death with a focus on the importance of the mitochondria.

1. Introduction

Investigation of different modes of cell death has become an important area of biomedical research. Recently, several cell death modalities have been characterized based on both morphological and biochemical criteria. In 2009, the Nomenclature Committee on Cell Death proposed unified criteria for the definition of twelve cell death modalities [1]. Among the best characterized of these modes of cell death are apoptosis, autophagy, and necrosis. Until recently, a requirement for gene expression was documented only for apoptotic and autophagic cell death. However, accumulating evidence suggests that necrotic cell death might also be mediated by a specific set of signal transduction pathways and degradative mechanisms. The interaction between the different forms of cell death is complex and still a matter of debate, although the mitochondria have been demonstrated to play a crucial role in the effectuation of several cell death modalities and the cross-talk between them.

The Ca^{2+} ion has long been known to be critically involved in both the initiation and effectuation of cell death. Hence, necrosis was early found to be associated with a perturbation of intracellular Ca^{2+} homeostasis, and key events in the apoptotic process are known to be triggered by Ca^{2+} signals [2]. Similarly, some forms of autophagic cell death and anoikis have been shown to be Ca^{2+}-dependent. In this review, we shall discuss current knowledge about the role of Ca^{2+} in both the initiation and effectuation of cell death with a focus on the interplay between Ca^{2+} and the mitochondria.

2. Mitochondria, Ca^{2+} and necrosis

Necrosis has long been regarded as the result of an accidental and uncontrolled process, usually caused by factors external to the cell or tissue, such as infection, toxins, heat or trauma. It is characterized by disruption of the plasma membrane, cell swelling, chromatin digestion, DNA hydrolysis and, finally, cell lysis. Necrosis is often associated with local inflammation, triggered by the release of factors from dead cells that alert the innate immune system [3]. Necrosis is known to play a prominent role in many pathological conditions, including ischemia/reperfusion injury (e.g. stroke and myocardial infarction), trauma, and some forms of neurodegeneration.

The involvement of mitochondria in necrotic cell death has been known for a long time. Thus, a common cause of necrosis is the collapse of mitochondrial energy metabolism, leading to a drastic drop in ATP level. This, in turn, can result in intracellular Ca^{2+} overload and stimulation of various Ca^{2+}-dependent catabolic enzymes – phospholipases, proteases and endonucleases. Historically, the role of the Ca^{2+} ion as a death trigger dates back to Fleckenstein's observation that excess Ca^{2+} entry into cardiomyocytes underlies cardiac pathology after ischemia [4]. Subsequent studies emphasized the general importance of this finding,

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as both receptor overstimulation and many cytotoxic agents were found to cause lethal Ca^{2+} influx into cells (see Ref. [2] for review).

Many forms of toxic cell death were initially thought to be of the necrotic type and related to a perturbation of intracellular Ca^{2+} homeostasis. Hepatotoxicity caused by carbon tetrachloride, acetaminophen, or bromobenzene are classical examples thereof. Acetaminophen- and bromobenzene-induced cell death was studied intensely in the 1970's and found to be preceded by cytochrome P450-mediated formation of reactive metabolites, glutathione depletion, and disruption of Ca^{2+} homeostasis. Cytotoxicity was usually monitored by cellular leakage of lactate dehydrogenase, or uptake of trypan blue, traditional assays of the increased plasma membrane permeability associated with necrotic cell death.

2.1. Mitochondrial Ca^{2+} handling

The ability of mitochondria to accumulate Ca^{2+} was demonstrated by Vasington and Murphy [5] in the beginning of the 1960's. They postulated that mitochondrial accumulation of this ion depends on respiration and phosphorylation. Later, it was shown that mitochondria take up Ca^{2+} electrophoretically from the cytosol through a "Ca^{2+} uniporter", which could be inhibited by lanthanides or ruthenium red (RR). They can release it again via several different routes, including a calcium/sodium (excitable tissues) or a calcium/proton exchanger [6]. The uptake of Ca^{2+} is driven by the mitochondrial membrane potential, whereas its release in exchange for protons or sodium is electroneutral. The affinity for Ca^{2+} of the uniporter is low, and the size of the mitochondrial Ca^{2+} pool is small under physiological conditions. However, mitochondria can accumulate much larger amounts of Ca^{2+} under pathological conditions, when intracellular Ca^{2+} concentrations rise [7]. Hence, for many years mitochondrial Ca^{2+} uptake was regarded primarily as a safety device in situations of temporary intracellular Ca^{2+} overload. However, this view changed after the development of novel indicators, which can sense Ca^{2+} fluctuations in specific intracellular compartments [8]. Thanks to this technology, it has become apparent that mitochondrial Ca^{2+} fluxes are integrated parts of intracellular Ca^{2+} signaling. The low affinity of the mitochondrial Ca^{2+} import system is overcome by the proximity of the mitochondria to the endoplasmic reticulum (ER), where the local concentration of Ca^{2+} released from ER can reach very high levels [9]. Subsequent uptake of Ca^{2+} by the mitochondria stimulates the Ca^{2+}-sensitive matrix dehydrogenases, which provide NADH for mitochondrial respiration and ATP production.

For many years, the mitochondrial Ca^{2+} uniporter resisted purification and molecular characterization. However, based on early findings by Carafoli and Lehninger [6] that yeast mitochondria lack the uniporter, Perocchi et al. [10] used a comparative genomics strategy to identify human MICU1, a component of the mitochondrial uniporter complex. Soon thereafter, the pore-forming component, MCU, was identified and shown to be responsible for the RR-inhibitable Ca^{2+} uptake by mitochondria, and was also found to be capable of Ca^{2+} transfer through artificial membranes [11,12]. Using MCU as a handle, it was now possible to affinity-purify the uniporter holocomplex.

The discovery of the gene encoding the MCU protein was recently followed by the generation of knockout mice lacking the MCU [13]. Unexpectedly, these mice were found to be fully viable and showed impairment only in their ability to perform strenuous work. Mitochondria from MCU-deficient mice failed to undergo Ca^{2+}-induced permeability transition and did not respond to the permeability transition pore (PTP) inhibitor, cyclosporin A (see below). However, it is important to note that removal of MCU in zebrafish significantly influenced the formation of the notochord axis by controlling blastomere convergence and extension movements during gastrulation. Interestingly, the function of MCU in zebrafish as well as intracellular Ca^{2+} trafficking is controlled by a new Bcl-2-related multidomain apoptosis accelerator, Bcl-wav [14].

2.2. Ca^{2+}-induced mitochondrial permeability transition

Certain conditions, notably mitochondrial Ca^{2+} accumulation and oxidative stress, can trigger the opening of a high-conductance pore in the inner mitochondrial membrane (IMM) (Fig. 1). This phenomenon has been termed mitochondrial permeability transition (MPT) and is associated with drastic changes in mitochondrial morphology and functional activity [15]. Pore opening is a Ca^{2+}-dependent process, but it can be facilitated by other factors, such as inorganic phosphate, ATP depletion, low pH and oxidative stress [16]. It is followed by osmotic swelling of the mitochondria and rupture of the mitochondrial membrane, leading to the release of mitochondrial proteins including cytochrome *c* into the cytosol. Such mitochondrial collapse might occur in several forms of necrotic cell death, for example cell death caused by oxidative stress, ischemia/reperfusion, and Ca^{2+} ionophores [17,18].

According to the traditional view, the permeability transition pore (PTP) represents a multimeric protein complex composed of the voltage dependent anion channel (VDAC) located in the outer mitochondrial membrane (OMM), the adenine nucleotide translocase (ANT), an integral protein of the IMM, and the matrix protein, cyclophilin D (CyPD). VDAC and ANT form contact sites between the OMM and the IMM. ANT was thought to be critical for pore opening, since it was demonstrated that the mitochondrial ADP/ATP carrier, when incorporated into lipid membrane, could be reversibly converted into a large channel by Ca^{2+} [19]. However, it was later found that deficiency of ANT failed to block Ca^{2+}-induced permeability transition [20]. Similarly, VDAC was also found to be dispensable for Ca^{2+}-induced MPT and mitochondria-dependent cell death [21]. In contrast, experiments with down-regulation of cyclophilin D revealed it to be critical for MPT-mediated cell death [22].

In addition to the potential PTP constituents mentioned above, other proteins residing in the mitochondrial membrane have been suggested to be involved in the regulation of MPT. For example, the mitochondrial phosphate carrier (PiC) has been reported to be able to regulate MPT through interaction with cyclophilin D [23] (Fig. 1). Indeed, CypD can directly bind PiC through its N-terminus [24]. However, studies using cardiac-specific mouse strains expressing

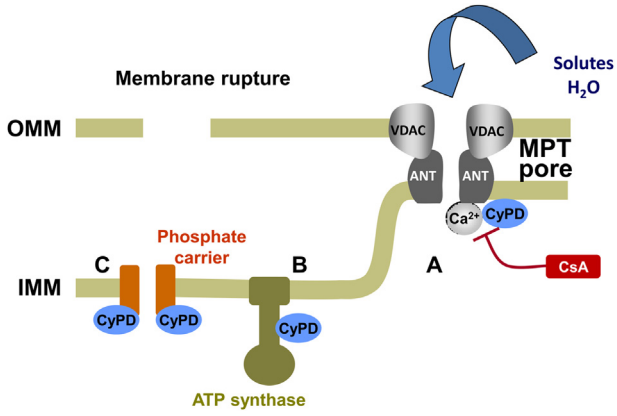

Fig. 1. Proposed models of mitochondrial permeability transition. The figure depicts the traditional view of the non-specific pore complex (A), as well as the more recently proposed involvement of the ATP synthase (B) or the phosphate carrier (C) in MPT induction.

different levels of mitochondrial PiC have indicated that the mitochondrial PiC plays a minor role in the regulation of MPT.

More recently, investigating the molecular mechanisms of MPT, Bernardi and colleagues found that PTP opening is regulated by matrix CyPD through its binding to the lateral stalk of the FOF1 ATP synthase [25]. When purified dimers of the ATP synthase, which did not contain VDAC or ANT, were reconstituted into lipid bilayers, addition of the ATP synthase inhibitor benzodiazepine 423 in the presence of Ca^{2+} triggered opening of a channel with currents that were typical of the mitochondrial megachannel, the electrophysiological equivalent of PTP [25]. This finding is in agreement with the observation that the c subunit of the FO ATP synthase is required for MPT, mitochondrial fragmentation and cell death induced by intracellular Ca^{2+} overload and oxidative stress [26]. Hence, it appears that the subunit of the FO ATP synthase is a critical component of the PTP complex.

3. Necroptosis

Our view that necrotic cell death is always an uncontrolled process has changed recently, since accumulating evidence now suggests that it might also be mediated by a specific set of signal transduction pathways and degradative mechanisms. Hence, like apoptosis (see below), cell death with a necrotic appearance can contribute to embryonic development as well as tissue homeostasis in the adult organism. In fact, apoptosis and necrosis might occur concomitantly in tissues or cell cultures exposed to the same lethal stimulus – often the intensity of the initial insult decides which mode of cell death will follow. Intracellular energy levels are rapidly depleted in necrosis, whereas ATP is required for the active execution of apoptosis [27].

Interestingly, often the inhibition of apoptosis does not completely block cell death, but rather results in a switch to caspase-independent mechanisms with morphological features resembling necrosis. One such mechanism is necroptosis [28]. Necroptosis is initiated via death receptors, such as tumor necrosis factor receptor1 (TNFR1); it requires the kinase activity of receptor-interacting protein 1 (RIP1; also known as RIPK1) and RIP3 (also known as RIPK3), and its execution involves the active disintegration of mitochondrial, lysosomal and plasma membranes (reviewed in Ref. [29]). Increased concentrations of cytoplasmic Ca^{2+} triggered necroptosis in several experimental models by activating calcium-calmodulin kinase (CaMKK) II, which phosphorylates and activates RIP1 [30]. In contrast, necroptosis induced by TNF in hippocampal neurons was reported to be independent of both ROS accumulation and Ca^{2+} influx [31].

It has been shown that necroptosis critically relies on mixed lineage kinase domain-like (MLKL), a pseudokinase that binds ATP but is catalytically inactive [32]. Relocalization of MLKL trimers to the plasma membrane promotes an influx of Ca^{2+} ions, which is mediated (at least in part) by transient receptor potential cation channel, subfamily M, member 7 (TRPM7), a non-voltage sensitive, poorly selective cation channel of the plasma membrane [33]. Unfortunately, this group did not directly investigate whether ions other than Ca^{2+} (notably Na^{+}) would be involved in this process. Indeed, using Na^{+}- and Ca^{2+}-sensitive fluorochromes and Ca^{2+}-depleted culture media, another group detected a rapid intake of Na^{+} (but not Ca^{2+}) ions by multiple cell types undergoing necroptosis and showed that Na^{+}-depleted culture media could selectively inhibit cell death in response to necroptotic, but not apoptotic, stimuli [34]. Additional work is required to better understand the precise role of calcium ions in the regulation of necroptosis.

Finally, it has been demonstrated that necroptosis was responsible for delayed ischemic brain injury in mice *in vivo* through a mechanism distinct from apoptosis. A specific and potent small-molecular inhibitor, necrostatin-1, was shown to block a critical step in necroptosis offering a potential new approach to neuroprotection [28].

4. Apoptosis or programmed cell death

Apoptosis has been defined as a form of cellular suicide, since death results from active processes within the cell itself. Morphologically, apoptosis is characterized by margination and condensation of nuclear chromatin (pyknosis), cell shrinkage, nuclear fragmentation, and blebbing of the plasma membrane. The cell subsequently breaks up into membrane-enclosed fragments, termed apoptotic bodies, which are rapidly recognized and engulfed by neighboring cells or macrophages.

The execution stage of apoptosis involves several enzyme systems activated through elaborate signaling pathways. The proteolytic activity of caspases provides a basis for the apoptotic phenotype [35]. Caspases are synthesized as pro-enzymes with very low intrinsic activity and require activation, either by proteolytic maturation or by interaction with an allosteric activator. Based on the size of the pro-domain, caspases are divided into long and short pro-domain containing enzymes. Long pro-domain caspases, *i.e.* caspase-2, -8, -9, and -10, belong to the group of initiator caspases, while short pro-domain caspases, *i.e.* caspase-3, -6, and -7, represent effector enzymes. The effects of caspases in apoptosis are accomplished by the cleavage of numerous proteins located in the cytoskeleton, cytoplasm and nucleus.

4.1. Involvement of mitochondria in apoptosis signaling

Several major signaling pathways lead to apoptosis in mammalian cells. In the *extrinsic pathway*, the ligation of surface receptors (e.g. CD95, TNFR1) results in the formation of the death-inducible signaling complex (DISC) and the activation of pro-caspase-8. In some cell types, caspase-8 directly activates pro-caspase-3, which cleaves multiple target proteins, leading to apoptosis. In other cells, caspase-8 cleaves the BH3-only protein, Bid, which, in turn, triggers the translocation and insertion of the pro-apoptotic Bcl-2 protein, Bax into the OMM. This leads to OMM permeabilization and the release of a host of proteins from the mitochondrial intermembrane space, including cytochrome *c* that forms the apoptosome complex in the cytosol together with apoptosis activating factor-1 (Apaf-1) and pro-caspase-9 in the presence of dATP. This results in the formation of caspase-9, which triggers the caspase cascade by activation of pro-caspase-3.

In the *intrinsic pathway*, death signals act on the mitochondria to cause the release of pro-apoptotic proteins from their intermembrane space. This pathway is controlled by Bcl-2 family proteins (Fig. 2). As discussed below, the intrinsic pathway may also operate via caspase-independent mechanisms, which involve the release from mitochondria and translocation into the nucleus of at least two proteins, Apoptosis Inducing Factor (AIF) and Endonuclease G (EndoG). Nuclear effects of AIF include chromatin condensation and formation of high-molecular-weight DNA fragments. The role of EndoG in cell death is still unclear. In certain experimental models, when DNA damage is the trigger of the apoptotic response, the initially activated caspase is pro-caspase-2. Its activation also leads to the release of cytochrome *c* and apoptosome formation, although the precise mechanisms involved are unclear.

4.2. Mechanisms of mitochondrial membrane permeabilization

The main mechanism of OMM permeabilization in apoptosis involves the pro-apoptotic members of the Bcl-2 family of proteins.

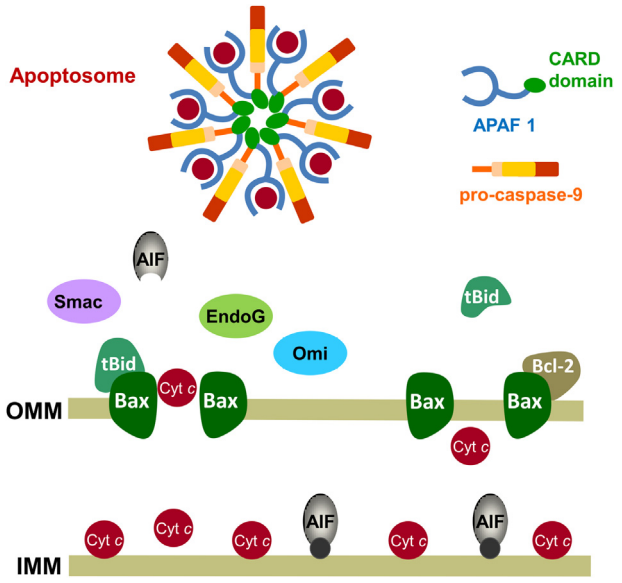

Fig. 2. Regulation of mitochondrial outer membrane permeabilization by Bcl-2 family proteins.

The first indication that genes and proteins, which play a role in tumorigenesis, might be involved in the regulation of cell death came from the study of Bcl-2 [36]. Overexpression of this protein was subsequently shown to inhibit cell death induced by a variety of stimuli, including IL-3 deprivation, chemotherapeutic agents and heat shock (reviewed in Ref. [37]).

Today, more than thirty members of the Bcl-2 family and related proteins have been identified. They can be divided into two groups: the Bcl-2-like survival factors (e.g. Bcl-2, Bcl-X_L, Bcl-w and Mcl-1), which all contain three or four characteristic regions of homology (BH1-4; Bcl-2 Homology domains), and the pro-apoptotic Bcl-2 proteins. The latter can in turn be divided into two subgroups: Bax, Bak, Bcl-X_S, and some others, contain two or three BH-domains, whereas another group of proteins, including Bad, Bid, Hrk/DP5, Bim, Noxa, and Puma, share only the short (9-16 amino acids) BH3-domain [38]. For many years it was widely assumed that the presence of BH4 distinguishes all anti-apoptotic Bcl-2 family proteins from pro-death members that lack BH4. However, recently performed structural comparison of vaccinia virus anti-apoptotic Bcl-2-like protein F1L with other Bcl-2 family members revealed a novel sequence signature that redefines the BH4 domain as a structural motif present in both pro- and anti-apoptotic Bcl-2 members, including viral Bcl-2-like proteins [39]. Moreover, most multiple alignment programs failed to align the BH4 motifs of the original trio (Bcl-2, Bcl-XL, and Bcl-w) with the putative BH4 of their anti-apoptotic homologs, Mcl-1 and Bfl-1/A1. These sequence differences are paralleled by the sensitivity of the same three BH4-containing proteins to the BH3 mimetic ABT-737, although ABT-737 does not directly contact the BH4 (for review, see Ref. [40]).

Permeabilization of the OMM requires the oligomeric form of Bax or Bak and usually also involves the truncated form of the BH3-only protein Bid (*t*Bid); Bid can be cleaved by multiple proteases, including caspase-8, caspase-2, calpain and granzyme B. Anti-apoptotic proteins, e.g. Bcl-2, Bcl-X_L, Mcl-1 and Bcl-w, can bind to the pro-apoptotic proteins, Bax or Bak, and prevent their oligomerization. Hence, the balance between the pro- and anti-apoptotic proteins in the OMM is critical for the determination of cell fate (Fig. 2).

Another mechanism of mitochondrial permeabilization involves the induction of MPT, described above. Although PTP opening has mostly been associated with necrotic cell death, several cytotoxic agents have been reported to trigger apoptosis via Ca^{2+}-mediated MPT. In some instances, cell death could be prevented by inhibitors of mitochondrial Ca^{2+} uptake or PTP formation, such as ruthenium red and cyclosporin A. Of particular interest is the observation that apoptotic stimuli, notably ceramide, can induce a switch in mitochondrial Ca^{2+} signaling at the beginning of the apoptotic process by facilitating Ca^{2+}-induced opening of the PTP [41]. This is in accordance with the observation that resistance of leukemic cells to 2-chlorodeoxyadenosine (CDA) was associated with an increased ability of their mitochondria to sequester Ca^{2+} without concomitant PTP opening [42]. The CDA-resistant cells were selectively cross-resistant to thapsigargin, but not to staurosporine or CD95-mediated apoptosis.

The role of CyPD in apoptotic cell death is still controversial. Cyclophilins represent a group of peptidyl-prolyl *cis*–*trans*isomerases (PPIase) with highly conserved protein sequences, which are important for protein folding [43]. For many years, CyPD was considered critical for the opening of the PTP. This view was based on the observation that Cyclosporin A (CsA) blocks the opening of the PTP at concentrations similar to those needed to inhibit the enzymatic activity of CyPD. Based on these properties, CyPD was thought to facilitate cell death. Indeed, overexpression of CyPD was found to sensitize the mitochondria to agents (Ca^{2+} and oxidants) that promote PTP formation in both stressed and unstressed cells, as well as in isolated mitochondria [44]. In addition, it was reported that CypD-deficient mitochondria do not undergo MPT. However, CypD-deficient cells died normally in response to several apoptotic stimuli, but showed resistance to necrotic cell death induced by either oxidative stress or Ca^{2+} overload [45].

Ischemia/reperfusion injury has often been associated with mitochondrial permeability transition in the damaged tissue, and pretreatment with CsA has been found to exert a protective effect, suggesting that PTP formation is the cause of the damage. Of interest, this was recently also found to be the case in wt mice subjected to ischemia/reperfusion, whereas MCU deficient mice suffered from similar tissue damage, which was however not prevented by CsA [13]. The reason for this discrepancy is unclear.

Various modes of OMM permeabilization can cooperate. Thus release of Ca^{2+} from ER was shown to initiate cytochrome *c*-dependent apoptosis through cooperation between Bax and the MPT in human leukemic CEM cells [46]. Inhibition of the MPT induction pharmacologically, or by knockdown of the MPT core component, CyPD, using small interfering RNA (siRNA) blocked cytochrome *c* release and caspase-dependent apoptosis, but it did not prevent Bax activation, translocation or N-terminal exposure in mitochondria.

The Bcl-2 family proteins are involved in the regulation of multiple modes of cell death. Moreover, the localization of these proteins to either the mitochondria or the endo/sarcoplasmic reticulum can influence ER-mitochondrial calcium cross-talk and thereby the mode of cell death. For example, the Bcl-2 family protein, Nix (also known as Bnip3L), is transcriptionally upregulated in cardiac hypertrophy and can trigger apoptotic death of cardiomyocytes [47]. However, its localization to the ER also affects Ca^{2+} signaling in a manner that can evoke PTP opening. Hence, cardiomyocytes undergoing Nix-mediated cell death exhibit characteristics of both apoptosis and necrosis, depending on the level of Ca^{2+} [48]. Genetic targeting of Nix specifically to the mitochondria or the ER revealed that its organelle localization determines whether Nix mediates cell death by apoptosis or MPT-mediated necrosis [49]. Interestingly, another protein from this family, namely Bnip3, contributes to cardiac dysfunction by localizing to the ER and increasing ER-mitochondrial calcium transport [50]. A link between Ca^{2+} and Bcl-2 family proteins in apoptosis was

suggested many years ago. Thus, it was shown that ionomycin-induced calpain activation promoted a decrease of Bcl-2 proteins, thereby triggering the intrinsic apoptotic pathway [51]. Moreover, the Ca^{2+}-induced dephosphorylation of BAD was correlated with its dissociation from 14-3-3 in the cytosol and translocation to mitochondria where Bcl-XL resides. In hippocampal neurons, L-glutamate, an inducer of Ca^{2+} influx and calcineurin activation, triggered mitochondrial targeting of BAD and apoptosis, which were both suppressible by coexpression of a dominant-inhibitory mutant of calcineurin or pharmacological inhibitors of this phosphatase [52]. Finally, cleavage of Bid by calpain led the tBid to activate mitochondria-dependent apoptosis in vitro and during myocardial ischemia/reperfusion [53].

Bcl-2 can control the release of Ca^{2+} from ER, where its N-terminal BH4 domain can directly bind to and inhibits the inositol 1,4,5-trisphosphate receptor (IP3R), the main intracellular Ca^{2+}-releasing channel [54]. Targeting the IP3R–Bcl-2 complex with cell-permeable peptide (stabilized TAT-fused IP3R-derived peptide (TAT-IDP(S)) that selectively targets the BH4 domain of Bcl-2 (but not that of Bcl-X_L), can reverse the inhibitory action of Bcl-2 on IP3Rs and stimulate the release of Ca^{2+} and subsequent activation of the mitochondrial pathway of apoptosis in chronic lymphocytic leukemia cells and diffuse large B-cell lymphoma cells. Interestingly, the efficiency of such targeting strongly correlated with their IP3R2-protein level, but not with IP3R1-, IP3R3- or total IP3R-expression levels [55].

4.3. Ca^{2+} -triggered AIF processing

Apoptosis Inducing Factor (AIF) was the first mitochondrial pro-apoptotic protein identified [56]. This 62 kDa flavoprotein is anchored to the IMM in the vicinity of Complex I by part of its peptide chain. Hence, AIF needs to be cleaved before a 57 kDa pro-apoptotic fragment can be released into the cytosol for further translocation into the nucleus, where it promotes large-scale DNA fragmentation and chromatin condensation by a not yet clearly defined mechanism. Responsible for AIF cleavage is calpain localized to the intermembrane space of the mitochondria, where it is activated by a sustained Ca^{2+} elevation [57] (Fig. 3). Proteolysis is further stimulated by the oxidative modification of AIF by mitochondrially produced ROS, leading to carbonylation of the protein and increased susceptibility to calpain cleavage [58]. In lung cancer cells as well as cortical neurons treated with protein kinase C inhibitors (staurosporine or PKC 412), calpain activation results from the import of extracellular Ca^{2+} via a hyperpolarization-activated cyclic nucleotide-gated (HCN2) ion channel in the plasma membrane [59]. Down-regulation of this channel blocks Ca^{2+} influx as well as AIF processing and apoptosis in both tumor cells and neurons.

It appears that AIF plays a critical role in the death of certain cell types, e.g. neurons and some tumor cells [60,61]. Moreover, the type of lethal agent also determines whether AIF will be important for subsequent cell death. Hence, agents that perturb intracellular Ca^{2+} homeostasis, or cause early lysosomal permeabilization, are most likely to make the AIF-mediated pathway the predominant mechanism of subsequent apoptosis. However, both these events are frequent components of cell death signaling, particularly in ischemia-reperfusion injury and after treatment with toxic drugs.

Several studies suggest a critical role for AIF in neuronal cell death. Hence, microinjection of neutralizing AIF antibodies, or downregulation of AIF, have been found to suppress glutamate-, hypoxia- and NMDA (N-methyl D-aspartate)-induced neuronal death in cultures. Further, knockdown of AIF in PC12 cells reduced the cytotoxic effect of MPP^+ (1-methyl-4-phenylpyridinium) [62]. There are also several *in vivo* observations demonstrating the importance of the AIF-mediated pathway in neuronal cell death. For instance, as compared to wt mice, AIF-deficient Harlequin (Hq) mice were protected from NMDA- and kainic acid-induced neuronal damage in the hippocampus [63]. Cell death was also found to be suppressed in Hq mice subjected to hypoxia/ischemia. Further neuroprotection was also observed in different mouse models, when AIF processing was prevented by oral administration of HIV protease inhibitors. Finally, it was reported that inhibition of the nuclear translocation of AIF caused neuroprotection in a rat model of retinal degeneration [64].

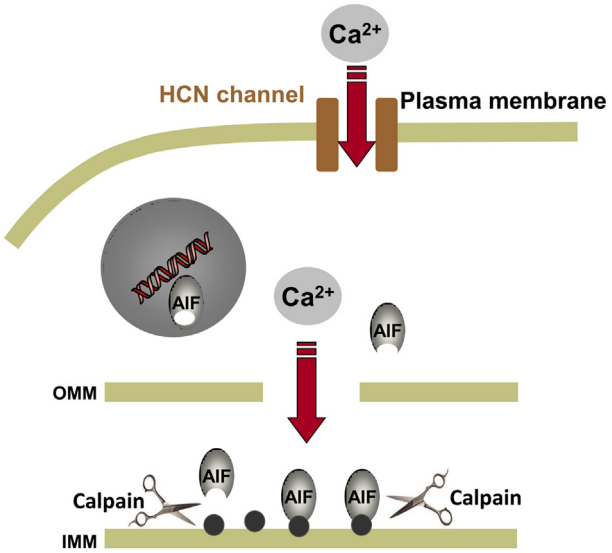

Fig. 3. Involvement of calcium/calpain in AIF processing.

5. Autophagic cell death

Autophagic cell death occurs in the absence of chromatin condensation, but is accompanied by massive autophagic vacuolization of the cytoplasm [65]. Autophagy was initially described as a survival mechanism, and some reports indicate that cells presenting features of 'autophagic cell death' can still recover upon withdrawal of the hazardous stimulus. Nevertheless, in some instances autophagy may be responsible for the deterioration of cells as a result of a protracted atrophy of the cytoplasm beyond a not yet clearly defined point-of-no-return.

Autophagy (macro-autophagy) is a regulated lysosomal pathway involved in the degradation and recycling of long-lived proteins and organelles within cells. Although the detailed mechanisms of autophagy-mediated cell death remain unclear, some evidence favors mitochondrial involvement in this process [66]. In particular, MPT and the associated permeabilization of the OMM can be responsible for stimulation of mitochondrial autophagy, a process thought to be important for the removal of damaged mitochondria [67]. In fact, the degradation of mitochondria via autophagy may be more tightly regulated than originally thought, which has led to the designation of this type of autophagy as "mitophagy" (reviewed in Ref. [68]). There is also evidence that mitochondrial morphology can influence the final outcome of autophagy. Hence, it was reported that mitochondria elongate during autophagy [69]. This elongation is triggered by PKA-mediated inhibition of the dynamin-related protein, DRP-1, which promotes mitochondrial fusion. Elongation was required to sustain cellular ATP level and cell viability.

Further, more recent findings have revealed that when the hepatic mammalian target of rapamycin complex 1 (mTORC1) signaling pathway, an important part of the mitophagy machinery, disengages, the mitochondrial network fragments, cristae density drops, and mitochondrial respiratory capacity decreases. Instead, mitochondria-ER contacts, which mediate Ca^{2+} and phospholipid fluxes between these organelles, double in length. These events are associated with the transient expression of two previously unidentified C-terminal fragments (CTFs) of Optic atrophy 1 (Opa1), a mitochondrial GTPase that regulates cristae biogenesis and mitochondrial dynamics [70]. A link between calcium and mitochondrial fusion/fission was also shown by downregulation of mitofusin 2 (Mfs2), which causes mitochondrial dysfunction and altered calcium homeostasis, resulting in delayed neuronal death [71]. These findings suggest that mitochondrial shape might influence the fate of the cell during autophagy and that this involves effects on calcium homeostasis.

ROS are involved in autophagy regulation, and starvation is known to stimulate the generation of H_2O_2. This appears to be essential for autophagy, as treatment with antioxidants can abolish the formation of autophagosomes and subsequent protein degradation. The cysteine protease, HsAtg4 was identified as a direct target for oxidation by H_2O_2 [72]. Evidence for a crucial role of mitochondrial hydrogen peroxide was confirmed by the finding that catalase targeted to mitochondria prevented cardiac hypertrophy, mitochondrial damage and autophagy induced by angiotensin II, whereas peroxisomally-targeted catalase was inefficient [73]. Thus, autophagy can mediate cell death by excessive self-digestion, e.g. in apoptosis-deficient cells subjected to various treatments, including radiation [74].

There are other examples of cross-talk between apoptosis and autophagy. Hence, the pro-autophagic function of Beclin 1 can be inhibited by both Bcl-2 and Bcl-X_L [75]. Notably, although Beclin-1 possesses a BH3-only domain, and all BH3-only proteins of the Bcl-2 family are known inducers of apoptosis, Beclin 1 fails to trigger apoptosis. In fact, by promoting autophagy it offers protection against pro-apoptotic agents. However, upon growth factor withdrawal, when autophagy precedes apoptosis, caspase-mediated cleavage of Beclin 1 inactivates autophagy and stimulates apoptosis by promoting the release of pro-apoptotic factors from the mitochondria. In this model, a caspase-generated fragment of Beclin 1 seems to trigger an amplifying loop enhancing apoptosis [76]. Recently it has been demonstrated that the anti-apoptotic Bcl-2 family members affect autophagy indirectly, owing to their inhibition of Bax and Bak [77]. Moreover, it was shown that different forms of the same Bcl-2 family protein, namely Mcl-1, had separable functions depending on its localization within the mitochondria [78]. When present at the OMM, a Mcl-1 isoform acted like other anti-apoptotic Bcl-2 proteins to antagonize apoptosis, whereas its amino-terminally truncated isoform, when imported into the mitochondrial matrix, promoted fusion of the mitochondria.

Detailed analysis of autophagy pathways in normal and tumor cells revealed that the Transient Receptor Potential Melastatin 3 (TRPM3) modulates autophagy by affecting Ca^{2+} influx, which in turn regulates CAMKK2 activity [79]. In addition, TRPM3 influences autophagy by regulation of the level of miR-204, which targets the autophagosome adaptor, LC3. Another link between calcium, miR and autophagy was demonstrated in a model of developmental cell death [80]. miR-14 regulates autophagy through its target, inositol 1,4,5-trisphosphate kinase 2 (ip3k2), thereby affecting inositol 1,4,5-trisphosphate (IP3) signaling and Ca^{2+} levels during salivary gland cell death, providing *in vivo* evidence of microRNA regulation of autophagy through modulation of IP3 signaling. Mikoshiba and colleagues have shown that a pleiotropic enzyme, transglutaminase type 2, targets the allosteric coupling domain of IP3R type 1 (IP3R1) and negatively regulates IP3R1-mediated Ca^{2+} signaling and autophagy by locking the subunit configurations, suggesting a pathogenic role of this modification in neurodegenerative disease [81]. It has also been shown that the IP3R regulates autophagy through its interaction with Beclin-1, establishing a functional link between the ER and mitochondria also in autophagy [82]. Another link between Ca^{2+} and autophagy was observed when cells were treated with the autophagy inducer, fluspirilene, an inhibitor of Ca^{2+} fluxes. Finally, lowering of intracellular Ca^{2+} prevents calpain-mediated cleavage of Atg5, which, in turn, increases the levels of full-length Atg5 and of the Atg5-Atg12 conjugate [83].

Through inhibitory serine phosphorylation of GSK-3β and inhibition of FBXW7 recruitment, calcium/calmodulin-dependent protein kinase IV (CaMKIV) prevents the proteasomal degradation of mTOR and thereby augments autophagy – at least in macrophages and the kidney [84]. CaMKIV-mTOR-dependent autophagy is important for IL-6 production and fundamental for the functional and adaptive responses to septic insult. A direct link between inflammation and autophagy was observed in acute pancreatitis [85]. Interleukin-1β was shown to induce autophagy by affecting Ca^{2+} homeostasis and trypsinogen activation in pancreatic acinar cells, suggesting calcium signaling as a promising therapeutic target in the treatment of pancreatitis.

Stimulated Atg7-deficient cells display impaired influx, but not efflux, of Ca^{2+}, and ER calcium storage is increased in these cells; Ca^{2+} mobilization by thapsigargin, ionomycin, or vitamin D led to autophagy [86]. Moreover, aggregation of GFP-LC3 was inhibited by BAPTA-AM, suggesting that autophagosome formation was Ca^{2+}-dependent. In fact, autophagy might have occurred by Ca^{2+}-dependent activation of AMP-activated protein kinase (AMPK), which requires upstream activation of the CaMKK2.

Another Ca^{2+}/calmodulin-regulated kinase, DAPK (death-associated protein kinase), also was shown to be an essential regulator of autophagy [87]. This enzyme interacts with the LC3-binding protein, MAP1B, which is involved in autophagosome trafficking along microtubules. It interacts with and phosphorylates Beclin-1, disrupting the inhibitory Beclin-1-Bcl-2/X_L interaction. On the other hand, interaction of MAP1B with TSC2, a negative regulator of mTOR, leads to DAPK phosphorylation and inactivation [88].

Lysosomes play a key role in autophagy, and there is substantial evidence that Ca^{2+} can accumulate also in lysosomes (for review see Ref. [89]). In animal cells, lysosomes exhibit Ca^{2+} uptake mechanisms that strongly depend on the activity of a vacuolar type of proton pump (V-H+-ATPase). This is selectively and potently blocked by Bafilomycin A1, an inhibitor of late phase autophagy. It has been suggested that the acidic environment generated by the proton pump, is used by a Na^+/Ca^{2+} exchanger to accumulate Ca^{2+} in lysosomes. Interestingly, there are additional Ca^{2+} permeable channels expressed in the lysosomes, i.e. TRPML1 and TRPM2 [90]. However, further work is required to better understand the role of lysosomal calcium in autophagy.

6. Calcium in anoikis

Anoikis is defined as a type of programmed cell death, which occurs when anchorage-dependent cells detach from the surrounding extracellular matrix. It is involved in a variety of tissue-homeostatic, developmental and oncogenic processes. The signaling events culminating in anoikis are still unclear; however, like in other cell death modalities, Ca^{2+} and ROS have been implicated also in the regulation of anoikis [91].

In certain tumor cells treated with anticancer drugs, the level of calpain was found to influence their detachment from the matrix

[92]. Notably, another study concluded that Ca^{2+}-activated channels are involved the regulation of anoikis [93]. Accordingly, down-regulation of the Ca^{2+}-activated chloride channel was shown to result in resistance to detachment-induced cell death in breast cancer cells. Transfection of cells with plasmids encoding this channel led to significant reduction of colony formation and to cell death by anoikis.

In an effort to identify mechanisms that can inhibit anoikis, β-catenin (a major oncoprotein) was found to down-regulate DAPK-2 (death-associated protein kinase-2), leading to anoikis resistance and promotion of anchorage-independent growth [94]. In contrast, connective tissue growth factor, CCN2, was shown to inhibit lung cancer metastasis by promoting DAPK-dependent anoikis and inducing EGFR degradation [95]. DAPK activity also can be regulated by protein phosphatase-2 (PP2A), although this pathway seems to be most relevant for ceramide-induced anoikis [96]. Hence, it seems that similar proteins with a requirement for Ca^{2+} might be involved in the regulation of multiple cell death modalities, including anoikis. Interestingly, depletion of calcium was found to be associated with the accumulation of an anoikis-resistant population of cells in mouse colonic epithelium [97]. Another link between calcium and anoikis was observed when ectopic expression of Double C2-like domain β (DOC2B) gene, encoding Ca^{2+}-binding protein, was found to result in anoikis-mediated cell death and repression of tumor growth in a nude mice xenograft model [98]. DOC2B expressing cells showed a significant increase in intracellular Ca^{2+} level, impaired AKT1 and ERK1/2 signaling, and remodeling of the actin cytoskeleton.

The Ca^{2+}-dependent cell adhesion molecule, E-cadherin, is important for hepatocyte spheroid formation, a mechanism preventing hepatocytes from undergoing anoikis [99]. Accordingly, treatment with an E-cadherin blocking antibody, or calcium chelators, can induce anoikis. Upregulation of adhesion molecules during the process of aggregate formation, as well as cell to cell contact in the aggregate, are mediated by Ca^{2+} [100]. These findings shed light on anoikis resistance mechanisms and might help develop new therapies that target the anoikis-resistant tumor cells in the metastasis process.

7. Mitochondria, oxidative stress and cell death

Reactive oxygen species can promote cell death. Mitochondria consume most of the molecular oxygen available and represent the major site of ROS production in aerobic cells [101]. ROS, if not detoxified, may cause cell dysfunction or death. In fact, mitochondria themselves are sensitive targets for the damaging effects of oxygen radicals. Hence, ROS produced by the mitochondria can oxidize proteins and induce lipid peroxidation, thereby compromising the barrier properties of the mitochondrial membrane. Another target of ROS is mitochondrial DNA (mtDNA), which is particularly susceptible to ROS-mediated damage due to its close proximity to the respiratory chain and the lack of protective histones. Thus, the level of oxidatively modified bases in mtDNA is 10- to 20-fold higher than that in nuclear DNA [102]. mtDNA encodes several proteins essential for the function of the respiratory chain and, hence, for ATP synthesis by oxidative phosphorylation. Oxidative damage caused by ROS is probably a major cause of mitochondrial genomic instability and respiratory dysfunction.

ROS production also markedly enhances Ca^{2+}-induced cellular deterioration. Thus, oxidative stress decreases the level of Ca^{2+} that is required to trigger MPT and the cellular damage thereof. This may be important in ischemia/reperfusion, when an increase in intracellular Ca^{2+} level caused by hypoxia is followed by enhanced ROS generation upon re-oxygenation.

Enhanced ROS production might also contribute to both MPT- and Bax/Bak-dependent mitochondrial membrane permeabilization and release of cytochrome *c*. ROS can modify thiol groups on the ANT and stimulate PTP opening [103]. Oxidation of mitochondrial pyridine nucleotides has also been shown to facilitate Ca^{2+}-induced PTP opening under conditions where glutathione was maintained in the reduced state [104]. Also Bax-mediated permeabilization of the OMM might be triggered by ROS, since oxidation of conserved cys-62 on the Bax molecule has been reported to lead to Bax activation and mitochondrial permeabilization [105].

ROS often mediate cell death induced by inhibitors of the mitochondrial respiratory chain. Some naturally occurring isothiocyanates, such as phenethylisothiocyanate (PEITC), benzyl isothiocyanate (BITC) and sulforaphane, are effective inhibitors of tumorigenesis in rodents treated with carcinogens [106]. For example, oral administration of PEITC significantly retarded growth of PC-3 xenografts in athymic mice [107]. The PEITC-induced death of PC-3 cells was associated with generation of ROS followed by disruption of the mitochondrial membrane potential and release of cytochrome *c* and other mitochondrial proteins. These changes were successfully blocked by superoxide dismutase mimetics and catalase. The pro-apoptotic effect of PEITC correlated with inhibition of Complex III activity, suppression of oxidative phosphorylation and ATP depletion. The Rho-0 mutants of PC-3 cells were more resistant to PEITC-mediated ROS generation and apoptosis as compared to wild-type cells [108].

PEITC-induced apoptosis was found to be dependent on p66Shc, a lifespan-regulating protein also known to modulate the mitochondrial apoptosis pathway. Thus, treatment of PC-3 cells with PEITC resulted in translocation of p66Shc to the mitochondria [109], where it generated hydrogen peroxide using reducing equivalents derived from the mitochondrial electron transport chain through the oxidation of cytochrome *c* [110]. In fact, p66Shc responds to a variety of pro-apoptotic stimuli by increasing ROS levels in the mitochondrial intermembrane space, which might trigger the mitochondrial apoptosis pathway [111]. Mouse embryonic fibroblasts derived from p66Shc knockout mice were significantly more resistant to PEITC-mediated growth inhibition and apoptosis than wild-type fibroblasts.

8. Concluding remarks

Our understanding of the mechanisms involved in the regulation of cell death has improved considerably during recent years, and the importance of Ca^{2+} and ROS signaling in the regulation of multiple modes of cell death has become increasingly obvious. However, it is also clear that the signaling networks involved in cell death regulation are highly complex, and that ample opportunities for cross-talk between the various pathways exist. The pathophysiological consequences of dysfunctional cell death regulation are also becoming apparent, as are the possibilities to modulate the susceptibility of cells to various death signals by the manipulation of Ca^{2+} fluxes and of mitochondrial redox state and energy metabolism. This knowledge might have important therapeutic implications in the future.

Conflict of interest

The authors declare no conflicts of interests.

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