BBA - Molecular Basis of Disease 1870 (2024) 167317

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

Permeabilization of the outer mitochondrial membrane: Mechanisms and consequences

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ARTICLE INFO

Keywords:
Permeabilization
Mitochondrial membrane
Cell death
Mechanisms
Disease

ABSTRACT

Permeabilization of the outer mitochondrial membrane is a physiological process that can allow certain molecules to pass through it, such as low molecular weight solutes required for cellular respiration. This process is also important for the development of various modes of cell death. Depending on the severity of this process, cells can die by autophagy, apoptosis, or necrosis/necroptosis. Distinct types of pores can be opened at the outer mitochondrial membrane depending on physiological or pathological stimuli, and different mechanisms can be activated in order to open these pores. In this comprehensive review, all these types of permeabilization, the mechanisms of their activation, and their role in various diseases are discussed.

1. Introduction

Cell death is an event in which a biological cell terminates performing its functions. For decades, researchers have mainly studied necrotic cell death, an accidental death caused by factors external to the cell or tissue such as trauma or infection. During necrosis, cells and intracellular organelles swell, the plasma membrane undergoes rupture, and cells die due to autolysis. Mitochondrial deterioration was initially regarded as a sign of necrotic cell death. Indeed, disruption of the plasma membrane during necrosis allows the entry of solutes and water into the cell, causing swelling of the cell and intracellular organelles. In this situation, cell death was previously regarded as accidental. The fact that cell death can be programmed drew scientists' attention at the beginning of the 1960s. Several groups found that the morphology of dying cells significantly differed from the well-known necrosis and that cell death is apparently biologically controlled (programmed) [1,2]. At that time, the most characteristic morphological and biochemical signs of apoptosis were chromatin condensation in the nucleus and internucleosomal DNA fragmentation, which can be demonstrated on a blot in the form of a characteristic “ladder” [3–6]. This allowed researchers to conclude that apoptosis is a purely nuclear event.

Mitochondrial permeabilization as a phenomenon either accompanying or triggering cell death was observed long ago. In 1952, Ashwell and Hickman [7] demonstrated the suppression of oxidative phosphorylation in mitochondria from the thymus and spleen of irradiated mice, which was not observed in radioresistant tissues. Later it was found that this was linked to a decline of cytochrome *c*, a component of the mitochondrial respiratory chain, content and, as a result, the alteration of the electron transfer between cytochromes *b* and *c* [8,9]. This conclusion was supported by the experiments demonstrating that the addition of exogenous cytochrome *c* restored oxidative phosphorylation in mitochondria isolated from radiosensitive, but not from radioresistant, tissues of irradiated rats [10,11]. These works became some of the first indications of the importance of permeabilization of the mitochondrial membrane in the implementation of cell death.

During the 1990s, publications appeared indicating the participation of mitochondria in the regulation of cell death [12,13]. Mitochondria are unique cellular organelles that are involved in the generation of metabolic energy, maintenance of calcium, and oxidative homeostasis. The suggestion that mitochondria were involved in cell death was astonishing. However, as it has been convincingly demonstrated by Wang and colleagues [14], cytochrome *c*, an important component of the mitochondrial respiratory chain, could stimulate the apoptotic program. Subsequent studies proved that these organelles could determine whether cells should die or not. Stimulation of apoptosis via targeting mitochondria is important when it comes to cancer cells, as it can help to overcome tumor cell resistance to treatment. As has been shown during the last few decades, permeabilization of the outer mitochondrial

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https://doi.org/10.1016/j.bbadis.2024.167317
Received 31 March 2024; Received in revised form 17 June 2024; Accepted 17 June 2024
Available online 21 June 2024

membrane (OMM) is in many cases a "point of no return" in cell death stimulation.

Apoptosis can be triggered by the interaction of an apoptosis inducer with a receptor on the plasma membrane (so-called an extrinsic, or receptor-mediated, pathway) (Fig. 1). This causes a sequence of events leading to the activation of one of the pro-apoptotic enzymes, caspase-8, which is capable of degrading and activating another member of the caspase family, caspase-3, responsible for the exhibition of a few biochemical and morphological signs of apoptosis. In addition to this extrinsic pathway, there is a pathway triggered by the release of cytochrome *c* from mitochondria. After leaving the mitochondria, cytochrome *c*, together with the cytoplasmic proteins APAF1 and procaspase-9, in the presence of dATP (or ATP), participates in the formation of the apoptosome complex, in which caspase-9 is processed and activated. This leads to the cleavage and activation of caspase-3, which, in turn, cleaves a set of targets responsible for the manifestation of the characteristic signs of apoptosis. Mitochondria are also involved in the extrinsic pathway of apoptosis. Among the targets of active caspase-8 is the cytosolic protein BID: after cleavage and formation of its truncated form (*t*BID), it migrates to the mitochondria and triggers the release of cytochrome *c*. In many (but not all) models of apoptosis, the release of cytochrome *c* is considered to be a point of no return in the induction of apoptosis. Thus, investigation of the mechanisms of cytochrome *c* release is important both for stimulation of cell death, in tumor cells for example, or to rescue cells, in which uncontrolled apoptosis causes deleterious consequences, such as during neurodegeneration. The present review describes multiple pathways of the outer membrane permeabilization and their significance for the fate of the cell.

2. Possible mechanisms of the permeabilization of mitochondrial membranes

2.1. Induction of mitochondrial permeability transition

Cytochrome *c* is localized in the intermembrane space of mitochondria, attached to cardiolipin of the inner mitochondrial membrane (IMM) either electrostatically or hydrophobically. There is also a pool of free releasable cytochrome *c*. Thus, the OMM should be permeabilized and cytochrome *c* should be detached from cardiolipin for the release

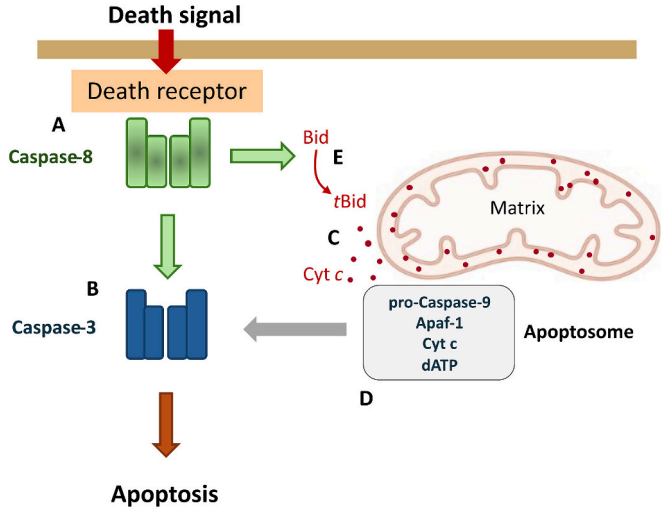

Fig. 1. Apoptotic pathways. A, Activation of caspase-8 via interaction of death signal (ligand) with death receptor, leading to activation of procaspase-8 within DISC complex; B, active caspase-8 cleaves and activates caspase-3, which triggers apoptosis execution; C, release of cytochrome *c* from mitochondria results in formation of apoptosome complex and activation of caspase-9 (D); E, caspase-8-mediated cleavage of pro-apoptotic protein BID (for the details, see the text).

(Fig. 2). Its release can be facilitated by mitochondrial permeability transition (MPT) due to the opening of a Ca^{2+}-dependent pore in the IMM [15], also called the mitochondrial permeability transition pore (MPTP). Opening the MPTP allows the passing of molecules not larger than 1.5 kDa through the IMM; it causes a drop in the mitochondrial membrane potential (MMP) and uncontrolled entry of water and solutes into the matrix, causing organelle swelling, which causes a rupture of the OMM and the release of various proteins from the intermembrane space into the cytosol. Opening the pore can be facilitated by several factors, particularly by reactive oxygen species (ROS) due to oxidation of thiols of adenine nucleotide translocase (ANT), an integral part of the MPTP [16]. In addition to ROS, several compounds and conditions, such as inorganic phosphate, pyridine nucleotide oxidation, ATP depletion, and low pH can stimulate Ca^{2+}-dependent pore opening.

Researchers have done a great deal of work to establish the structure of MPTP and the mechanisms of its regulation. The work was mainly carried out on mitochondria isolated from various mammalian tissues, mainly rats or mice. According to the traditional point of view, the main components of nonspecific pores are the voltage-dependent anion channel (VDAC) located in the outer membrane, ANT in the inner mitochondria membrane, and a soluble matrix protein cyclophilin D (CyPD). In the presence of Ca^{2+}, a complex is formed between ANT, Ca^{2+}, and CyPD, which leads to a conformational change in ANT, which turns this protein into a non-specific pore (for a recent comprehensive review, see [17]).

2.2. Components of the MPTP

2.2.1. VDAC

As a component of the MPTP, the VDAC resides in the OMM and regulates the transport of metabolites through the last. Keeping the VDAC open is vital for mitochondrial function, while the closure of VDAC and subsequent abrogation of metabolite entry into mitochondria may cause deleterious consequences and even kill cells. Earlier, it was shown that VDAC has a greater permeability for Ca^{2+} in the closed than in the open state [18], i.e., the closure of the VDAC can facilitate the entry of calcium, leading to the opening of pores and MPT stimulation. However, the binding of certain proteins, such as Hexokinase [19], keeps VDAC in the open state and hence prevents cell death.

There are three isoforms of VDAC. In general, the spreading of VDAC isoforms is abundant in tissues, although there is a prevalence of the VDAC1 isoform. A high-sequence similarity between VDAC1 and VDAC3 provides a similar pore-forming capacity. However, VDAC3 displayed a reduced affinity to cytosolic proteins which supports its isoform-specific role in mitochondrial physiology [20]. An important mitochondrial function in which VDAC plays a key role is accumulation of Ca^{2+}. The permeability of all three isoforms for Ca^{2+} slightly but statistically significantly differs. Among the three isoforms, VDAC3 is more Ca^{2+} selective and demonstrated the highest Ca^{2+} permeability followed by VDAC1 and VDAC2 [21].

Even though VDAC was considered a structural component of the MPTP, its significance for pore opening is apparently minimal: mitochondria from *Vdac1*-, *Vdac3*-, and *Vdac1/Vdac3*-null mice underwent a Ca^{2+} and oxidative stress-induced MPT similarly to mitochondria from wild-type animals. Mouse embryonic fibroblasts lacking all three VDAC isoforms also showed similar or even enhanced levels of cell death, upon stimulation with hydrogen peroxide, ionomycin, staurosporine, and TNFα [22]. Nevertheless, modulation of MPT can be achieved by targeting VDAC. The isoforms of VDAC and their biochemical characteristics were recently discussed in a comprehensive review [23].

2.2.2. ANT

Some of the first evidence for the involvement of ANT in MPTP formation was an experiment where ANT, incorporated into a lipid membrane, acquired the properties of a nonspecific pore in the presence of Ca^{2+}. Carboxyatractyloside, an inhibitor of ANT, as well as the

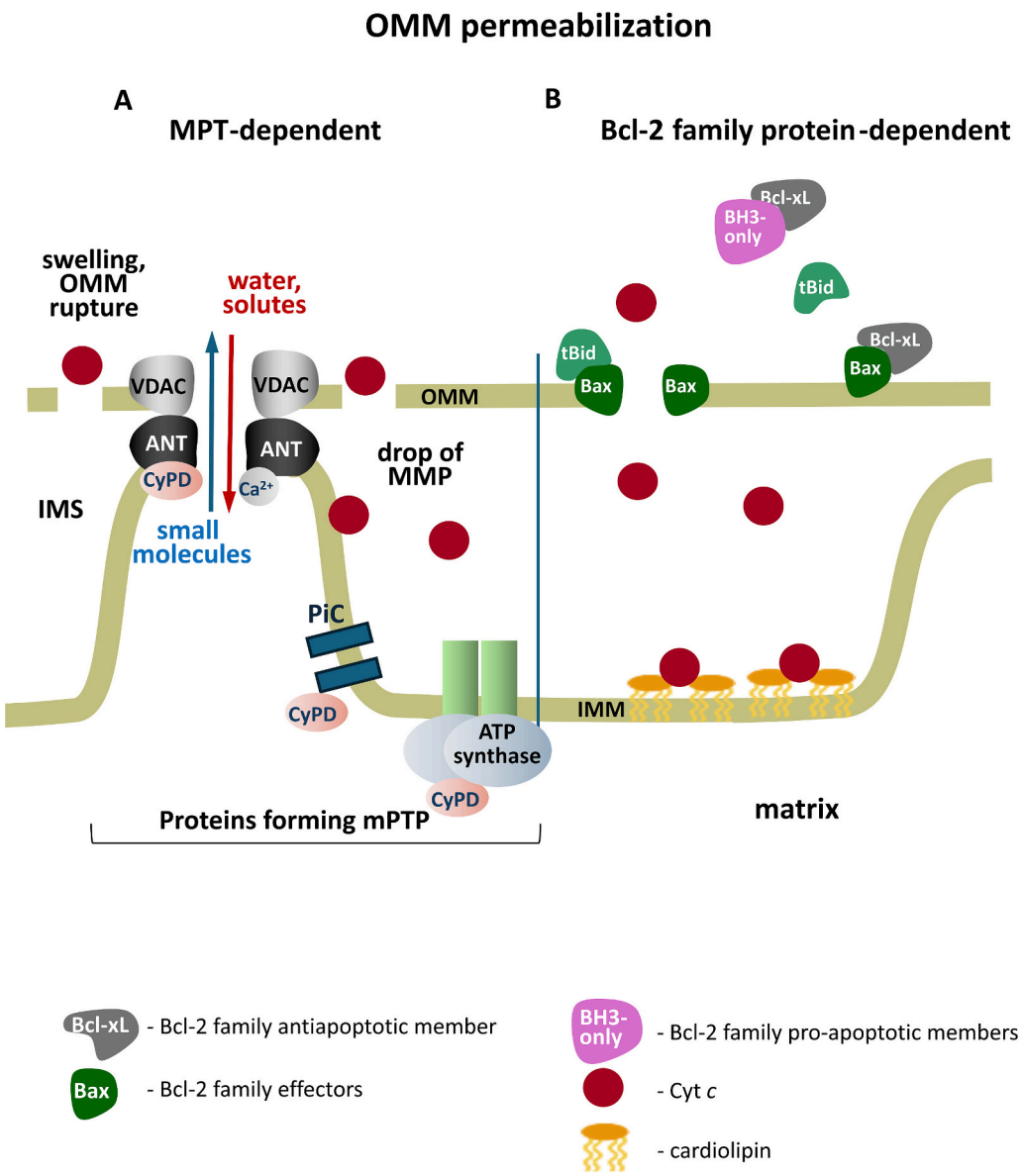

Fig. 2. Modes of the OMM permeabilization. A, MPT-dependent permeabilization; B, BCL-2 family protein-dependent permeabilization (for the details, see the text).

removal of adenine nucleotides, sensitized the pore to calcium ions [24].

ANT exists in three isoforms, the expression of which is tissue-specific and depends on the state of cellular differentiation. Thus, ANT1 is expressed mainly in terminally differentiated tissues like skeletal muscle, heart, and brain. Expression of ANT2 is characteristic of proliferating and regenerating tissues such as the kidneys, spleen, liver, fibroblasts, and lymphocytes. The expression of ANT3 is relatively weak in any tissues examined.

To investigate the role of ANT in MPTP formation, two ANT isoforms were genetically inactivated in mouse liver, and it was shown that MPTP can be induced in mitochondria without ANT; although, higher Ca^{2+} loading was necessary to trigger MPTP, and various ANT ligands were not able to modulate the pore [25]. In addition, even in the absence of ANT, hepatocytes remained sensitive to various cell death inducers. This may be explained by the incomplete knockout of ANT isoforms or the existence of another protein complex that plays an important role in the formation of the MPTP [26]. Thus, this work showed that ANT molecules are not key components of the MPTP, although they are involved in the regulation of the pore.

Other constituents of the IMM can be considered regarding the regulation of MPTP opening. While investigating MPT regulation, Bernardi and colleagues [27] found that dimers of mitochondrial ATP synthase can form the MPTP. In this model, MPTP opening requires Ca^{2+} binding to catalytic sites of the F1. The authors showed that as in the case of ANT, CyPD regulates pore opening via binding to the oligomycin sensitivity-conferring protein (OSCP) subunit of ATP synthase. According to another hypothesis, MPTP is formed within the c-ring of the F0 subunit of the F1F0 ATP synthase monomers. MPTP formation is caused by structural changes in ATP synthase leading to the opening of the ATP synthase leak channel [28,29]. It should be, however, mentioned that the role of ATPase in the MTPT was questioned in a recent publication [30]. The authors found that Ca^{2+}-induced pore opening was markedly sensitized by loss of the mitochondrial ATP synthase. The latest advances and controversies in the identity, structure, and function of the MPTP were recently discussed in a comprehensive review [17].

Along with the possible MPTP-forming proteins mentioned above, other IMM proteins have been suggested as parts of the MPTP complex. For instance, the mitochondrial phosphate carrier (PiC) has been reported to be involved in MPTP formation [31]. However, although CyPD can interact with PiC through its N-terminus, further studies using

cardiac-specific mouse strains expressing different levels of mitochondrial PiC revealed that the contribution of PiC in MPT regulation is minor [32]. Therefore, among possible components of the MPTP, CyPD is currently considered the main regulatory element of MPTP opening.

2.2.3. CyPD

CypD is a peptidyl-prolyl *cis-trans* isomerase, which is expressed in the nucleus as a ~22 kDa protein containing a mitochondrial targeting sequence. Upon import into the mitochondrial matrix, it undergoes cleavage with the formation of a ~19 kDa product [33].

The ability of CyPD to interact with all of the candidates for pore-forming proteins (ANT, ATP synthase, and PiC) makes CyPD a key component in MPTP regulation, and several studies have demonstrated the possibility of regulating pore opening through post-translational modification of CyPD. Thus, deacetylation of CyPD by the deacetylase sirtuin3, a member of the sirtuin family found in mitochondria, suppresses MPTP opening [34,35]. Another mechanism of regulation is the phosphorylation of CyPD at the Ser191 residue, which facilitates its binding to OSCP of ATP synthase and thus sensitizes MPTP opening leading to subsequent cell death [36].

The efforts to explain cytochrome *c* release during apoptosis by MPT induction did not satisfy most mitochondrial researchers. Opening MPTP has deleterious effects on mitochondria. Indeed, in pathological situations, when Ca^{2+} enters the cytosol, the mitochondria take it up, attempting to restore Ca^{2+} homeostasis. This accumulation is driven by the MMP. If the damaging factor is not eliminated and Ca^{2+} loading is high, Ca^{2+} stimulates the opening of MPTP, which leads to mitochondrial energetic collapse. The MMP drop stimulates mitochondrial ATPase activity and instead of producing ATP, mitochondria start hydrolyzing ATP which leads to the drop in ATP content in the cytosol, causing necrotic cell death. Indeed, it has been demonstrated that the opening of the MPTP is a cause of cardiomyocyte death during ischemia/reperfusion [37]. Under these circumstances, inhibition of CyPD, as a key component of the MPTP, prevents MPT and affords cardioprotection [38].

3. Physiological role of MPTP

Opening of the MPTP appears to be crucial in necrotic/necroptotic cell death as a consequence of a pathological impact causing uncontrolled elevation of Ca^{2+} concentration in the cytosol, whereas under physiological conditions MPTP opening should not occur. However, when analyzing the membrane potential in cardiomyocytes using the fluorescent indicator tetramethylrhodamine ethyl ester (TMRE), Duchen et al. [39] showed spontaneous short-term depolarizations in a subpopulation of mitochondria. This phenomenon was later called flickering. Such transient mitochondrial depolarization can be caused by the accumulation of Ca^{2+} and brief MPTP opening. The average concentration of Ca^{2+} in the cytosol is low to stimulate Ca^{2+} uniporters and hence is insufficient for triggering MPT. However, near the endoplasmic reticulum (ER), the concentration of Ca^{2+} released from the ER during physiological signaling (so-called Ca^{2+} hot spots) may be high enough to activate Ca^{2+} uniporters and cause accumulation of this ion in the mitochondrial matrix. Indeed, flickering was shown to be prevented by inhibition of calcium accumulation in the mitochondria, either by preliminary depletion of the calcium pool of the sarcoplasmic reticulum (for example, by thapsigargin) or by blocking the release of Ca^{2+} from the reticulum (for example, by ryanodine) [40]. Flickering was also prevented by CyPD inhibitor cyclosporin A (CsA), showing that this phenomenon is caused by the opening of a nonspecific pore in the IMM. If flickering can occur under physiological conditions, what might be the physiological role of this phenomenon?

The possibility of physiological transient MPTP opening in single mitochondria in situ in cardiac myocytes has been demonstrated directly [41]. The authors monitored MPTP opening during spontaneous releases of Ca^{2+} from the sarcoplasmic reticulum. They found that the duration of pore opening (~60 s) and its size were distinct from the well-known pathological MPTP parameters, although transient PTP (tPTP) was sensitive to CsA, the presence of Ca^{2+}, and inhibitors of the mitochondrial Ca^{2+} uniporter. The transient opening was a quite rare phenomenon, occurring in <0.1 % of myocyte mitochondria at any moment. The tPTP size was much smaller than typical for permanent MPTPs, indicating that proteins or even molecules the size of NADH (663 Da) should be retained during tPTP opening. The authors suggested that tPTP can be beneficial to mitochondria, since tPTP opening allows the release of accumulated Ca^{2+} and/or other harmful factors, such as ROS, which accumulate in mitochondria as byproducts of normal metabolism, especially under pathological conditions [42]. According to the results obtained by Pavlov and colleagues [43], the number of MPTPs per mitochondrion is low and ranges from one to nine, although the authors admit that these results may be slightly underestimated.

While it has been convincingly demonstrated that the Ca^{2+}-dependent opening of MPTP can involve both ANT and ATP synthase, the molecular details of the MPTP are still incompletely understood. Using holographic and fluorescent microscopy, Pavlov and colleagues demonstrated CsA-sensitive membrane depolarization but not high-conductance MPTP in cells lacking either ATP synthase or ANT. The authors proposed that both ATP synthase and ANT are required for high-conductance MPTP, whereas depolarization is apparently based on the activation of the low-conductance MPT, the molecular nature of which differs from ANT- or ATP synthase-mediated MPTP opening [44].

Physiological mitochondrial Ca^{2+} oscillations were shown to initiate mitophagy (the selective degradation of mitochondria by autophagy) through the PINK1-Parkin pathway essential to Parkinson's disease [45]. Whereas in rotenone-treated SH-SY5Y cells, mitochondrial Ca^{2+} oscillation was suppressed to a single-time Ca^{2+} spike and the mitophagy index decreased significantly.

If flickering of the PTP is impaired, it can cause SPG7 spastic paraplegia [46], a group of rare genetic neurological diseases characterized by degeneration of the corticospinal tract. The *SPG7* gene encodes paraplegin, an essential and conserved component of the MPTP [47]. Paraplegin mutations increase the expression of sirtuin3, which deacetylates CypD, suppressing transient openings of the MPTP with destructive effects on synaptic transmission.

Thus, the opening of the MPTP cannot be attributed solely to pathological conditions. Its involvement in physiological processes is undoubtedly important and requires further investigation.

4. Formation of pores by BCL-2 family proteins

When it became clear that cytochrome *c* is a key component of apoptosis machinery, there was a need to identify the mechanisms of its release and to determine how the OMM can be permeabilized. Initially, attempts were made to explain the release of cytochrome *c* from mitochondria during apoptosis by the opening of the MPTP pore with subsequent swelling of mitochondria and rupture of the OMM [48,49]. However, as mentioned above, opening the MPTP has deleterious consequences for mitochondria. It causes mitochondrial depolarization, converting mitochondria from ATP-producing machines to ATP-consuming in attempts to build up the MMP. This should cause another type of cell death namely necrosis. This is why scientists have made numerous attempts to resolve this issue.

One of the first steps that helped to solve this problem was the discovery of the BCL-2 family of proteins, which play a contrasting role in apoptosis regulation. Back in 1984, Tsujimoto and colleagues found a chromosome translocation (t(14;18)) that linked the immunoglobulin heavy chain locus to a *bcl-2* gene located on chromosome 18 [50]. Later it has been shown that the expression of *bcl-2* promotes haemopoietic cell survival and provides modest growth advantage [51]. In addition, *bcl-2*-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation [52]. The pro-survival protein BCL-2 promoted B-cell proliferation and inhibited cell death

induced by deprivation of IL-3. BCL-2 and its homolog BCL-X_L were shown to inhibit cell death induced by various stimuli, such as heat shock, serum deprivation, chemotherapy agents, hypoxia, respiratory chain inhibition, and calcium overload [53–56]. These proteins were able to block most forms of apoptosis and some types of necrotic cell death. BCL-2 family proteins contain one to four BCL-2 homology (BH) domains — BH1, BH2, BH3, and BH4 [57]. The BH4 domain is critical for the suppression of apoptosis, as the BH4-peptide from BCL-X_L exerts antiapoptotic activity [58,59]. It was later shown that the protective effect of BCL-2 is associated with its localization in mitochondria, attracting attention to this organelle, although the authors mistakenly assumed BCL-2 localization in the IMM [12].

Subsequently, researchers have found that some BCL-2 family proteins do not prevent but rather facilitate cell death, and the fate of the cell depends on the balance between the members of these two groups. Incubation of pro-apoptotic protein BAX with isolated mitochondria in purified cytosol led to the release of cytochrome *c* from the intermembrane space of mitochondria followed by caspase activation. Likewise, supernatants from BAX-treated mitochondria were able to induce the processing and activation of caspases. Importantly, the antiapoptotic recombinant protein BCL-X_L blocks both the release of cytochrome *c* and caspase activation induced by BAX [60]. The importance of BAX and BCL-2 antagonist/killer (BAK) for apoptosis execution was confirmed by the fact that double knockout of BAX and BAK prevents cytochrome *c* release and apoptosis in response to multiple apoptotic stimuli [61]. In mice, double *bax/bak* knockout promotes apoptosis resistance but enhances autophagic cell death. Respectively, *Atg5* knockout decreases embryo death in these mice [62].

According to the current view, the BCL-2 family proteins functionally and structurally are divided into three subgroups: anti-apoptotic/pro-survival (e.g., BCL-2 and BCL-X_L); pro-apoptotic effectors/the executioners (e.g., BAX and BAK) and pro-apoptotic BH3-only. An important property of these proteins is their heterodimerization with each other via BH domains. The anti-apoptotic BCL-2 proteins, such as BCL-2, BCL-X_L, MCL1, and BCL-w, contain four BH-domains and via the hydrophobic groove in their structure directly bind BH3 domains of pro-apoptotic BAX and BAK, preventing their oligomerization upon physical sequestration at the OMM [63]. They also bind BH3-only proapoptotic activators [64]. The interaction between pro- and anti-apoptotic proteins is strictly defined: for example, BID, BIM, and PUMA bind BCL-2; BAD binds BCL-2, BCL-X_L, and BCL-w; NOXA binds Mcl1, and HRK binds BCL-X_L [65].

In addition to binding proapoptotic proteins at the OMM, BCL-X_L promotes retranslocation of BAX to the cytosol [66]. This retranslocation of BAX between the mitochondria and cytosol is enhanced by another pro-apoptotic protein, BAD. Quantitative fluorescence resonance energy transfer (FRET) imaging in living cells and co-immunoprecipitation analyses have shown that the interaction of BCL-X_L with BAD is stronger than that with BAX. Using isolated mitochondria, the authors showed that ABT-737, a BH3 mimetic, dissociated BAX from BCL-X_L, suggesting that mitochondrial BAX was directly released to the cytosol after BAD binding to BCL-X_L [67].

BCL-2 family proteins are engaged in OMM permeabilization in response to extrinsic apoptotic stimuli. As mentioned above, caspase-8 can cleave not only caspase-3 but also the cytosol-located protein BID. *t*BID can cause oligomerization of other pro-apoptotic proteins, BAX or BAK, which are integrated into the mitochondrial membrane, forming a pore. Through this pore, pro-apoptotic factors such as cytochrome *c*, apoptosis inducing factor (AIF), and second mitochondria-derived activator of caspase (SMAC) are released. In this model, anti-apoptotic proteins prevent permeabilization via binding to pro-apoptotic proteins such as BAX or BAK, blocking pro-apoptotic regulatory proteins from interacting with pro-apoptotic proteins that form the pore.

The major proteins forming pores in the OMM are BAX and BAK. They contain three BH3-domains and a conserved transmembrane domain. BAX exists in the cytosol in a monomeric or an inactive dimeric form and upon activation inserts into the OMM. Effector proteins can be directly or indirectly activated by the second BCL-2 subgroup — BH3-only activators. *t*BID, BIM, and to a lesser extent PUMA and NOXA, physically (but transiently) interact with the mitochondrial pool of BAX and/or BAK and promote conformational changes and dissociation from inhibitory proteins [65]. There are BH3-only proteins that are not able to activate BAX and BAK directly but sequester antiapoptotic proteins. This subgroup includes BAD, BMF, HRK, and BIK, and is called sensitizers. PUMA and NOXA also inhibit antiapoptotic BCL-2 proteins. It is now considered to be a continuum with potent activators such as *t*BID and Bim at one side and other proteins such as PUMA with less activator activity [68]. The activity of *t*BID, BIM, and to a lesser extent PUMA and NOXA, is controlled by direct binding to the 14-3-3σ protein [69]. Activated JNK-kinase promotes 14-3-3σ dissociation from BAX, allowing its translocation to the OMM [70].

An attempt to explain in detail how BAX and BAK assemble the apoptotic pores was made recently. Upon activation, BAX and BAK assemble oligomers at the OMM (homodimers further assembled into multiple homo- and hetero-oligomers) to form toroidal pores. At the supramolecular level, these pores consist of rings or linear/arc-shaped oligomers that perforate the OMM [69]. The authors have demonstrated that although the structure of BAX and BAK is quite similar, these proteins have distinct oligomerization properties. BAK forms smaller structures with faster kinetics than BAX. It has even been demonstrated that BAX and BAK can regulate each other as they co-assemble into the same pore in the OMM. BAK recruits and accelerates BAX assembly into oligomers that continue to grow during apoptosis. Large pores reach hundreds of nanometers in size, while 2 nm would be enough for cytochrome *c* release. The authors suggested that low-order oligomers form small pores for cytochrome *c* and other small proteins, while large — for mtDNA [71,72]. Although the authors mentioned mtDNA release, in principle, it may not leave the mitochondrial matrix through a pore in IMM. Instead, the formation of large pores in the OMM allows the IMM to herniate into the cytosol, carrying with it fragments of mtDNA [73].

There are various mechanisms of pro-apoptotic protein activation. Thus, the activation of BAK by BH3-only proteins includes a hit-and-run mechanism by transient binding of BH3-only proteins. In addition, the possibility of BAK autoactivation has been suggested recently [74]. Apparently, the structural changes induced by BH3 domains are equally important for OMM permeabilization. Activation of BAK can be induced by peptides from the human proteins BID, Bim, and Puma. Studies based on computer modeling identified 10 new peptides from the human proteins BNIP5 and PXT1, and three non-native peptides capable of BAK activation [75]. These results indicate the diversity of peptide sequences capable of regulating BAK. Such peptides can serve to develop strategies for finding therapeutic agents that can, if necessary, modulate the activity of BAK.

The ability of BID to induce cytochrome *c* release depends on the composition of the mitochondrial membrane. The addition of *t*BID to non-synaptosomal brain mitochondria induced partial cytochrome *c* release which was inhibited by antibodies against BAK. At the same time, *t*BID failed to release cytochrome *c* from rat liver mitochondria lacking both BAK and BAX [24]. Another atypical effector protein that supposedly can trigger apoptosis in the absence of both BAX and BAK is BOK [76]. However, since BOK is predominantly localized to the ER and interacts with IP3R, its role as a killer has been questioned recently. Moreover, in the absence of IP3Rs, BOK is highly unstable and rapidly eliminated via proteasomal degradation [77].

Interestingly, it has been suggested that *t*BID can cause OMM permeabilization by itself even in the absence of BAX and BAK [78]. This leads to the release of cytochrome *c*, caspase activation, and subsequent cleavage of PARP. In these experiments, *t*BID was overexpressed in HCT cells in which all the BH3-only proteins were knocked out. In addition, the authors reported that the expression of *t*BID can cause permeabilization of the IMM, assessed by mtDNA release. This seems unlikely and the appearance of mtDNA in the cytosol (as was mentioned

above) should be explained by mitochondrial herniation facilitated by *t*BID-mediated pore formation, as shown earlier for pores created by BAK/BAX [73]. Keeping in mind that *t*BID is the only pro-apoptotic protein that can bind to VDAC [79], it cannot be excluded that the effects observed by the authors can be explained by the closure of VDAC, which causes mitochondrial deterioration.

In some cases, permeabilization of the OMM involves other subcellular structures, such as the ER. Thus, as mentioned above, BIK protein is in the ER, where it interacts with antiapoptotic proteins that promote ER calcium release. It has been suggested that BIK is localized at both the ER and ER membranes associated with mitochondria where it attaches and transports the mitochondrial BCL-X_L to the mitochondria-associated ER membrane [80].

Another member of the apoptotic BCL-2 protein family, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP-3), can induce cell death through permeabilization of the OMM but is also involved in mitophagy. Thus, under hypoxic conditions, the accumulation of BNIP3 and BNIP3L (also known as NIX), facilitates receptor-mediated mitophagy and prevents apoptosis through the elimination of pro-apoptotic mitochondria [81].

There is increasing evidence of an interplay between different modes of cell death and various physiological processes. Parkin, a protein playing a critical role in ubiquitination, labeling molecules with ubiquitin for subsequent degradation in proteasomes or lysosomes, can ubiquitinate BAK, inhibiting its proapoptotic activity. This is because the hydrophobic patch of ubiquinone binds to the canonical hydrophobic groove of BAK, preventing binding of *t*BID and hence activation of BAX [82].

Although the importance of BCL-2 proteins in the regulation of OMM permeabilization is not in doubt, the role of MPTP in regulating cytochrome *c* release in apoptosis has not lost its significance. In some cases, inhibition of MPTP opening can rescue cells from death. Thus, the release of cytochrome *c* and subsequent apoptosis induced by tumor necrosis factor alpha (TNFα) in HeLa cells was shown to be inhibited not only by overexpression of BCL-2, but also in the presence of CsA, an inhibitor of MPTP or oligomycin, an inhibitor of H^+-ATP-synthase [78]. However, when apoptosis was induced by staurosporine, only BCL-2 was able to suppress cell death. The authors suggested that in addition to ANT, the oligomycin-sensitive F0 component of H^+-ATP-synthase might be involved in TNF-induced MTPT opening [83]. This was proved later by Bernardi and colleagues [24].

Earlier, it was shown that pro-apoptotic BCL-2 family proteins can interact with MPTP machinery. The overexpression of BAX triggers cell death in Jurkat cells. Inhibitor of MPTP, cyclosporin A in combination with aristolochic acid (ArA), an inhibitor of phospholipase A2, prevented apoptosis [84]. Although BAX is not directly involved in Ca^{2+}-induced MPT [85], addition of the recombinant protein BAX to Ca^{2+}-loaded mitochondria has been shown to facilitate MPT induction [86]. This conclusion was supported by experiments in which inhibition of the anti-apoptotic BCL-2 family by BH3 mimetics sensitized the MPTP through BAX and BAK [87]. Similarly, inhibition of BCL-2 was found to sensitize MPTP opening in ischemia-damaged mitochondria [88]. Thus, cytochrome *c* release can occur via Ca^{2+}-dependent and Ca^{2+}-independent mechanisms; both are regulated by BAX.

Cytochrome *c* release from mitochondria does not necessarily lead to apoptosis. Release of cytochrome *c* due to sublethal OMM permeabilization has been shown to cause caspase-independent initiation of an activating transcription factor 4 (ATF4)-dependent, drug-tolerant persister phenotype [89]. Although it is accepted that permeabilization can be called sublethal if only some mitochondria in a cell undergo OMM permeabilization, it is still not clear how the extent of permeabilization can be evaluated.

The formation of pores in the OMM mediated by pro-apoptotic BCL-2 family proteins is a key step in the mitochondrial apoptosis pathway, defining the cell's commitment to death by allowing the release of apoptogenic factors such as cytochrome *c* and AIF. In the case of BCL-2-mediated pore formation, in contrast to MPTP opening, the IMM remains intact, and mitochondria maintain their membrane potential and are capable of ATP synthesis.

5. Cardiolipin and OMM permeabilization

Most of the cytochrome *c* in the intermembrane space is bound to cardiolipin, a unique phospholipid located almost exclusively in the IMM. To stimulate the release of cytochrome *c*, the interaction between cytochrome *c* and cardiolipin should be breached. This can be facilitated by cardiolipin oxidation. Experiments performed on isolated mitochondria have revealed that the fraction of releasable cardiolipin increases in the presence of prooxidants. The amount of cytochrome *c* released from mitochondria in the presence of recombinant BAX and prooxidant decreased in the presence of the antioxidant butylated hydroxytoluene. The release of cytochrome *c* therefore represents a two-step process that is undermined when either step is compromised [90]. The two-step mechanism of cytochrome *c* release was later confirmed [91]. Given that the mitochondrial respiratory chain is an important source of ROS in mitochondria [92], enhancement of ROS production due to targeting mitochondrial respiratory complexes will facilitate the detachment of cytochrome *c* with its subsequent release upon OMM permeabilization.

It has been suggested that cardiolipin enhances the pore-forming activity of pro-apoptotic members of BCL-2 family proteins [93,94]. As mentioned above, cardiolipin is located almost exclusively in the IMM, while BCL-2 family proteins reside at the OMM. However, the presence of cardiolipin at the contact sites between IMM and OMM cannot be excluded [94].

Oxidation of cardiolipin, causing breaching of cytochrome *c*, can be fulfilled by cytochrome *c* itself. Kagan and colleagues showed that cardiolipin oxidation can be due to the peroxidase activity of cytochrome *c* in a complex with cardiolipin [95]. They found that binding with cardiolipin and its subsequent partial unfolding switches cytochrome *c* function from electron transfer to peroxidase activity [96]. Cytochrome *c* content was shown to be essential for the release of other proapoptotic factors and apoptosis execution. Thus, in radiation-induced apoptosis, accumulation of hydroperoxides was mostly found in cardiolipin and to a lesser extent in other phospholipids. Lowering cytochrome *c* content using an siRNA approach attenuated the level of peroxidized cardiolipin, and at the same time proportionally decreased the release of the pro-apoptotic factor Smac/DIABLO [97]. The antibiotic minocycline exhibits antiapoptotic activity by binding cytochrome *c* and preventing its interaction with cardiolipin [98]. On the other hand, the viral protein PB1F2, which binds to cardiolipin, promotes the dissociation of cytochrome *c* from cardiolipin and stimulates apoptosis upon OMM permeabilization [99]. Thus, the formation of complexes between cardiolipin and cytochrome *c* as well as cytochrome *c* peroxidase activity play a key role in OMM permeabilization and apoptosis.

Another pathway of cytochrome *c* involvement in OMM permeabilization depends on the protein p66Shc, a redox enzyme, which regulates ROS metabolism and apoptosis. p66Shc generates hydrogen peroxide in mitochondria via the oxidation of cytochrome *c*. The generation of mitochondrial ROS is blocked when p66Shc is mutated. Mutation abrogates swelling of mitochondria in vitro and inhibits mitochondrial pathways in apoptosis in vivo [100].

Cardiolipin facilitates apoptosis in multiple ways. Although under normal conditions cardiolipin is mostly found in the IMM, during apoptosis distribution of cardiolipin in mitochondrial membranes is altered and its content on the OMM increases [101]. Cardiolipin microdomains on the OMM were shown to provide a platform for procaspase-8 activation [102]. This step facilitates cleavage of BID by caspase-8 and subsequent involvement of *t*BID in OMM permeabilization. Moreover, cardiolipin facilitates cleavage of BID by caspase-8, due to electrostatic interactions of anionic cardiolipin and positively charged amino acids of BID [103]. Using isolated OMM, it has been

demonstrated that the presence of cardiolipin is required for the activation of monomeric BAX by *t*BID, or its BH3-domain peptide. Activation results in the formation of large pores allowing the passage of very large (2 MDa) dextran molecules [104].

Cardiolipin-containing membranes exhibit complex behaviors depending on various factors and research modeling systems, which complicates a complete understanding of its interactions with main apoptotic players and their significance during apoptosis.

6. An interplay between MPT- and BCL-2 proteins in OMM permeabilization

6.1. VDAC

The involvement of ANT, VDACs, and CyPD in pore formation machinery does not exclude their participation in apoptosis execution (Fig. 3). It was observed that in VDAC1-deficient mitochondria isolated from yeast cells, BAX and BAK failed to induce the loss of membrane potential and cytochrome *c* release [105]. This suggests that VDAC1 might interact with BAX in pore formation. Apparently, BAX can form pores alone or together with VDAC. The size of VDAC is not enough to allow leakage of cytochrome *c* from the intermembrane space; however, VDAC has been shown to undergo Ca^{2+}-dependent oligomerization to form pores sufficient for the release not only of cytochrome *c* but also mtDNA fragments from oxidatively-stressed mitochondria. Chelation of intracellular Ca^{2+} using the cell-permeable Ca^{2+}-chelating reagent 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA-AM), suppressed VDAC1 oligomerization and apoptosis, while increasing cytosolic Ca^{2+} concentration using the Ca^{2+} ionophore, facilitated VDAC1 oligomerization and apoptosis induction even without apoptosis inducer [106,107].

It is tempting to explain OMM permeabilization by BCL-2 family proteins by their interaction with MPTP components. It has been shown in experiments with VDAC incorporated into liposomes loaded with cytochrome *c* that the recombinant pro-apoptotic proteins BAX and BAK triggered the release of this protein. The release was prevented by the anti-apoptotic protein BCL-X_L, which interacts with VDAC and closes it. In accordance with this, BAX and BAK failed to trigger a loss in membrane potential and cytochrome *c* release from VDAC1-deficient mitochondria isolated from a mutant yeast [105]. The role of VDAC in mitochondrial permeabilization during apoptosis is currently an unresolved issue, and research data shows conflicting results. In one of the earliest review papers on this topic [108], the authors suggested that the permeability of VDAC can indeed be regulated by the members of BCL-2 family proteins: BAX or BAK open the VDAC to induce cytochrome *c* release, while BCL-2 or BCL-X_L close this channel. Additionally, BAX was unable to induce cytochrome *c* release from mitochondria isolated from VDAC mutant yeast cells. Transfection with VDAC1 restored the ability of BAX to stimulate cytochrome *c* release. In contrast to this observation, it was shown when the expression of two isoforms of VDAC (VDAC1 and VDAC3) was suppressed, mitochondria were still able to undergo MPT similarly to mitochondria from wild-type cells. Moreover, wild-type and VDAC-deficient mitochondria both demonstrated the same level of cytochrome *c* release, caspase activity, and cell death in response to the pro-apoptotic BCL-2 family members BAX and BID. In other words, these results demonstrate that VDACs do not play any significant role in cell death mediated by either MPTP or the proapoptotic members of the BCL-2 family [22].

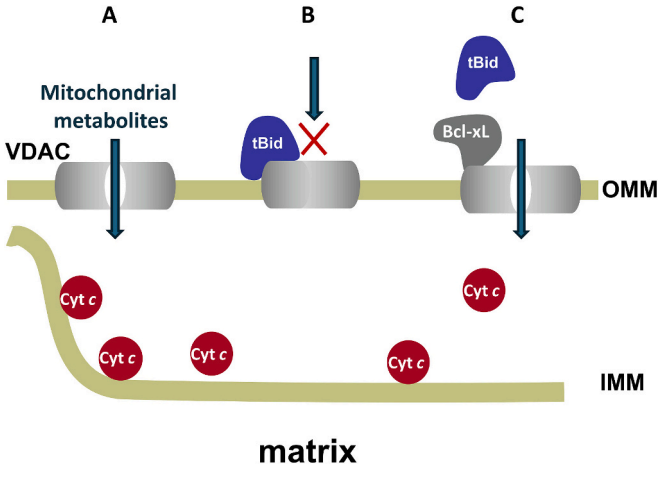

Fig. 3. Involvement of VDAC in OMM permeabilization. A, Transport of metabolites through VDAC; B, *t*BID interacts with VDAC causing its closure and mitochondrial deterioration; C, BCL-X_L binds to VDAC preventing interaction of *t*BID with the channel (for the details, see the text).

Another model of the VDAC-dependent OMM permeabilization is based on the possible existence of the tertiary VDAC1–BAX–BCL-X_L complex demonstrated in vitro. According to this model, the balance between BAX and BCL-X_L can modulate the VDAC1 state and mitochondrial membrane permeabilization [109].

Despite plentiful data on the opening of VDAC by pro-apoptotic members of BCL-2 family proteins, Rostovtseva and colleagues [79] demonstrated that among pro-apoptotic proteins only *t*BID can interact with VDAC, and this interaction causes not the opening, but the closure of the channel. The authors suggested that closing VDAC by *t*BID should reduce the transport of metabolites into mitochondria causing mitochondrial dysfunction. Indeed, experiments performed on rat liver mitochondria showed that closure of VDAC causes oxidative stress and stimulates the Ca^{2+}-induced MPT [110]. This effect of *t*BID can be prevented by the antiapoptotic protein BCL-X_L, which binds to VDAC and keeps it in the open state preventing mitochondrial dysfunction and cell death initiation [111].

Interestingly, although VDAC is a part of the MPTP and exogenous overexpression of VDAC makes cells more prone to apoptosis; nevertheless, its overexpression can be observed in tumor cells. These cells do not undergo apoptosis, largely because of overexpression of hexokinase, and antiapoptotic proteins, which bind to VDAC and keep it in an open, antiapoptotic state [112]. VDAC1 can be considered a pro-survival protein with pro-apoptotic activity, the function of which is controlled by the expression level and the interacting proteins.

6.2. ANT

The involvement of ANT-1 in apoptosis was demonstrated in the search for dominant genes responsible for this mode of cell death. Overexpression of ANT-1 triggers various manifestations of apoptosis. Mutations that impair the ability of ANT-1 in the transport of ADP and ATP, as well as mutations of the N-part of the protein, are still able to trigger apoptosis, which indicates that the proapoptotic properties of ANT-1 are independent of the transport activity of the protein. In contrast to ANT-1, the apoptosis-stimulating properties of ANT-2 are lower. Unlike BAX, ANT-1 is unable to induce cell death in yeast cells. This, and the repression of apoptosis by the ANT-interacting protein CyPD, leads to the conclusion that ANT-1 induction of apoptosis is mediated through a specific protein-protein interaction within MPTP [113]. The involvement of ANT in apoptosis stimulation was demonstrated in experiments with cytomegalovirus-infected cells. These cells use apoptosis as a defense mechanism. Human cytomegalovirus produces the anti-apoptotic protein viral mitochondria-localized inhibitor of apoptosis (vMIA). In infected cells, vMIA was found in mitochondria, where it is associated with ANT and thereby blocked Fas-mediated apoptosis [114]. However, according to another explanation, vMIA can also bind to BAX to prevent its pore-forming capacity. Mutant vMIA, which was unable to bind BAX, did not affect cytochrome *c* release and apoptosis induction [115].

Despite the data indicating the importance of ANT in apoptosis, the

role of ANT in this form of cell death and the formation of MPTP is currently quite controversial. Several attempts have been made to explain the OMM permeabilization through the interaction of BAX and BCL-2 with ANT [116]. The authors described a model of regulation of mitochondrial apoptosis in which ANT acts together with either BAX or BCL-2, which both modulate ANT function in an opposing manner. BCL-2 maintained the translocase activity at high levels, whereas BAX suppressed the translocase function of ANT. However, BAX and ANT do not form an active channel when they cooperate with BCL-2. ANT is a unique pore-forming protein whose interaction with BAX or BCL-2 determines the fate of the channel [117]. This model of OMM permeabilization based on the interaction of BCL-2 family proteins with ANT has an important obstacle: it remains unclear how BAX and BCL-2 both localized on the OMM interact with the IMM protein — ANT. According to another model, BAX (to manifest its effects) must interact not with ANT alone, but with the VDAC–ANT complex at some stages of its action [118]. The possibility of regulation of ANT by BCL-2 family proteins via interaction with VDAC appears to be more realistic.

6.3. CyPD

Similarly to VDAC and ANT, CyPD has also been shown to regulate apoptosis; however, the consequences of its expression for necrotic and apoptotic modes of cell death are different. Although CyPD promotes MPT, it has opposite effects on cell death triggered by apoptotic or necrotic inducers. The overexpression of CypD in some tumors can be viewed as an additional mechanism of apoptosis suppression in tumor cells. The mechanisms of apoptosis suppression by CyPD are poorly understood. It has been demonstrated that in CyPD-overexpressing glioma cells, the suppression of apoptosis correlates with the amount of mitochondria-bound hexokinase II, which binds to VDAC and keeps it open. Inactivation of endogenous CyPD by siRNA or its inhibition causes the detachment of hexokinase II from the mitochondria and facilitates BAX-mediated apoptosis [119].

In another study, based on immunoprecipitation data, the authors suggested that CyPD interacts with BCL-2 and enhances its inhibitory effect on the *t*BID-induced release of cytochrome *c* from mitochondria [120]. Although in both cases it is hard to explain a spatial interaction between matrix protein CyPD and proteins that reside at the OMM, it can apparently be carried out at contact sites between the IMM and the OMM.

Interestingly, a BH3 mimetic ABT-737 in addition to its ability to deactivate antiapoptotic BCL-2 family proteins, sensitizes curcumin-induced anti-melanoma cell activity by facilitating MPTP-mediated cell death [121]. These data clearly demonstrate an interplay between the two main pathways of OMM permeabilization and should be taken into consideration when analyzing the mechanisms of possible regulation of OMM permeabilization.

Although it is well documented that the main reason for myocardial infarction by ischemia and reperfusion is MPT, recent studies point to the involvement of pro-apoptotic BCL-2 family proteins. Genetic deletion of BAX, or both BAX and BAK, in mice, reduces infarct size following ischemia/reperfusion. Similarly, in BAK-deficient mice and isolated cardiomyocytes, the size of the infarct was markedly reduced, which is caused by the inhibition of necrosis. These observations suggest that BAK may be a therapeutic target to cure the consequences of ischemia/reperfusion [122]. How BAK contributes to MPT-mediated heart deterioration needs to be further investigated.

7. Mitochondrial membrane permeabilization and other cell death modalities

In addition to apoptosis and necrosis, other modes of programmed cell death (PCD) also involve mitochondria. During pyroptosis, cleavage of the Gasdermin E (GSDME) protein by caspase-3 liberates the GSDME-N domain, which forms pores in the plasma membrane triggering this form of cell death. Interestingly, the cleavage product GSDME-N has been shown to be able to permeabilize the OMM with the release of cytochrome *c* and subsequent formation of the apoptosome. The mitochondria-activated caspase cascade is significantly suppressed in cells lacking Gasdermin E compared to wild-type cells. The pore-forming capacity is abrogated in cells with mutated Gasdermin E [123]. Mitochondrial damage is independent of the BCL-2 family proteins but requires GSDMD-NT binding to cardiolipin. Silencing of cardiolipin synthase or the scramblase that transfers cardiolipin to the OMM suppresses mitochondrial damage, pyroptosis, and inflammatory cytokine release [124]. Recently it has been shown that the oxidation of cardiolipin by ROS derived from Complex II of the mitochondrial respiratory chain, and its redistribution from the IMM to the OMM, facilitates GSDMD-N-mediated pore formation [125]. It also has been suggested that GSDMD-mediated formation of the pore in the OMM releases mitochondrially produced ROS, promoting a switch to RIPK1/RIPK3/MLKL-dependent necroptosis [126]. This example serves as one of the proofs of the ability of mitochondria to be a switch between various modes of cell death.

Another mode of PCD, necroptosis, depends on receptor-interacting serine/threonine-protein kinase 3 (RIPK3) and mixed lineage kinase domain-like protein (MLKL). Although the role of mitochondria in necroptosis needs to be further investigated, there are some indications that MMP can regulate this process. Thus, cells or mice deficient in BAX/BAK or CyPD, a protein that regulates MPTP opening, were shown to be resistant to cell death induced by necroptotic mediators. The authors concluded that necroptotic cell death requires oligomerization of BAX/BAK and that this oligomerization facilitates MPTP opening [127]. Another study demonstrates that in acute lymphoblastic leukemia (ALL) cells in which caspase-8 is either inhibited by pan-caspase inhibitor, zVAD.fmk, or its expression is blocked, Smac mimetic BV6 and dexamethasone collaborate to trigger necroptosis, which can be prevented by silencing or pharmacological inhibition of RIPK3 or MLKL. BV6/Dexa treatment was shown to hyperpolarize the IMM followed by loss of MMP, stimulation of ROS production, and subsequent activation of BAK. Knockdown of *BAK* delayed cell death while scavenging ROS prevented BAK activation. The authors conclude that mitochondria may serve as an amplification step in BV6/Dexa-induced necroptosis [128].

8. OMM permeabilization and diseases

One of the hallmarks of cancer cells is their resistance to cell death and antiproliferative signals [129]. Evading apoptosis facilitates tumor development, whereas stimulation of apoptosis (or other forms of PCD) is a promising strategy for tumor cell elimination. Cell death stimulation by targeting mitochondria and OMM permeabilization is important when it comes to cancer cells, as it can help to overcome tumor cell resistance to treatment. However, tumor cells develop various strategies to avoid death. In cancer cells, the ratio between pro- and anti-apoptotic proteins is shifted towards anti-apoptotic proteins, due to the upregulation of anti-apoptotic BCL-2 proteins to ensure cancer cell survival, growth, and resistance to various anti-cancer treatments. As mentioned above, cytosolic BAX upon activation is translocated to the mitochondria triggering mitochondrial dysfunction and apoptosis. Interestingly, the formation of dimer inactivates BAX due to autoinhibition. The autoinhibited BAX dimer dissociates to BAX monomers before BAX can be activated [130]. The intensity of apoptosis can therefore be regulated by the content of dimeric conformation of cytosolic BAX. Chemical modulation of the cytosolic BAX homodimer potentiates BAX activation and apoptosis [131]. BDM19, a small-molecule modulator, binds and activates cytosolic BAX dimers and can trigger apoptosis in tumor cells either alone or in combination with Navitoclax, an inhibitor of BCL-2/BCL-X_L.

Resistance to tumor growth is due to the activity of the transcription factor p53. This protein acts as a tumor suppressor by inducing growth arrest, apoptosis, senescence, and inhibition of angiogenesis. However,

in many tumors, p53 is either mutated or poorly expressed. In such a situation, the efficiency of DNA-damaging antitumor agents declines, as these cells lack a p53-mediated apoptotic response. In the last two decades, compounds under the general name mitocans (MITOchondria and CANcer) have appeared. One of these compounds, alpha-tocopheryl succinate (α-TOS), was shown to successfully eliminate human colon carcinoma HCT116 and osteosarcoma U2OS cells by triggering OMM permeabilization under hypoxic conditions, which usually makes tumor cells resistant to treatment [132]. α-TOS kills tumor cells independently of the status of oncogene N-MYC [133]. α-TOS was shown to stimulate ROS production in the mitochondrial respiratory chain through the suppression of Complex II, followed by activation of BAX [134]. α-TOS not only triggers ROS production but also stimulates rapid Ca^{2+} entry into the cells and its subsequent uptake by mitochondria, which is a prerequisite step for MPT induction [135]. Tagging α-TOS with triphenylphosphonium significantly (with IC_{50} lower by up to two orders of magnitude) enhances its cytotoxic effects on cancer cells [136].

Alteration of the permeability of the OMM by targeting pore-forming proteins plays an important role in disease development. Cardiac fibrosis is a serious disorder leading to chronic cardiac remodeling and dysfunction. It is caused by dysregulation of fatty acid β-oxidation (FAO). Overexpression of VDAC1, which serves as a long-chain fatty acid transporter, improves cardiac fibroblast activation initiated by transforming growth factor beta 1 (TGFβ1) while silencing of VDAC1 results in the opposite effect. In vivo, overexpression of VDAC1 in myofibroblasts or treatment mice with the VDAC1-derived R-Tf-D-LP4 peptide significantly alleviates transverse aortic constriction (TAC)-induced cardiac fibrosis and rescues cardiac function in mice [137]. In addition to forming large pores in OMM through oligomerization, VDAC1 can also hetero-oligomerize with various proteins, such as Aβ peptide, α-synuclein (αSyn), tau, polyglutamine, and superoxide dismutase, which may result in various diseases [138,139]. For example, oligomerization of VDAC1 with Aβ peptide is associated with Alzheimer's disease and the induction of apoptosis [138]. Recent studies have introduced several compounds with the capacity to prevent VDAC1 oligomerization [140]. The benefits of these and other molecules as effective therapeutic interventions against various neurodegenerative diseases are worthy of investigation and are currently being explored in animal models.

For many years, mitochondrial outer membrane permeabilization (MOMP) was attributed to the initiation of cell death, either apoptotic or necrotic, especially when MOMP is due to MPTP opening and is accompanied by a severe drop in ATP content. In contrast, partial permeabilization of OMM, leading to limited caspase activation, which is insufficient to induce cell death, can trigger various consequences. In addition to the generation of drug-tolerant persister cells mentioned above [89], MOMP occurring in a subpopulation of mitochondria (minority MOMP, miMOMP, not sufficient for apoptosis induction), causes cellular senescence [141]. The formation of macropores enables the release of mitochondrial mtDNA into the cytosol. Once in the cytosol, mtDNA activates the GAS-STING pathway, a major regulator of the senescence-associated secretory phenotype (SASP). Inhibition of MOMP in vivo was found to decrease inflammatory markers and improve the health span of aged mice. In case of inadequate capacity of caspase activation to initiate cell death, the limited caspase activity might cause DNA damage leading to genomic instability, cellular transformation, and tumorigenesis [142]. Thus, in contrast to its well-known ability to initiate cell death, MOMP may also have an oncogenic potential and the fate of the cell will be regulated by the extent of MOMP.

Many lines of evidence suggest that mitochondria play a key role in neurodegenerative diseases caused by aging. Oxidative stress and mutations in mitochondrial DNA both contribute to mitochondrial deterioration [143]. Under these circumstances, antioxidants targeted to mitochondria may have a beneficial effect. Various antioxidants tagged to triphenylphosphonium have turned out to be an effective weapon against various manifestations of aging [144–146].

9. Conclusions

MOMP is a decisive factor in cell fate, leading to various neurodegenerative diseases, particularly related to aging. MOMP results in the release of various intermembrane space proteins capable of triggering apoptotic cell death. Purposeful targeting of mitochondria aiming to permeabilize the OMM stimulates apoptotic cell death and can be regarded as a promising strategy for tumor cell elimination. MOMP is a complex process that depends not only on the proteins that form the pore but also on the lipid environment. Moreover, oxygen radicals facilitate MOMP through lipid and/or protein oxidation. Studies carried out during the last decade have revealed multiple mechanisms and consequences of OMM permeabilization, not restricted to cell death only. Undoubtedly, work aiming at studying possible pathways regulating the integrity of the outer membrane will contribute to the development of new strategies in the fight against numerous mitochondrial diseases.

Disclosures

None.

Sources of funding

This work was supported by a grant from the non-commercial organization “The Russian Science Foundation” (23-74-30006). The work in the authors' laboratories is supported (to BZ) by grants from the Swedish Cancer Society (22 2013) and Stockholm Cancer Society (181301).

CRediT authorship contribution statement

Maria A. Yapryntseva: Writing – original draft, Visualization, Validation, Formal analysis, Data curation. **Boris Zhivotovsky:** Writing – review & editing, Funding acquisition, Conceptualization. **Vladimir Gogvadze:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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