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The mitochondrial permeability transition pore and its involvement in cell death and in disease pathogenesis

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Abstract Current research on the mitochondrial permeability transition pore (PTP) and its role in cell death faces a paradox. Initially considered as an *in vitro* artifact of little pathophysiological relevance, in recent years the PTP has received considerable attention as a potential mechanism for the execution of cell death. The recent successful use of PTP desensitizers in several disease paradigms leaves little doubt about its relevance in pathophysiology; and emerging findings that link the PTP to key cellular signalling pathways are increasing the interest on the pore as a pharmacological target. Yet, recent genetic data have challenged popular views on the molecular nature of the PTP, and called into question many early conclusions about its structure. Here we review basic concepts about PTP structure, function and regulation within the framework of intracellular death signalling, and its role in disease pathogenesis.

Keywords Apoptosis · Mitochondria · Permeability transition pore

1 Introduction

Mitochondria have crucial roles in diverse cellular functions, such as energy production, modulation of redox status, osmotic regulation, Ca^{2+} homeostasis, inter-organelle communication, cell proliferation and senescence, and cell responses to a multiplicity of physiological and genetic stresses. They also orchestrate a wide number of signals to determine cell commitment to death or survival [1–7]. Extensive investigation in the last decades is making clear that these biochemical routines work as an integrated system. However, given the complexity of these intertwined signaling networks, their functional and molecular interplay is still the matter of intense investigation. Most of these processes are dynamic, and the same biochemical devices can be used for different and sometimes antinomic biological operations, possibly in different subcellular locations. For instance, mitochondria contribute to cellular Ca^{2+} level regulation by coupling and coordinating mitochondrial and endoplasmic reticulum Ca^{2+} fluxes, so that Ca^{2+} signals may be defined by the spatial organization of mitochondrial populations within cells [8].

Mitochondria are obligate participants in intrinsic apoptotic signaling, and play important roles also in extrinsic, receptor-mediated apoptosis and in non-apoptotic forms of cell death [1, 5, 6, 9, 10]. When a stress stimulus tips the death/survival balance towards a lethal outcome, several changes affect mitochondrial physiology and ultrastructure [7, 11]. Depending on the intensity and persistence of the stimulus, these alterations may drive the cell to a point of no return in its death path, eventually leading to the release of proteins that acquire key apoptogenic functions, such as cytochrome *c* (cyt *c*; [12]), apoptosis inducing factor (AIF; [13]), endonuclease G (Endo G; [14]), high temperature requirement A2 (HtrA2/Omi; [15]), and second mitochondria derived activator of caspase/direct IAP binding protein with low pI (SMAC/Diablo; [16]). Since the size of these factors largely exceeds the pore diameter of outer mitochondrial membrane (OMM) channels, some alternative form of OMM permeabilization is mandatory for their release.

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Several models were proposed to explain OMM permeabilization. These models are not necessarily mutually exclusive, and the possibility exists that different mechanisms, or different combinations of subroutines, may cause the release of intermembrane space proteins in different apoptotic conditions and cell types. On the whole, the main mechanisms can be summarized as the direct OMM permeabilization model and the permeability transition (PT) model. In the former, proapoptotic Bcl-2 family proteins such as Bax and Bak promote, directly or indirectly, the opening of pores on the OMM that are large enough to allow the channelling of apoptogenic proteins. In the latter, rupture of the OMM and release of the intermembrane space components follow the opening of an inner membrane channel termed the PT pore (PTP). A prolonged PTP opening, by eliciting inner mitochondrial membrane (IMM) depolarization and matrix swelling, would lead to cristae unfolding and subsequently to breaches in the OMM. This review will focus on the analysis of the PTP, of its regulation and of its involvement in cell death and in disease. The direct OMM permeabilization model will also be analyzed in correlation with PTP and cell death.

2 The mitochondrial permeability transition

2.1 General features

The inner mitochondrial membrane (IMM) possesses an intrinsically low permeability to ions and solutes, whose fluxes are tightly regulated by a set of channels and transporters [17]. Charge separation across the IMM generates a proton electrochemical potential difference (Δp) whose major component is the membrane potential difference ($\Delta\psi_m$, negative inside). The Δp is essential to store the energy required for the synthesis of more than 90% of the cellular ATP by the F_0F_1 ATP synthase [18].

The mitochondrial PT can be defined as a sudden increase of IMM permeability to solutes with molecular masses up to 1500 Da, and is due to the opening of a voltage- and Ca^{2+}-dependent, cyclosporin A (CsA)-sensitive, high-conductance channel [19–21]. In its fully open state the apparent diameter of the PTP is 3 nm, and the pore open–closed transitions are strictly regulated by a number of effectors. The PT and its association with Ca^{2+} overload and with large amplitude swelling of mitochondria [22] was initially thought to result from a damage to the IMM due to the production of lysophospholipids by a mitochondrial phospholipase (reviewed by [23]). Later on, several studies [24, 25] proposed what is the present consensus model: mitochondrial PT is originated by a unique supramolecular complex, the PTP, composed or regulated by components of all mitochondrial compartments [20, 26]. The molecular composition of the PTP could also not to be fixed, but rather dynamically regulated by a variety of stimuli and conditions [27]. An alternative view postulates that the PTP forms by aggregation of mitochondrial membrane proteins damaged by diverse stresses. Clustering of these misfolded protein would be blocked by chaperone-like molecules. When protein clusters exceed a certain threshold, they would overcome the effect of chaperones and cause opening of unregulated pores [28].

2.2 Consequences of pore opening

The primary consequence of a prolonged PTP opening is mitochondrial depolarization due to equilibration of the proton gradient, which may be followed by respiratory inhibition, as matrix pyridine nucleotides (PN) are lost [29, 30]. Equilibration across the IMM of ions and of solutes with molecular masses below the pore size induces massive release of the Ca^{2+} stored in the matrix and extensive swelling of mitochondria, given the colloidal osmotic pressure exerted by the high concentration of matrix proteins. As a consequence, the unrestricted cristae unfolding causes breaches in the OMM and release of intermembrane proteins.

It should be stressed that for individual mitochondria the PT is an all-or-nothing phenomenon. In a cell, a subpopulation of mitochondria may have a lower threshold for opening (e.g. those spatially closer to the triggering signal) and therefore open the PTP first. Ca^{2+} or other diffusible signals released by these mitochondria might then propagate a wave of PTP openings that eventually culminate in the spreading of the PT to the whole mitochondrial population [26, 31, 32].

Transient PTP openings, recorded electrophysiologically as conductance "flickerings", are not associated with a catastrophic permeability transition and suggest that the PTP has physiological roles unrelated to death stimuli. These PTP functions might encompass matrix volume and pH regulation, redox equilibrium, protein import [33], and a fast Ca^{2+} release mechanism. The latter would be regulated by matrix [Ca^{2+}] fluctuations, resulting in a dynamic steady-state distribution of the mitochondrial populations with open and closed pores [34–36]. Consistent with this idea, transient PTP openings eventually induce entry of radiolabelled sucrose in all mitochondria even for very low values of matrix [Ca^{2+}] [37]. Moreover, transient PTP openings allow PN funnelling in both directions across the IMM. In the matrix of adrenal cortex cell mitochondria, PNs take part in steroidogenesis, leading to the 11-β-hydroxylation of deoxycorticosterone [38–40], whereas a Ca^{2+}-dependent release of matrix PN into the cytosol [29] may support DNA repair by poly-ADP ribose polymerase [41]. Importantly, temporary PTP openings might also contribute to death signalling through release of cyt *c* [37]. The mechanism would be recruitment

of pro-apoptotic Bcl-2 family members onto mitochondria, providing a permeability pathway for cyt *c* [42].

2.3 Molecular nature of the PTP

A passionate debate surrounds the molecular composition of the PTP, which at presently remains an unsolved conundrum. Nonetheless, based on partial purification by a variety of methods, a restricted set of proteins was proposed to take part in the PTP. These include: the IMM adenine nucleotide translocator (ANT) [33]; the large and unselective OMM voltage-dependent anion channel (VDAC) [43, 44]; and the matrix cyclophilin D (CyP-D), a mitochondrial member of the cyclophilin family that is the target of the desensitizing effects of CsA on the PTP [45].

Additional proteins that may play a regulatory role, but are generally not considered as part of the pore itself, are both antiapoptotic and proapoptotic Bcl-2 family members on the OMM [46, 47]; mitochondrial creatine kinase (MtCK), which shuttles high energy phosphate groups in the intermembrane space of muscle and heart mitochondria [48]; mitochondrial hexokinases (HK), which catalyze the first step of glycolysis and are associated to the cytosolic side of the OMM [49, 50]; and the OMM peripheral benzodiazepine receptor (PBR), known to promote the transport of cholesterol into the matrix during steroidogenesis [21]. Some of these proteins will be further discussed below in the frame of the cross-talk between PTP regulation and cell death signalling.

An unambiguous *in vitro* reconstitution of the PTP would greatly help to unravel its molecular structure, but several problems must be tackled at the same time: the mitochondrial membrane proteins must be highly purified and maintain their activity all along purification; they must be correctly inserted in sealed liposomes, to which they should confer properties consistent with those displayed by the PTP in isolated mitochondria; and the detection method must be sensitive enough to allow measurements of pore opening in minute amounts of purified material [26]. A complementary and more telling approach is to knock out the genes of suspected PTP components, and then test PTP properties in mitochondria isolated from the mutant cells and organisms. So far, this technique has been used for the ANT [51], CyP D [52–55] and VDAC1 [56], and altogether the obtained data do not support the idea that the PTP is composed by either of these proteins. Further, mitochondria from the anoxia-tolerant brine shrimp *Artemia franciscana* do not undergo a PT despite a remarkable Ca^{2+} uptake capacity and the presence of ANT, VDAC and CyP-D [57].

The major points concerning the role played by ANT, VDAC and Cyp-D in PTP will be summarized here; more information can be found in recent reviews [21, 58].

2.3.1 Adenine nucleotide translocator

The PTP is strikingly modulated by ligands of the ANT. Atractylate, which inhibits the ANT stabilizing it in the "c" conformation, favors PTP opening while bongkrekate, which inhibits the ANT stabilizing it in the "m" conformation, favors PTP closure [59]. These findings led to the suggestion that the PTP may be directly formed by the ANT. However, transition of the translocase from the "m" to the "c" conformation is accompanied by a large decrease of the surface potential [60]. This might easily explain pore opening by atractylate and pore closure by bongkrekate within the framework of the PTP voltage dependence independently of a direct ANT involvement [17].

Unequivocal evidence that the ANT is not essential for PTP formation was obtained in a detailed analysis of liver mitochondria prepared from mice lacking both ANT isoforms. The $ANT^{-/-}$ mitochondria underwent a Ca^{2+}- and oxidant-dependent, CsA-sensitive PT with matrix swelling [51], indicating that the ANT is neither the binding partner of CyP-D nor the site of action of oxidants. The only difference between wild type and ANT null mitochondria was that the latter required a larger Ca^{2+} load to trigger the PT and had expectedly lost sensitivity to ligands of the ANT (atractylate and ADP, which like bongkrekate inhibits the pore) [51].

It has been suggested that ANT deficiency is compensated in the IMM by other ANT-like channels of the same mitochondrial carrier family, and that the relative contribution of ANT-containing and ANT-less forms to the PTP might depend on specific conditions [61]; however, these ANT-like molecules should be able to promote a CsA-sensitive PT and yet not respond to atractylate and ADP.

2.3.2 Voltage-dependent anion channel

The earliest indication that the OMM could be involved in the PT was the finding that swelling induced by sulfhydryl reagents is not observed in mitoplasts, *i.e.* mitochondrial preparations lacking the OMM [62]. A number of findings support the hypothesis that the relevant OMM component of the PTP is VDAC. Indeed, purified VDAC forms channels with a pore diameter of 2.5–3.0 nM that possess electrophysiological properties strikingly similar to those of the PTP [63, 64], and VDAC is modulated by many factors that also affect the PTP, such as NADH, Ca^{2+}, glutamate and binding of hexokinase [65–69]. It should be noted that these intriguing analogies do not represent a proof of mechanism, and that the PTP of mitochondria prepared from $VDAC1^{-/-}$ mice was indistinguishable from the PTP of strain-matched wild-type mitochondria [56]. However, in mammals there are three VDAC isoforms, and in the absence of VDAC1 its PTP-forming activity might be compensated by VDAC2 and/or VDAC3. Unfortunately, this cannot be fully tested

with genetic approaches, as elimination of VDAC2 is embryonically lethal [70].

2.3.3 Cyclophilin D

The PTP is sensitized to inducing agents by CyP-D, a matrix peptidyl-prolyl *cis-trans* isomerase that appears to modulate the PTP affinity for Ca^{2+} [71, 72]. CyP-D is inhibited by its high affinity ligand CsA in the same range of concentrations that desensitize the PTP [45, 71, 73].

Several groups inactivated the *Ppif* gene encoding CyP-D in the mouse [52–55]. In all studies (i) the Ca^{2+}-dependent PT still took place; (ii) CyP-D ablation increased the threshold Ca^{2+} load required to open the pore (which became identical to that of CsA-treated, strain-matched wild type mitochondria); (iii) CsA had no effects on the PT, which instead retained its normal response to other inhibitors including Ubiquinone 0. Further, the PTP was sensitized to oxidative stress [53]. These findings conclusively demonstrate that CyP-D is a regulator, but not a component, of the PTP, and that the effect of CsA is best described as desensitization, rather than inhibition, of the pore.

2.4 Regulation of the PTP

PTP regulation by pathophysiological effectors has been the subject of many reviews over the years [17, 19–21, 23, 26, 74–77], and the reader is referred to these for detailed information. Here we have singled out points that are particularly important for an understanding of PTP regulation in pathophysiology, and recent contributions that may not be found in previous review articles.

2.4.1 PTP regulation by the proton gradient (Δp)

A key feature of the PTP is its regulation by both components of the Δp, matrix pH and the IMM potential difference. The optimum for pore opening is observed at matrix pH 7.4 [78], whereas the opening probability sharply decreases both by lowering matrix pH (through reversible protonation of histidyl residues) and by increasing it (through an unknown mechanism) [79].

The PTP is voltage-dependent, and a high (inside-negative) membrane potential stabilizes it in the closed conformation [78]. A variety of pathophysiological effectors can move the threshold voltage at which opening occurs either closer to the resting potential (PTP inducers), or away from the resting potential (PTP inhibitors) [17]. We have postulated the existence of a sensor that translates the changes of both (i) the transmembrane voltage (through redox-sensitive sites affected by electron flux) and of (ii) the surface potential (through a set of charged residues) into changes of the PTP open probability.

(i) At least three different sites are regulated by redox equilibria: one is modulated by matrix PN, with oxidation promoting PTP opening [80, 81]; another by the GSH pool through vicinal protein thiols [81, 82], and the last is activated by the thiol oxidant copper-*o*-phenanthroline [83]. Pore modulation by these redox-sensitive sites probably explains the inducing effects of p66Shc, which directly oxidizes cyt *c* to produce superoxide anions and to induce PTP-dependent cell death [84].

(ii) Regulation through the surface potential is supported by observations that amphipathic anions such as arachidonic acid favour the PT, whereas polycations (like spermine), amphipathic cations (sphingosine, trifluoroperazine), and positively charged peptides inhibit the pore. These data imply that the effects of amphipatic compounds depend on their net charge that would affect the PTP voltage sensor [75].

Current evidence indicates that the PTP voltage sensor is regulated by critical arginine residues. Indeed, glyoxals of identical chemical reactivity towards arginines modulate the PTP voltage-dependence in a manner that is entirely consistent with the net charge and hydrogen bonding capacity of the adduct, suggesting that crucial arginines are functionally linked with the opening/closing and voltage sensing mechanisms [85–88].

2.4.2 PTP regulation by Ca^{2+}

In energized mitochondria, Ca^{2+} uptake into the matrix is achieved via the ruthenium-red-sensitive mitochondrial Ca^{2+} uniporter [89, 90] and/or the "rapid-mode" of uptake that activates in response to fast changes of cytosolic Ca^{2+} [91]. Ca^{2+} efflux occurs through the Na^+-Ca^{2+} antiporter [92] that exchanges 3 Na^+ per 1 Ca^{2+} ion [93], Na^+ being then extruded by the H^+-Na^+ exchanger; and through a Na^+-insensitive Ca^{2+} efflux pathway [94] that is inactivated by depolarization [95, 96]. The interplay between the rate of Ca^{2+} influx and efflux tightly modulates the matrix Ca^{2+} content, which is in turn widely considered to be a key factor for regulation of the PTP open-closed transitions [17, 97]. Matrix Ca^{2+} acts as a permissive factor for most pore inducers; its activity can be competitively inhibited by other Me^{2+} ions, such as Mg^{2+}, Sr^{2+} and Mn^{2+}.

The PTP dependence on matrix Ca^{2+} represents somewhat of a paradox, however, because there is no obvious correlation between matrix free [Ca^{2+}] and onset of the PT. Indeed, decreasing [Pi] from 5 mM to 2 mM, increased the apparent threshold for PTP opening from 1.8 μM to 5.0 μM matrix free [Ca^{2+}] in rat brain mitrochondria [98]. Based on

these results, we suspect that the PTP Ca^{2+}-binding sites saturate at very low matrix free [Ca^{2+}], and that PTP opening is not caused by Ca^{2+} overload as such, but by additional factors that still need to be characterized.

2.4.3 Other PTP regulators

Any PTP model must take into account the puzzling fact that the pore is affected by a large variety of unrelated compounds that may inhibit or stimulate opening [23]. Among the inhibitors we find positively charged peptides (e.g. mastoparan, BH3-Bax), some anti-apoptotic proteins (Bcl-2 and Bcl-X_L), proteins involved in antioxidant defences (like catalase, superoxide dismutase and glutathione) and a wide array of small molecules (PK11195, CsA, sanglifehrin A, bongkrekic acid, ADP and ATP, Ubiquinone 0, 4,40-diisothiocyanatostilbene- 2,20-disulfonic acid, Ro 68–3400, and NADH); among the inducers, matrix Ca^{2+}, the GD3 ganglioside, arsenite, pro-oxidants (like *tert*-butylhydroperoxide, diamide, phenylarsine oxide), and atractylates [99].

The molecular targets and mechanisms of actions of some of these molecules have been discussed in the preceding paragraphs, and we must refer the reader to previous reviews for further details [17, 19–21, 23, 26, 74–77].

3 The PTP and cell death regulation

The hypothesis that PTP could have a role in cell death was already proposed nearly 20 years ago [100]. Albeit a rigorous test of whether a PT takes place in organisms is still lacking, a number of experimental findings has supported a PT occurrence in diverse death-promoting conditions, such as hepatocytes subjected to oxidative stress [101], anoxia [102] or treatment with ATP [103], and in cardiomyocytes [104] and isolated hearts [105] exposed to ischemia followed by reperfusion. We also found that arachidonic acid plays a key role in Ca^{2+}-dependent death signalling through activation of the PTP [106, 107]. In addition, several PTP inhibitors (bongkrekate, CsA and its derivatives) were reported to protect from cell death both *in vitro* and *in vivo*. *In vitro*, these inhibitors abolish cyt *c* release and protect different cell types from apoptosis induced by glucocorticoids, neurotoxins and tumor necrosis factor α (TNFα) [108–111]; *in vivo*, CsA is effective in a variety of settings that are covered more in detail below (see also [21]).

3.1 Cyclophilin D and cell death

As CsA delays PTP opening by binding to CyP-D, one would expect that CyP-D favors PT and cell death. However, CyP-D overexpression reportedly *desensitizes* cells from apoptosis induced by the overexpression of caspase-8 (but not Bax) or by exposure to arsenic trioxide [112, 113]. Thus, CyP-D may play a role as a survival molecule, possibly acting on target(s) other than the PTP. This dual function of CyP-D could lead to a balance of its pro- and anti-apoptotic effects in animals lacking CyP-D. Consistently, $Ppif^{-/-}$ mice clearly demonstrate that CyP-D is dispensable for embryonic development and viability of adult animals. Various cell types isolated from CyP-D-deficient mice normally undergo apoptosis in response to various stimuli, including etoposide, staurosporine, and TNFα, and tBID or Bax caused cyt *c* release from isolated mitochondria [52, 54, 55]. Instead, CyP-D-deficient MEFs and hepatocytes are significantly more resistant to necrosis induced by a Ca^{2+} ionophore (A23187) or by H_2O_2, and cardiac ischemia/reperfusion injury causes less damage in $Ppif^{-/-}$ animals, similarly to what is observed following treatment of wild-type animals with CsA [52, 54]. These studies were used to conclude that the PTP only plays a role in necrotic, rather than apoptotic, responses [114, 115]. However, it must be highlighted that results obtained on $Ppif^{-/-}$ mice or cells can only be interpreted in terms of the role of CyP-D, not of the PTP, in cell death. In addition, the PTP could be involved in apoptosis triggered by stimuli that differ from those utilized in these works. Indeed, the inference that PTP opening cannot take place because CyP-D is absent has not been documented *in vivo*. It remains therefore undetermined what is the physiological role played by the PTP in the different cell death pathways.

3.2 PT-dependent permeabilization of the OMM

An increase in the permeability of the OMM is central to cell death, as it is mandatory for the release of proteins with key functions in cell dismantling (Fig. 1). As stated above, different models compete to explain how the OMM permeabilizes during cell death induction, postulating either the exclusive involvement of outer membrane components or the opening of the PTP on the inner membrane. These models are not mutually exclusive, and the choice among them could depend on several variables, including the cell type, the stress stimulus and the energetic conditions of the cell, and the same molecules could be involved in different OMM rupture paradigms. It is however important to underline that PTP openings can induce rupture of the OMM only as a result of matrix swelling, and therefore cyt *c* and the other apoptogenic molecules do not exit mitochondria through the PTP itself. In the next sections, we will focus on PTP regulators that affect death signalling.

3.2.1 Bcl-2 family proteins

A role of Bcl-2 family proteins as PTP regulators was put forward by several groups. The pro-apoptotic Bax protein

triggers a CsA-inhibitable death in lymphoma cells [116], and a CsA-sensitive PT in isolated mitochondria [68, 117], even though other studies have concluded that Bax-mediated release of cyt *c* is independent of the PTP and occurs without IMM permeabilization [118–120]. Bax (and perhaps Bak and Bid [121]) may induce mitochondrial PT and cyt *c* release by regulating PTP components. For instance, after mitochondrial translocation Bax would form a pore upon interactions with ANT [122]. When ANT and Bax were reconstituted into liposomes or planar lipid bilayers, they formed a bongkrekate- and CsA-sensitive channel with cationic selectivity, whose opening was elicited by the ANT ligand atractylate [122, 123]. Coherently, anti-apoptotic Bcl-2 family members (Bcl-2 and Bcl-X_L) were reported to antagonize the PT and to inhibit a reconstituted PTP-like complex [122]. It was proposed that Bcl-2 would stimulate the ATP/ADP translocator function and abolish the pore function of ANT, whereas the pro-apoptotic Bax would act in the opposite way [33]. Bax and Bcl-2/X_L could also interact with VDAC to open or close it, respectively, suggesting that they modulate the PTP via interaction with VDAC [124–126]. However, others failed to detect interactions between components of the PTP and Bcl-2 family proteins [127, 128]. Since the role of both ANT and VDAC in PTP assembly is still disputed (see above), these results must be considered with extreme caution when trying to establish a Bcl-2 family role on PTP modulation.

Our laboratory has recently described a Bcl-2-binding molecule, EM20-25, that is able to induce PTP opening, to disrupt the Bcl-2/Bax interactions *in situ* and to activate apoptosis in Bcl-2-overexpressing cells [129]. It is tempting to speculate that the effects of EM20-25 on the PTP are related to Bcl-2 binding, raising the intriguing possibility that EM20-25 interaction with Bcl-2 plays a mechanistic role in PTP regulation.

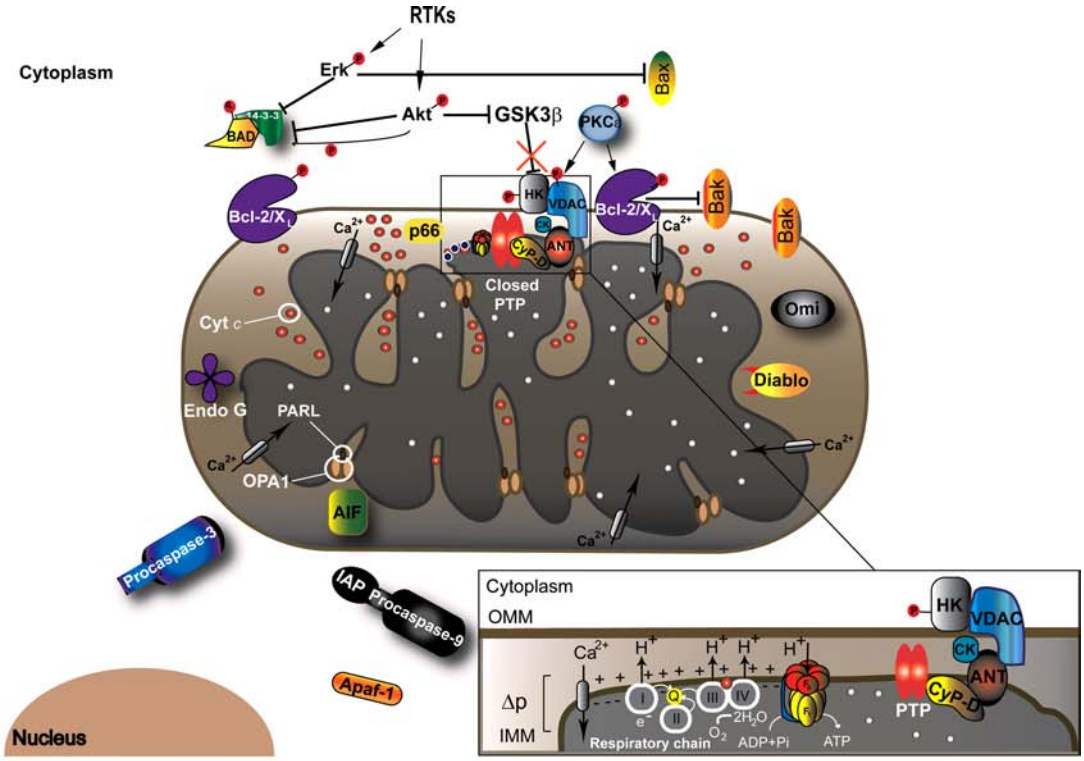
Bcl-2 family members are also localized in ER membranes, where they contribute to the modulation of intracellular Ca^{2+} homeostasis in a complex and subtle interplay with mitochondria [5]. Certain apoptotic stimuli were shown to induce PTP opening by promoting release of Ca^{2+} from endoplasmic reticulum (ER) stores [130]; this Ca^{2+} would be taken up by mitochondria, overloading the matrix and eventually prompting the PT [4, 131–133]. In this context, the PTP could integrate different Ca^{2+} signals, switching their output towards death or survival in a fashion dependent on the activity of Bcl-2 family members both on mitochondria and on the ER [11, 134, 135]. Waves of CsA-sensitive mitochondrial depolarization and Ca^{2+} release would propagate through the cell, resulting in cyt *c* release and apoptosis [32, 136].

3.2.2 (De)phosphorylation reactions

Dynamic networks of kinase/phosphatase pathways, which are known to transmit localized signals to subcellular compartments, could regulate the PT, either directly or through intermediate adaptors [137, 138]. For instance, mitochondrial Ca^{2+} homeostasis, and therefore the threshold for PTP opening, can be modulated by the stress-activated p38 MAP kinase through phosphorylation of the Ca^{2+} uniporter [139]. PTP inhibition might involve mitogen-activated protein kinase kinase 6 [140], whereas the stress-activated kinase Jnk inhibits Bcl-2/X_L and promotes the release of cyt *c* and of Smac/DIABLO and a decrease in $\Delta\psi_m$ [141–144]. Cyt *c* oxidase, whose activity could affect the PT through modulation of the membrane potential, is phosphorylated at two different sites, with opposite effects on its activity [145–147].

Possible direct PTP modulators include PKCδ and PKCε. Activated PKCδ translocates onto mitochondria in several cell models and in a variety of apoptogenic settings, where it triggers IMM depolarization, release of cyt *c* and the subsequent apoptosis induction through unknown mechanisms

Fig. 1 Synopsis of the main molecules involved in mitochondrial membrane permeabilization during cell death induction and of their network of interactions. (A) The mitochondrion in a healthy cell. Several cytosolic kinase pathways regulate mitochondrial permeability: following growth factor binding, receptor tyrosine kinases (RTKs) activate Erk MAPK and Akt, which (i) contribute to keep some apoptogenic Bcl-2 family proteins away from the OMM and (ii) inactivate GSK3β, thus allowing hexokinase (HK) interaction with VDAC on the OMM. Several pro-apoptotic molecules (cyt *c*, Endo G, Omi/HtrA2, Smac/Diablo) are segregated in the intermembrane mitochondrial space (IMS), where they display diverse, non-apoptogenic functions. Caspases are kept in their zymogenic, inactive form in the cytosol. The respiratory chain is working in the IMM to produce the proton electrochemical gradient, which is then used to make ATP by the FoF1 ATPase. Respiratory activity is stimulated by a regulated Ca^{2+} influx into the matrix (inset). The PTP is depicted in the closed state, and several putative PTP regulators/components are indicated: HK/VDAC on the OMM, creatine kinase (CK) in the IMS, ANT in the IMM and cyclophilin-D in the matrix. (B) The mitochondrion exposed to death stimuli. A plethora of stress signals converges on mitochondria to induce the release of the IMS-stored apoptogenic proteins. Lack of growth factors abrogates "survival kinase" activity, favouring relocalization of pro-apoptotic Bcl-2 family proteins on the OMM and HK detachment from VDAC. The balance between pro-and anti-apoptotic Bcl-2 proteins on the OMM is tipped towards death induction. This is achieved either through changes of the OMM itself, allowing release of cyt *c* and of other death inducers through proteinaceous pores (e.g. Bax channels), or through opening of the PTP in the IMM and consequent matrix swelling, leading to protein release in the cytosol. PARL and Opa1 regulate cristae remodelling and facilitate mitochondrial protein release. Once released, cyt *c* induces apoptosome aggregation and caspase activation; Endo G and AIF target DNA; Omi/HtrA2 and Smac/Diablo inhibit a class of apoptosis inhibitors (IAPs). Inset: the electrochemical gradient across the IMM is dissipated and oxidative phosphorylation is disrupted. p66Shc oxidizes cyt *c* and contributes to PTP opening. Protein composition of the PTP remains unsolved


RTKs
Cytoplasm
Erk
Akt
GSK3β
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Bax
BAD
14-3-3
Bcl-2/X$_L$
HK
VDAC
CK
ANT
CyP-D
p66
Bak
Closed PTP
Omi
Diablo
Cyt c
Endo G
PARL
OPA1
AIF
Ca^{2+}
Procaspase-3
IAP
Procaspase-9
Apaf-1
Nucleus
OMM
IMM
Δp
Respiratory chain
ADP+Pi
ATP
PTP
(A)
BH3
Open PTP
caspase-3
caspase-9
Apaf-1
(B)

Fig. 1

[148–150]; PKCε was reported to prevent PT in cardiomyocytes by phosphorylating VDAC [151].

In addition, these and/or other kinase pathways might impinge upon PTP regulation by indirect means, mainly interacting with Bcl-2 family members. PKCε activates the anti-apoptotic Bcl-2 and inactivates the pro-apoptotic Bad in several cell types and conditions, thereby maintaining mitochondrial membrane potential and preventing cyt *c* release [138, 152]. Garlid and coworkers have proposed that two pools of PKCε participate in inhibition of the PTP, one pool acting directly on the pore after activation by ROS and the other acting on the K_{ATP} channel, which would in turn inhibit the PTP [153].

Bad is inactivated also by PKA, extracellular signal-regulated kinases (ERKs) and Akt [138, 154–156]. The effect of ERKs on mitochondrial death pathways seems however more complex, as they have also been implicated in the apoptogenic translocation of Bax to mitochondria [157]. Conversely, growth factor-activated Akt exerts a coherent survival action at multiple levels, either in the cytosol or following translocation into mitochondria. Among the actions that may relate more directly to the PTP, Akt was found to influence the expression of putative PTP components or regulators [158] and to phosphorylate glycogen synthase kinase 3β (GSK3β) [159]. Various protective pathways could impinge on GSK3β, whose inhibition would result in a decreased probability of PTP opening [160].

3.2.3 Hexokinase

Further evidence indicates that Akt might be a central knot in the network of cross-regulations between energy metabolism, mitochondrial membrane integrity and cell death through its regulation of hexokinase (HK). The mammalian HK isoforms HKI and HKII bind to VDAC on the OMM and catalyse the first glycolytic step by using ATP to convert glucose into glucose-6-phosphate. The dynamic movement of HK between mitochondrial and cytosolic compartments is regulated by cycles of association/dissociation with VDAC [161] and influenced by a variety of factors (ATP, divalent cations, P_i, intracellular pH and glucose-6-phosphate), suggesting that HKs have specific functions of metabolic sensing: as HKs on the OMM selectively utilize intramitochondrial ATP for glucose phosphorylation, they directly couple glycolysis to oxidative phosphorylation.

HK metabolic functions are connected to the regulation of mitochondrial membrane permeability. In a reconstituted system, an enhanced association between HK and VDAC correlated with PTP closure and *vice versa* [162]. The HK/VDAC interaction might either propagate a conformational change that alters the conductive properties of the PTP, or prevent the interaction between pro-apoptotic Bcl-2 family members and PTP regulators, such as VDAC itself or other proteins [50]. For instance, mtCK associates with VDAC in the intermembrane space only when HK is not externally bound [162, 163]. mtCK might therefore compete with HK for the modulation of VDAC activity and VDAC–ANT interaction [48, 164].

Akt modulates mitochondrial HK activity in several ways. The interaction between HK and VDAC is abrogated by a GSK3β-dependent phosphorylation of VDAC, and promoted by Akt, which inhibits GSK3β [69]; HK ectopic expression and its association with mitochondria mimics the ability of growth factors and Akt to maintain OMM integrity and to inhibit cyt *c* release and apoptosis [68, 165–168]. In tumors, which usually are highly glycolytic even if oxygen is available (the Warburg effect) [169], mitochondrial HK activity is generally increased [50, 170], and disruption of HK–VDAC binding enhances apoptosis induction [161].

Mitochondrial HKs prevent the apoptogenic activity of Bax [68, 168]. Active Bax/Bak might compete with mitochondrial HKs for a binding site on the OMM. In the absence of growth factors, a prolonged HK dissociation would promote OMM association of the activated Bax/Bak, leading to permeabilization of the OMM. However, HK dissociation from mitochondria was reported to induce OMM permeabilization even in the absence of Bax and Bak, when the majority of apoptotic signals are not effective [168]. As this OMM permeabilization is Ca^{2+}-independent, an additional, Bax/Bak-independent and PTP-independent mechanism might exist by which HK dissociation prompts loss of mitochondrial membrane integrity and apoptosis. Nonetheless, cells doubly deficient for Bax and Bak seem to have a functioning PTP, which would be responsible for cyt *c* release and cell death observed in response to Ca^{2+}-dependent apoptotic stimuli [132].

An alternate model proposes that a prolonged VDAC closure, and not opening, would lead to mitochondrial matrix swelling, OMM rupture, and release of apoptogenic proteins [165, 168, 171, 172], either as a consequence of HK dissociation from mitochondria [165, 173], or following HK/VDAC interaction, as HK inhibits apoptosis by PTP inhibition [161] and decreases the conductivity of purified VDAC reconstituted into planar lipid bilayers [174]. However, these observations are inconsistent with evidence that mitochondrial HKs selectively utilize intramitochondrial ATP, which is funnelled through an open VDAC, to phosphorylate glucose [49].

3.3 PT-independent permeabilization of the OMM

Several PTP-unrelated mechanisms of apoptogenic protein release probably exist (Fig. 1). These would exclusively involve an increase in OMM permeability. A wealth of evidence indicates that mitochondrial depolarization follows cyt *c* release in several types of apoptosis [46, 175], and

that a number of compounds (thiols, gangliosides, peptides) can induce OMM permeabilization in a Ca^{2+}- and CsA-insensitive manner [27]. OMM would permeabilize either by formation of large channels by Bax and/or Bax-related proapoptotic proteins [176], or by association of these proteins with VDAC or ANT to form pores of adequate size [125], or by protein funnelling through pores entirely formed by lipids [177].

According to the hypothesis of a channel composed by Bcl-2-family members, it was shown that several of these proteins resemble the pore forming domain of diphteria toxin and bacterial colicins [178–181], and that they exhibit ion channel activities in synthetic lipid bilayers. These channels display multiconductance levels, are voltage- and pH-dependent, and are poorly ion selective [182–184]. However, in most cases, channel activities were recorded in non-physiological conditions, and their relevance, if any, in apoptosis signalling remains undetermined. It was also shown that recombinant, tetrameric Bax alone was sufficient to release fluorescein-labeled cyt *c* from plain liposomes [176] and from isolated mitochondria in a Bcl-X_L-sensitive fashion [118], whereas it was reported not to be involved in Ca^{2+}-induced PT [185]. In a cell-free system composed either of mitochondria, OMM vesicles or liposomes, permeabilization required a mixture of tBid and Bax, whereas neither protein alone was sufficient, and it was inhibited by Bcl-X_L. Remarkably, permeabilization occurred without the need for IMM or matrix components [186].

Bax or Bak could form the so-called mitochondrial apoptosis-induced channel, MAC. MAC was identified as a high conductance, voltage-independent and slightly cation-selective channel that forms during early apoptosis and putatively releases cyt *c*. MAC is large enough to allow the passage of cyt *c*, which in turn modifies MAC properties in a way consistent with entrance of cyt *c* into the pore. MAC is not detected in cells lacking both Bax and Bak, and its openings are abrogated by overexpression of Bcl-2 [187]. Recombinant active Bax (BaxΔC20) channels have electrophysiological properties that are similar to those of MAC. Unlike PTP opening, MAC formation does not cause loss of OMM integrity or mitochondrial depolarization. However, MAC and PTP might function sequentially to maximize cyt *c* release. In this model, PTP would break the OMM after MAC activation, thus completing the release of apoptogenic factors [11, 188].

BH3-only proteins (e.g. Bid) induce oligomerization of Bax/Bak on the OMM, resulting in Bax activation [189] and OMM permeabilization [127, 190, 191]. Oligomerized Bax on the OMM has been shown to generate high-conductance channels following interaction with ANT (even if the two are located into different mitochondrial membranes; [122]) or VDAC [125]. In this latter study, neither Bax nor VDAC alone could trigger the efflux of cyt *c* from liposomes, but Bax widened the VDAC pore just enough to allow efflux of cyt *c* [120]. The interaction with Bcl-2 family members could modulate the VDAC oligomeric state, suggesting that it is the multimeric form of VDAC that mediates cyt *c* release [44]. In addition, observations that cyt *c* is recruited at the intermembrane space side of VDAC [192], and that anti-VDAC antibodies prevent both Ca^{2+}-induced and Bax-induced cyt *c* release, mitochondrial depolarization and apoptosis [175], whereas VDAC overexpression induces apoptosis [161], argue for a central role of VDAC in OMM permeabilization.

Once on the OMM, Bax might promote the formation of lipidic pores. Other proteins, such as VDAC or molecules involved in mitochondrial fusion/fission (e.g. Drp1 or Mfn2), might facilitate this process by destabilizing the membrane. Bax could interact with Drp1/Mfn2 to destabilize the OMM through mitochondrial fission-like mechanisms [133].

Other observations indicate that the network of Bcl-2 family proteins that impinges upon OMM permeabilization is extremely intricate. Mcl-1 complexes with Bak and suppresses its pore forming activity [193]. The BH3-only proteins PUMA and NOXA, which are expressed in a p53-dependent manner upon DNA damage, were shown to cause OMM permeabilization [194, 195]. Interestingly, cytosolic p53 can directly activate Bax and thereby cause permeabilization of the OMM, although the mechanism of this activation is still unclear [196].

Other investigators used *in vitro* translated proteins to show that Bax, but not Bcl-X_L, could break planar lipid bilayers [177], possibly through lipid-dependent membrane destabilization due to an increase of the local membrane curvature, eventually resulting in the formation of lipidic pores [197].

Caspases could also control mitochondrial membrane permeability. In models of UV-irradiated or staurosporine-treated cells, cyt *c* is released, but IMM damage is only consequent to caspase-dependent events [119]. Similarly, addition of recombinant caspase-3 following Bax/Bak-dependent permeabilization of the OMM caused changes to the IMM and to mitochondrial morphology [198]. Hence, activated caspases might target the permeabilized mitochondria, increasing apoptosis through a positive feedback loop. Caspase-2, which is activated in response to genotoxic stress, can be directly involved in the release of cyt *c* from mitochondria [199]; in isolated mitochondria caspase-2 stimulates mitochondrial release of cyt *c* and of Smac/DIABLO, but not of AIF, independent of several Bcl-2 family proteins [200–202].

3.4 Mitochondria remodelling and cell death

The PTP could also induce cyt *c* release through remodeling of mitochondrial cristae. Tomographic analyses after high-voltage electron microscopy have shown that mitochondrial cristae are pleiomorphic tubular structures connected

through a narrow hose to the intermembrane space at regions called contact junctions [203]. The intra-cristae regions may form a barrier to the free diffusion of proteins, and contain approximately 85% of the total cyt *c* pool, while only 15% is found in the intermembrane space [204]. It follows that two steps could be required for substantial release of cyt *c*: first, cristae remodelling in order to eliminate the diffusion barrier and to redistribute cyt *c* in the intermembrane space; this step is at least partially controlled by PARL and Opa1 [205, 206], two proteins known to regulate mitochondrial fusion [207]; second, cyt *c* exit into the cytosol via either formation of pores (e.g., Bax/Bak channels) or rupture of the OMM (e.g. following matrix swelling). CsA appears to block the first step of cyt *c* release from mitochondria by inhibiting the structural remodeling of cristae [208, 209]. Therefore, the structural reorganization of mitochondria was proposed to be regulated by PTP [58, 210]. In this model, the second step of cyt *c* release would instead be triggered by Bax/Bak channels on the OMM [208].

In several apoptotic settings mitochondrial morphology changes from a tubular networks to a fragmented phenotype, suggesting that mitochondrial fission is also related to cyt *c* release [211]. Accordingly, proteins involved in fission (e.g. hFis1 or DLP1) regulate cyt *c* release and apoptosis [212, 213].

Cyt *c* binds to cardiolipin, a lipid selectively found on IMM, and it was shown that cyt *c* release can be favored by cardiolipin peroxidation [214], a phenomenon that can follow the burst of ROS associated with PTP opening [215], or by Ca^{2+} interaction with cardiolipin [216]. Based on these findings a somewhat different "two step" model of cyt *c* release was proposed where cyt *c* first mobilizes from cardiolipin [215] in association with cristae remodeling (see above and [217]), and is then released in the cytosol through permeabilization of the OMM by Bax-like proteins.

Mitochondrial swelling has been reported to occur following many apoptotic stimuli, including growth factor withdrawal, heat shock, sustained increase in intracellular Ca^{2+} levels, TNFα treatment and ischemia [218]. Nonetheless, it must be pointed out that a matrix volume increase and the subsequent release of intermembrane space proteins could also rely upon PTP-independent mechanisms not associated to a general increase of inner membrane permeability (the high-energy swelling of [219]). In this paradigm, swelling occurs without impairment of mitochondrial function and with maintenance of intracellular ATP levels, which in turn are required for the apoptotic pathway to proceed. In fact, PTP openings are pro-apoptotic when a high intracellular ATP level is maintained, whereas a low ATP level switches the cell death subroutine towards necrosis (e.g. [220–222]).

Mitochondrial volume is controlled by K^+ transport across the IMM, which is regulated by a balance of inward electrophoretic flux with outward electroneutral K^+/H^+ exchange [17]. Indeed, stimulation of net K^+ influx (e.g. by valinomycin or by openers of mitochondrial K_{ATP} channels) induces matrix swelling and cyt *c* release without loss of membrane integrity [223–225]. Intriguingly, K^+ uptake, swelling of mitochondria and cyt *c* release can be inhibited by Bcl-2 and stimulated by tBid [153] possibly through upregulation of the K^+/H^+ exchanger with the ensuing reduction of net influx of K^+ [226, 227].

4 The PTP in pathology

Mitochondria are involved in more than 40 known human diseases. The effects of CsA in treatment have implicated PTP-dependent mitochondrial dysfunction and Ca^{2+} deregulation in many of these conditions (see Table 1), including ischemia-reperfusion (I/R) injury of the heart [30, 228], ischemic and traumatic brain damage [229, 230], muscular dystrophy caused by collagen VI deficiency [231], amyotrophic lateral sclerosis [232], acetaminophen hepatotoxicity [99], hepatocarcinogenesis by 2-acetylaminofluorene [233], and fulminant, death receptor-induced hepatitis [234].

In cardiac I/R injury, overload of matrix Ca^{2+} leads to opening of the PTP, cyt *c* release, and cell death [20]. During ischemia, lactic acidosis prompts the exchange of extracellular Na^+ with cytosolic H^+. The increased cytosolic Na^+ stimulates the plasma membrane Na^+/Ca^{2+} exchanger, resulting in cytosolic Ca^{2+} overload that eventually lead to an augmented matrix Ca^{2+} load. In addition, ATP hydrolysis increases cell P_i [20]. Recovery of respiration in the presence of high intracellular and intramitochondrial $[Ca^{2+}]$ and $[P_i]$ provides ideal circumstances for promoting PTP opening, which would be further favored by overproduction of ROS and recovery of neutral pH. Hence, ischemia *per se* does not appear to cause PTP opening, but it creates the conditions for PTP opening at reperfusion [105]. The partial recovery of mitochondrial function generates an amount of ATP that is sufficient for contraction, but not for relaxation, ultimately resulting in sarcolemmal rupture [21, 37, 235]. Myocyte viability is maintained when PTP opening is prevented not only by CsA, but also by CsA analogues that lack immunosuppressive activity, further supporting the importance of PTP opening in I/R injury [30, 236, 237]. In keeping with this model, mice lacking CyP-D display a reduced susceptibility to ischemic injury [52, 54, 55].

Tumor cells are more resistant to the breakdown of the OMM [238], and PBR, HKII, mtCK, CyP-D, VDAC isoforms and ANT-2 were found to be dysregulated in a number of neoplastic tissues and tumor cell lines [113, 170]. An involvement of an altered PTP opening in cancer is suggested by several pieces of evidence: (i) PTP opening can be both the cause and the consequence of increased ROS unbalance, and part of the amplification loop of apoptosis;

Table 1 Involvement of permeability transition in pathological conditions

System or organ	Disease	References
Cardiac/circulatory system	Ischemia/reperfusion injury	[30, 105, 228, 236, 237]
Nervous system	Ischemic and traumatic brain damage	[229, 230]
	Hyperglycemia	[229, 248]
	Hypoglicemia	[230, 249]
	Trauma	[250, 251]
	Facial motoneuron axotomy	[252]
	Photoreceptor apoptosis	[253]
	Amyotrophic lateral sclerosis	[232]
	Middle cerebral artery occlusion	[55]
Muscle	Muscular dystrophy caused by collagen VI deficiency (mouse)	[231]
	Ullrich congenital muscular dystrophy	[259]
Liver	Acetaminophen toxicity	[99, 254]
	Cholestasis	[243]
	Fulminant, death receptor- or viral-induced hepatitis	[234, 255, 256, 257]
	Hepatitis C virus-induced hepatocyte apoptosis	[244]
	Alcohol-induced damage	[245]
	Acute endotoxemia	[258]
	Graft rejection	[260]
Tumors	Hepatocarcinoma?	[21]
	Hepatocarcinogenesis by 2-acetylaminofluorene	[233]
	Sensitivity/resistance to chemotherapeutics	[239–241]
	Resistance to hypoxia or anoikis	[241]
	Transformed cell apoptosis by jasmonates	[242]

notably, inhibitors of chemotherapy-induced apoptosis include several antioxidant agents; (ii) resistance to chemotherapeutics is related to a reduced release of Ca^{2+} from intracellular stores upon apoptosis induction [239]; (iii) cell treatment with chemotherapeutic agents reduces the interaction of the antiapoptotic Bcl-2 with mitochondria [240, 241]; (iv) tumor cells escape apoptosis elicited by hypoxia and matrix-detachment (anoikis), both of which activate the PTP [241]; (v) the plant lipids jasmonates, which target the PTP, selectively induce apoptosis in transformed tumor cells [242], making the PTP itself a potential target in cancer therapy.

Resistance to PTP opening displays hepatoprotective effects in a variety of conditions. CsA abrogates the lethal effects of a combination of *E. coli* lipopolysaccharide plus D-galactosamine, a treatment that kills hepatocytes through TNF-α [234]. In a model of hepatocarcinogenesis, the arylamine 2-acetylaminofluorene (AAF) causes onset of liver tumors preceded by a sequence of alterations that closely resembles the clinical course of chronic hepatitis. We found that PTP desensitization early during AAF feeding induces a tumor-promoting adaptive response that selects apoptosis-resistant hepatocytes [233], and a similar adaptive response of the PTP *ex vivo* has also been demonstrated after bile duct ligation in rats [243]. A core protein of the hepatitis C virus increases ROS production and possibly PTP opening by inhibiting respiratory chain complex I [244] in a fashion that is reminiscent of what is observed in liver chronic alcohol exposure [245]. As both alcoholic liver disease and chronic hepatitis C are leading causes of hepatocarcinoma, inhibition of liver apoptosis through PTP adaptation might play a role in the onset of liver cancer in these conditions.

In the nervous system, CsA protects from brain damage provoked by hyperglycemia, hypoglycemia, ischemia, trauma, from cell death caused by facial motoneuron axotomy in neonatal rodents and from photoreceptor apoptosis. Furthermore, *Ppif*$^{-/-}$ mice have a striking decrease of the damage induced by middle cerebral artery occlusion (see [21] for a review).

PT plays a key role in the pathogenesis of muscular dystrophy in a mouse model of collagen VI deficiency, indicating that collagen VI myopathies have an unexpected mitochondrial pathogenesis [231]. In humans, mutations of collagen VI genes cause either Bethlem myopathy [246] or Ullrich congenital muscular dystrophy [247]. Collagen VI-deficient mice display a phenotype strongly resembling Bethlem myopathy, with loss of contractile strength associated with major alterations of sarcoplasmic reticulum and mitochondria, and spontaneous apoptosis. These defects are caused by inappropriate PTP openings, as CsA rescues a normal muscle ultrastructure and dramatically decreases apoptosis *in vivo* [231]. We have recently found that the same defect can be

demonstrated in myoblast cultures from patients with Ullrich congenital muscular dystrophy [259].

5 Conclusions and perspectives

Despite the uncertainties about its molecular composition, sound evidence indicates that the PTP plays a role in several disease paradigms *in vivo*, and that the pore represents a viable target for drug development. Many well-characterized death signalling pathways affect mitochondrial function in a variety of ways, and these include modulation of the PTP. Whether Bcl-2 family members, VDAC and HK affect the release of apoptogenic proteins through PT-dependent or-independent pathways, or both, remains a controversial issue that may depend on the intrinsic complexity of the system and on the dynamics of the protein interactions involved. The challenge of clarifying these issues will greatly benefit from the structural definition of the PTP, a task that represents one of the major efforts of our laboratory.

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