Review

Mitochondria-dependent apoptosis and cellular pH regulation

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Abstract

Mitochondria play a critical role in apoptosis induction in response to myriad stimuli. These organelles release proteins into the cytosol which trigger caspase activation or perform other functions relevant to apoptosis, including cytochrome *c* (cyt-*c*), caspases, AIF, and SMAC (Diablo). The mechanisms by which these proteins escape from mitochondria remain enigmatic. Moreover, it is unclear whether release of these proteins *versus* disturbances in core mitochondrial functions represents the cell death commitment mechanism. In this regard, suppression of apoptosis using broad-spectrum caspase inhibitory compounds has been reported in many circumstances to prevent the morphological and biochemical manifestations of apoptosis, and yet not protect cells from death and not preserve clonigenic survival. Thus, while mitochondrial damage can be coupled to caspase activation pathways, cell death commitment often occurs upstream of caspase activation when mitochondria-dependent cell death pathways are invoked. Here, we review evidence implicating dysregulation of cellular pH as a component of the cell death mechanism involving mitochondria.*Cell Death and Differentiation* (2000) **7, 1155–1165.**

Introduction

The mitochondria of healthy cells maintain an electrochemical gradient ($\Delta\psi$) across their inner membrane which is created by pumping protons from the matrix to the inter-membrane space (IMS) of these organelles in conjunction with electron transport through the respiratory chain (reviewed in[1]). The electrochemical gradient consists of ΔpH and ΔV_m compo-

nents, but fundamentally is caused by a net efflux of H^+ ions from matrix to the outer surface of the inner membrane, with the membrane acting like a capacitor that builds up charge and is poised to discharge when conditions are permissive. Influx of protons back into the matrix is thought to be mediated chiefly by the F_0F_1-ATPase/H^+ pump. This multi-subunit protein complex includes a H^+ channel coupled to an ATPase.[2] Proton accumulation on the external surface of the inner membrane provides the proton-motive force that permits H^+ ions to flow through the channel and into the matrix, using an elegant biomechanical process to drive the ATPase, converting ATP from ADP and PO_4^{-3} (P_i). While operating normally in a single direction with H^+ entering the matrix for the purpose of generating ATP, the F_0F_1-ATPase is a bidirectional H^+ pump. Under conditions where ΔV_m is low, the F_0F_1-ATPase can operate in reverse direction, effluxing H^+ and consuming ATP.[1] Also, up to a certain point, the F_0F_1-ATPase can pump protons against a H^+ gradient when ADP or P_i is in limiting supply and ATP is present in relative excess.[3-5]

Additional ion-channels have been discovered in the inner membrane which are capable of modulating mitochondria pH gradients, including a H^+/K^+ antiporter, Na^+/H^+ exchanger, Cl^-/HCO_3^- antiporter, uncoupling proteins, and the P_i/OH^- exchanger[6] (reviewed in[7-10]). In contrast to the inner membrane, the outer membrane of mitochondria is completely porous to H^+, presumably due to the presence of porin (VDAC), a β-sheet-type transmembrane channel protein which is involved in transporting metabolites, including ADP and ATP, between mitochondria and cytosol. Even in its 'closed' confirmation, this family of channel proteins (the best characterized of which is the Voltage-dependent Anion Channel [VDAC]) creates a pore of approximately 1.8 angstroms diameter, which is adequate for passage of H^+ and other biologically relevant ions.[11]

Disturbances in mitochondrial membrane potential during apoptosis

Depolarization of mitochondria and loss of the electrochemical gradient ($\Delta\psi$) is universally associated with apoptosis and cell death (reviewed in[12]). Controversy reins however as to mechanisms responsible and the kinetics of $\Delta\psi$ disruption relative to other events involved in mitochondrial apoptosis. Depending on the stimulus employed, for example, release of cytochrome *c* (cyt-*c*) can either precede or follow membrane depolarization. The emerging consensus from the literature suggests that excessive Ca^{2+} influx represents a proto-typical example of a cell death stimulus where mitochondrial membrane depolarization precedes cyt-*c* release. In response to elevations in cytosolic Ca^{2+}, mitochondria are

known to import Ca^{2+} from the cytosol, where Ca^{2+} is drawn to the electronegative matrix via the Ca^{2+} uniporter, creating transient depolarizations of the inner membrane, followed by efflux of H^+ and restoration of $\Delta\psi$.[13] Typically, mitochondria can tolerate a few to several pulses of Ca^{2+}, but ultimately their capacity to adapt to Ca^{2+} loads is overwhelmed and the mitochondria depolarize irreversibly due to a profound change in the inner membrane permeability representing the so-called 'permeability transition'.[14] The molecules responsible for permeability transition (PT) are unknown, but the ability of compounds such as bongekreic acid and atractyloside, which modulate the adenine nucleotide translocator (ANT), has implicated this transporter involved in exchanging ADP and ATP between mitochondria and cytosol (reviewed in[14]). When PT occurs, the associated ~ 1.5 kDa ($2.0-2.6$ nm diameter) channel opens, protons and ions equilibrate freely across the inner membrane, and osmotic disequilibrium ensues, resulting in swelling of the matrix space (reviewed in[15]). Because the inner membrane with its folded *cristae* has a larger surface area than the surrounding outer membrane, eventually matrix swelling causes rupture of the outer membrane, thereby spilling the contents of the IMS into the cytosol, including cyt-*c* and other proteins normally sequestered in this compartment of mitochondria. This Ca^{2+}-based mechanism for cell death, involving mitochondrial swelling and release of apoptogenic proteins, may be particularly relevant to ischemia-reperfusion injury and neuroexcitotoxicity involving NMDA-family receptors, a family of plasma membrane Ca^{2+} channels that open in response to glutamate and related neurotransmitters and which have been implicated in cell death during stroke and several other neurological diseases (reviewed in[16,17]).

In contrast to pathological elevations in Ca^{2+}, most studies suggest that a wide-variety of apoptotic stimuli induce release of cyt-*c* from mitochondria prior to membrane depolarization, including growth factor or neurotrophin withdrawal, DNA-damaging drugs and radiation, inhibitors of protein kinases such as staurosporine, elevations in pro-apoptotic Bcl-2 family proteins, and various agonists or antagonists of the retinoid/steroid hormone family of nuclear receptors (reviewed in[18,19]). When cells are insulted with these sorts of stimuli, mitochondrial membrane depolarization typically occurs as a late event, after caspase activation. Indeed, caspase-inhibitors markedly delay or even prevent the ultimate depolarization of mitochondria in these scenarios, revealing that cyt-*c* release occurs in advance of any detectable fall in $(\Delta\psi)$.[20–23] Up until the event of cyt-*c* release, mitochondrial functions appear to be largely intact, inasmuch as oxygen consumption continues (respiration) and ATP levels remain near normal.[22] After cyt-*c* release, however, electron chain-transport may become interrupted, if insufficient cyt-*c* remains docked to its high-affinity sites on cyt-*c*-oxidase. Thus, loss of $(\Delta\psi)$ represents a rather late event in many models of apoptosis, probably occurring after commitment to cell death. Moreover, using protonophores to pharmacologically depolarize mitochondria in intact cells, it has been possible to create conditions *in vitro* where cells sustain their survival through anaerobic glycolysis and to demonstrate that depolarization is insufficient for triggering cyt-*c* release.[24,25] Thus, cyt-*c* release can be uncoupled

from membrane depolarization, separating these events mechanistically.

Hyper-polarization of the inner membrane: an early event associated with mitochondria-dependent apoptosis

Not only does depolarization occur after cyt-*c* release in many models of apoptosis, but it has been suggested that the inner membrane of mitochondria may undergo a transient hyperpolarization during apoptosis. Using cationic dyes that partition across the inner membrane of mitochondria in accordance with the Nernst equation, several groups have reported evidence of an initial rise in $\Delta\psi$, occurring before or contemporaneously with cyt-*c* release, and preceding caspase activation and subsequent membrane depolarization.[20,22,24,26,27] Moreover, in some of these reports, the mitochondria of the same cells were observed to undergo hyperpolarization in response to staurosporine but depolarization in response to Ca^{2+} influx, with both types of stimuli triggering cyt-*c* release.[28] Several difficulties however exist with interpreting results derived from use of cationic dyes for monitoring $\Delta\psi$ (reviewed in[29,30]). For example, the amount of these dyes which accumulate in mitochondria is dependent not only on $\Delta\psi$ but also the volume of the organelles, thus complicating interpretation when organellar swelling occurs. Some of the commonly used dyes also can leak out of mitochondria when PT occurs, due to simple diffusion rather than partitioning in accordance with membrane potential. Other problems include auto-quenching when intra-mitochondrial concentrations of dye are too high and destruction by oxidation, a likely problem in the face of elevated reactive oxygen species production in ailing mitochondria. Also, dye entry into cells is regulated at the plasma membrane, in addition to mitochondrial membranes.

For these reasons, we recently sought an alternative approach to monitoring pH locally within mitochondria, applying Green Fluorescent Protein (GFP) technology to this question of membrane hyperpolarization. In this regard, Tsien and colleagues have described mutants of GFP which emit light in a pH-dependent fashion.[31] These pH-dependent GFPs can be directed to various organellar compartments by expression in cells as fusion proteins, appended with targeting peptides. One such GFP mutant, which produces a near-linear increase in its fluorescence emissions within the pH range $7.5-8.5$, was directed to the matrix space of mitochondria by expression with an N-terminal COX-IV leader sequence.[24,31] The pH-GFP protein remains in the matrix, regardless of how apoptosis is induced, and appears to retain its near-linear pH-dependent fluorescence emission characteristics within intact cells under a wide variety of conditions.

Using pH-GFP to monitor matrix pH, it was observed that mitochondria-dependent apoptotic stimuli such as Bax over-expression and staurosporine induce rapid alkalinization of the matrix, with concomitant acidification of the cytosol. This change in pH was substantial, estimated at ~ 0.5 pH units in most experiments. Thus, the H^+ gradient becomes attenuated within minutes after confrontation with these apoptotic stimuli, suggesting that the net efflux of H^+

from matrix to IMS has increased. This increase in ΔpH may account for the hyperpolarization noted when using cationic dyes. Though contrary conclusions were reached by others studying the effects of Bcl-2 in isolated rat mitochondria,[32] those observations were based on use of protonophores at apparently sub-optimal concentrations.

Importantly, both hyperpolarization (as measured with cationic dyes) and matrix alkalinization are blocked by Bcl-2, an anti-apoptotic protein that localizes to mitochondria and which is known to prevent cyt-c release (reviewed in[12,33,34]). Also, hyperpolarization and matrix alkalinization are caspase-independent, inasmuch as treatment of cells with broad-spectrum irreversible caspase inhibitors does not prevent these changes in mitochondrial physiology.[20,22,24,27] In contrast to mitochondria-dependent cell death stimuli, apoptosis induced by anti-Fas antibody can occur without either hyperpolarization or matrix alkalinization,[24] indicating that an alternative pathway which bypasses mitochondria is involved with this Tumor Necrosis Factor Receptor (TNFR) family member (reviewed in[35]).

As in many other models of apoptosis, eventually hyperpolarization and matrix alkalinization are followed (typically several h later) by mitochondrial depolarization and an equilibration of mitochondrial and cytosolic pH, indicative of the phenomenon of permeability transition (PT).[24] This transition is markedly delayed (typically by a day or more) when caspase-inhibitors are included in cultures, suggesting that PT induction in this setting is a post-caspase activation event. Thus, a change in mitochondrial H^+ transport resulting in an increase in the H^+ gradient represents an early event associated with mitochondria-dependent apoptosis, preceding caspase activation and PT. How these changes in pH regulation relate to the mechanism of cyt-c release remains unclear to date. Experiments using protonophores to dissipate the H^+ gradient of mitochondria suggest that cyt-c release induced by staurosporine occurs independently of hyperpolarization or matrix alkalinization, implying that these events are separable mechanistically.[24] However, this issue requires further evaluation in the future, given limitations of sustaining survival of animal cells in the presence of protonophores and other potential confounding effects of these agents on cell physiology.

Conservation of mechanisms in yeast

Ectopic expression of mammalian Bax protein in budding (*S. cerevisiae*) or fission (*S. pombe*) yeast induces cell death (reviewed in[36]). Similar to its actions in mammalian cells, when expressed in yeast, the Bax protein targets mitochondria and induces cyt-c release.[37,38] Moreover, co-expression of Bcl-2, Bcl-X_L or other anti-apoptotic Bcl-2 family members can rescue yeast from the cytotoxic effects of Bax. Studies of the effects of Bax on mitochondria in intact yeast have provided evidence of hyperpolarization (based on cationic dyes) and matrix alkalinization (based on pH-GFP), with concomitant cytosol acidification.[24,26,39,40] Furthermore, Bcl-2 or Bcl-X_L prevents these effects of Bax on mitochondria and pH regulation.

These observations suggest that the mechanism employed by Bax for triggering mitochondrial alterations leading to cell death are at least partly conserved in yeast and animal cells, and they demonstrate that Bax can profoundly affect the regulation of mitochondrial H^+ transport even in simple eukaryotes. Thus, the effects of Bax on mitochondrial H^+ regulation may reflect an intrinsic property of this protein and/or indicate conservation of an ancient cell death mechanism. Furthermore, this cell death mechanism is caspase-independent, since no caspases have been identified in the genome of the yeast, *S. cerevisiae*. Interestingly, ectopic expression of Bax in bacteria has been reported to cause increased efflux of H^+, resulting in marked acidification of the extracellular medium and alkalinization of the cell interior,[41] thus mimicking Bax's effects upon mitochondria in mammalian cells. This Bax-induced H^+ efflux from bacteria is accompanied by increased O_2 consumption, implying an intact membrane and electron transport chain. Given the similarities between bacteria and mitochondria, it is therefore tempting to speculate that Bax directly or indirectly triggers a process that results in increase efflux of H^+ from mitochondria.

Mutagenesis studies are consistent with, but do not prove, that the lethal effects of Bax in yeast correlate with its ability to either form pores in membranes or at least to insert into membranes.[36,42,43] In this regard, Bax is predicted to share structural similarity with other Bcl-2 family proteins which are strikingly similar to the pore-forming domains of bacterial toxins (reviewed in[44]). The Bax protein inserts in the outer membrane of mitochondria, and may either directly create channels or collaborate with other proteins in the outer membrane such as VDAC to create protein-translocation channels.[36,45−51] It has also been suggested that Bax may translocate to the inner membrane of mitochondria, interacting with inner membrane proteins such as ANT.[52]

Debate reins as to whether the cytotoxic effects of Bax in yeast require an intact respiratory chain, but the consensus appears to be that Bax can kill yeast regardless of whether grown on fermentable *versus* non-fermentable substrates – though the kinetics and efficiency of cell death may be superior under conditions where respiration is maintained.[53] Regardless of the details of the mechanism, the ability of Bax to confer a lethal phenotype when ectopically expressed in yeast has permitted functional analysis of the relevance of several mitochondrial proteins using genetic approaches in which various yeast genes are ablated. Since yeast can grow anaerobically, it has been possible to approach certain questions in yeast which cannot be addressed in animal cells. The resulting picture however has been confusing, with widely varying results reported. For example, rho-zero cells lacking mitochondrial DNA (and hence containing mitochondria incapable of executing oxidative phosphorylation), ANT-deficient, and VDAC (porin)-deficient yeast have been reported to either retain sensitivity or display resistance to Bax, depending on the nutrients on which cells are grown and the conditions used to induce Bax expression.[37,39,40,43,52−56]

The F_0F_1-ATPase and cell death regulation

Classical techniques of yeast genetics have been applied for analysis of mechanisms of Bax induced cell death, revealing a role for the F_0F_1-ATPase/H^+ pump.[57] For these experiments, yeast were randomly mutagenized using chemical mutagens, and Bax-resistant strains were identified. Complementation cloning indicated that yeast genes encoding a specific subunit of the F_0F_1-ATPase (*atp4*) could restore Bax-sensitivity, implying that the Bax-resistance phenotype was due to a defect in the F_0F_1-ATPase. Analysis of independently derived mutant strains in which homologous recombination was used to knock-out the same subunit (*atp4*) or the *atpδ* subunit of the F_0F_1-ATPase also revealed a requirement for Bax-induced lethality in yeast. Subsequent studies confirmed that yeast lacking the *atp4*- or *atp2*-encoded subunits of the F_0F_1-ATPase display resistance to Bax-induced lethality.[39,40] Moreover, by expressing mitochondria-targeted pH-sensitive GFP in yeast, evidence has been obtained indicating that the F_0F_1-ATPase is required for Bax-induced mitochondrial matrix alkalinization and cytosol acidification,[24] based on use of a mutant strain in which the *atp4*-encoded subunit of the F_0F_1-ATPase was ablated by homologous recombination.[58]

Oligomycin binds subunits comprising the F_0 portion of the F_0F_1-ATPase and potently inhibits this H^+ pump.[59] Using oligomycin as a pharmacological probe, it was demonstrated that Bax-induced cell death and cyt-*c* release in both yeast and mammalian cells can be attenuated when the H^+ pump is shut-down.[24,43,57,60] Inhibition of Bax-induced cyt-*c* release from isolated mammalian mitochondria was also inhibited.[60] Oligomycin also inhibited cell death induced by p53, in a cell line where p53 is known to induce transcription of the endogenous *bax* gene and to trigger apoptosis through a *bax*-dependent mechanism.[57] Similar conclusions have been reached using oligomycin-treated cell lines in which cyt-*c* release and apoptosis were induced by DNA-damaging anticancer drugs, or UV-irradiation.[21,61] Importantly, while oligomycin attenuates cyt-*c* release and apoptosis induced by Bax, staurosporine, and DNA damaging agents, it does not interfere with Fas-induced cyt-*c* release or apoptosis (a mitochondria-independent stimulus),[24] suggesting a causal relation between the F_0F_1-ATPase and execution of the mitochondrial cell death pathway.

In addition to cyt-*c* release and apoptosis, a role for the F_0F_1-ATPase in apoptosis-associated mitochondrial hyperpolarization and mitochondrial matrix alkalinization has also been supported by experiments using oligomycin in mammalian cells. Though oligomycin itself causes some hyperpolarization and matrix alkalinization by precluding H^+ re-entry into the matrix, the apoptosis-associated increases in membrane potential and matrix pH are greater than those induced by oligomycin. Moreover, oligomycin reportedly prevents the rise in membrane potential and matrix pH induced by mitochondria-dependent cell death stimuli such as Bax or staurosporine.[24] Thus, the F_0F_1-ATPase is implicated in the changes in mitochondrial H^+ transport witnessed in cells stimulated to undergo apoptosis.

Use of any pharmacological agent, including oligomycin, for dissecting cellular mechanisms is subject to caveats, and thus the results reported thus far should be viewed as preliminary. For example, at higher concentrations, oligomycin can also interfere with the Na^+-K^+ ATPase.[62] Unlike oligomycin, however, oubain, a specific Na^+-K^+/ATPase inhibitor, failed to attenuate Bax-induced cell death when used at sublethal concentrations,[57] suggesting that Na^+-K^+-ATPase suppression is unlikely to explain the effects of oligomycin and cyt-*c* release. Nevertheless, other complementary approaches are needed to assuage concerns about the specificity of oligomycin, such as conditional ablation of expression of F_0F_1-ATPase subunits using antisense technology or other approaches.

What role precisely does the F_0F_1-ATPase play in mitochondria-dependent apoptosis? Given that matrix pH rises while cytosolic pH falls and that F_0F_1-ATPase inhibitors oppose these changes, the simplest interpretation is that apoptotic stimuli such as Bax and staurosporine cause the H^+ pump to run backwards, effluxing H^+ and consuming ATP. In this regard, the F_0F_1 complex can pump protons in reverse from the matrix across the inner membrane into the IMS (which is essentially contiguous with the cytosol where ions are concerned) under circumstances where ΔV_m is low or the matrix [ATP]/[ADP][P_i], ratio becomes high such that ADP or P_i is in limited supply.[3,4] Under these conditions, the F_0F_1 complex consumes ATP, resulting in extrusion of protons from mitochondria. Though hyperpolarization of mitochondria would seem incompatible with reverse operation of the F_0F_1-ATPase, it is actually unknown how high a negative voltage the F_0F_1 complex can pump against, due to the technical difficulties of working with sub-mitochondrial particles that contain functional F_0F_1-ATPase (reviewed in[1]). Also, given the many artifacts associated with use of membrane-permeant cationic dyes for *in situ* estimation of V_m (reviewed in[29,30]), the actual V_m of mitochondria in cells stimulated to undergo apoptosis is unclear. Probably all that can be safely said is that a gross depolarization of the inner membrane is not an early event when apoptosis is induced by Bax, staurosporine, or related stimuli, but whether ΔV_m is truly elevated from basal conditions is potentially arguable.

With respect to [ATP]/[ADP] (ï) [P_i] ratios, ATP/ADP exchange between the mitochondrial matrix and cytosol reportedly becomes impaired early in apoptosis (reviewed in[19]). ATP/ADP exchange is mediated largely by the adenine nucleotide translocator (ANT) in the inner membrane, in collaboration with the voltage-dependent anion channel (VDAC) in the outer-membrane.[14] Physical and functional interactions have been reported between anti-and pro-apoptotic Bcl-2 family proteins and the ANT, VDAC or both. If ATP/ADP exchange is shut-off, one would expect matrix ATP/ADP ratios to rise, favoring reverse operation of the F_0F_1/ATPase. However, if this hypothesis is correct, then other mechanisms must also come into play, since reverse operation of the F_0F_1/H^+-pump should eventually reach a steady-state condition where H^+ influx and efflux were equalized. Thus, the changes in pH observed in cells undergoing apoptosis in response to

Bax, staurosporine, and related stimuli probably are not entirely attributable to reverse-operation of the F_0F_1/H^+-pump, and could derive from other parallel events such as trapping of organic bases in the mitochondrial matrix, counter-ion movements, increased exchange of OH^- for P_i via the mitochondrial OH^-/P_i antiporter as a result of ATP hydrolysis, or other processes[19] (Figure 1).

The F_0F_1-ATPase is capable of modulating apoptosis responses, but how central is it for cell death induced by stimuli such as Bax? Though debatable,[38-40] some reports indicate that Bax can kill even rho-zero or rho $(-)$ yeast.[37,57] Furthermore, it has been shown that mammalian rho-zero cells are still capable of undergoing apoptosis in response to mitochondria-dependent cell death stimuli and that Bcl-2 retains its ability to suppress cell death in these cells.[63,64] Since rho-zero cells lack mitochondrial DNA and since the mitochondrial genome encodes two of the subunits of the F_1 portion of the F_0F_1-ATPase, it would seem that H^+ pumping by this multi-subunit protein complex is not critical, implying that proteins comprising the F_0F_1-ATPase participate in some other way in apoptosis. However, even without these two mitochondrial genome-encoded subunits, the incomplete F_0F_1-ATPase retains ATPase activity and is required for maintenance of mitochondrial membrane potential in rho-zero cells due to electrogenic ADP^{3-}/ATP^{4-} exchange.[5,65] Thus, the incomplete F_0F_1-ATPase of rho-zero cells could theoretically participate in the mitochondrial membrane hyperpolarization observed in Bax-expressing cells, including Bax-expressing rho-zero yeast.[39] Nevertheless, using Bax-resistant yeast in which complementation cloning experiments suggest defects in components of the F_0F_1-ATPase, we have identified gain-of-function mutations which can be engineered into the Bax protein, allowing it to kill under conditions were wild-type Bax does not.[42] These mutations fall into two central α-helices within the Bax protein which are known to be important for pore-formation in vitro,[57] removing polar residues in these α-helices, which presumably makes it easier for Bax to insert into membranes.[42] This observation implies that the F_0F_1-ATPase may be expendable, if the function of Bax is enhanced. Because the specific defect in the Bax-resistant mutant strain of yeast used for these studies is undetermined, these findings imply the results should be interpreted with caution. Nevertheless, these findings imply that the role of the F_0F_1-ATPase in Bax-induced cell death may be indirect, with the F_0F_1-ATPase perhaps creating a permissive state through effects on pH gradients or V_m which is required for optimal function of wild-type Bax. Also of possible relevance, Bax association with mitochondrial membranes in yeast was reported to be dependent on ATP,[43] raising the possibility that the F_0F_1ATPase might indirectly influence membrane insertion and function of Bax via its effects on mitochondrial ATP levels.

Cytosol acidification during apoptosis

Several reports indicate that acidification of the cytosol occurs in mammalian cells undergoing apoptosis. The extent of the change in pH observed varies among reports, but typically represents a drop of $\approx 0.3-0.4$ pH units. The stimuli which induce cytosol acidification include over-expression of Bax (in both mammalian cells and yeast), staurosporine, UV-irradiation, etoposide, anti-Fas antibodies, growth factor
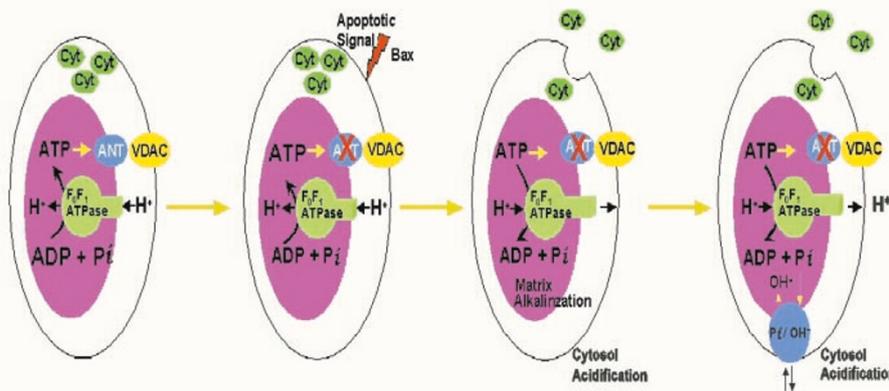

Figure 1 Model for F_0F_1-ATPase participation in mitochondria-dependent apoptosis. Normally, the F_0F_1-ATPase transports H^+ into the matrix, producing ATP which is then exchanged for cytosolic ADP via ANT at the inner-membrane and VDAC at the outer-membrane (left). Apoptotic signals such as Bax may somehow inhibit ATP/ADP exchange through effects either on ANT (shown) or on VDAC (not shown), thus causing the [ATP]:[ADP] ratio to rise (second panel). When the ATP:ADP ratio reaches a critical value, F_0F_1-ATPase theoretically, could reverse direction (third panel). The resulting hyperpolarization or other related events may trigger cyt-c release. For reverse operation of the F_0F_1-ATPase to be sustained, other unidentified mechanisms must come into play (fourth panel). For example, P_i is normally rapidly exchanged for OH^- via an antiporter. Though entirely speculation, depletion of P_i from the matrix could deny the F_0F_1-ATPase the substrate it requires (in combination with ADP) to produce ATP, thus favoring reverse operation

deprivation, somatostatin, and p53.[66−73] Studies using pharmacological inhibitors of the plasma membrane Na^+/H^+ antiporter and vacuolar H^+/ATPase suggest that these H^+ transporters remain functional during apoptosis, though they do not evidently compensate sufficiently for the decreased pH of the cytosol so as to hold pH near neutral. Nor do these particular H^+ transporters appear to be the cause of the observed cytosolic acidification, though they may allow growth factors or other cyto-protective stimuli to oppose cytosol acidification.[71,74,75]

Cytosol acidification can be either caspase-dependent or caspase-independent.[24,69,76−78] With death receptors such as Fas, caspase recruitment to the activated receptor complex occurs, resulting in direct activation of these cell death proteases (reviewed in[79,80]). Fas induces cytosol acidification as a downstream consequence of caspase activation, perhaps due to cleavage of unidentified regulators of cellular pH located at the plasma membrane or other non-mitochondrial sites. In contrast, mitochondria-dependent stimuli, including over-expression of Bax, p53, staurosporine, and growth factor deprivation also induce cytosol acidification, but where tested, appear to do so through a caspase-independent mechanism which is not suppressible by broad-spectrum irreversible inhibitors of these proteases, such as benzoyl-valine-alanine-asparate-fluoromethylketone (zVAD-fmk).[24,69,78] Moreover, the decrease in cytosolic pH precedes caspase activation in cells undergoing apoptosis in response to mitochondria-dependent cell death stimuli (Bax, staurosporine, UV-irradiation), whereas the opposite is observed in Fas-induced apoptosis.[24,77]

While caspase inhibitors fail to prevent cytosol acidification in response to mitochondria-dependent cell death stimuli, over-expression of Bcl-2 does.[24,69,73,77] Oncogenic v-Abl also prevents cytosolic acidification and apoptosis in hematopoietic cells deprived of growth factors.[70] Furthermore, apoptosis suppression by v-Abl is reportedly lost when cytosol acidification is induced by pharmacological inhibitors of the plasma-membrane Na^+/H^+ antiporter. Though cause-and-effect data are lacking to implicate pH as the explanation, it may also be of relevance that hematopoietic cells expressing oncogenic Abl can maintain their survival, failing to undergo apoptosis, even though cyt-c release occurs after growth factor withdrawal.[81]

Is cytosol acidification important for apoptosis? Several lines of evidence suggest it at least contributes. For example, when F_0F_1-ATPase inhibitors (oligomycin) were employed, cytosol acidification induced by Bax or staurosporine was impaired and apoptosis was reduced.[24] Similarly, when using protonophores or membrane-permeant organic bases to equilibrate H^+ across biological membranes, thus 'clamping' the pH at 7.1−7.4, apoptosis induced by Bax, staurosporine, UV-irradiation, growth factor deprivation, and lovastatin can be reduced.[24,68,69,82] Neither F_0F_1-ATPase inhibitors nor pH clamping completely prevents apoptosis of cells, however, suggesting that while pH changes make contributions, they are not essential for apoptosis in most instances – perhaps having more to do with the kinetics of apoptosis than whether or not it occurs. Thus, rectifying the problem of cytosol

acidification does not uniformly prevent apoptosis.[71] Nevertheless, it remains possible, though untested, that differences in the kinetics of apoptosis could be important determinants of clonigenic survival *in vivo*, when death stimuli are transient in nature and survival factors/signals (either endogenous or exogenous) are applied shortly thereafter. In this regard, many growth factors which deliver survival signals have been shown to induce cytosol alkalinization through effects on Na/H^+ exchange at the plasma membrane.[70−72,83] Effects of growth survival factors on the vacuolar H^+/ATPase have also been documented, thus opposing acidification and sustaining cell viability.[68,74] Thus, it may be speculated that one of the mechanisms by which growth factors promote cell survival is by maintaining cytosolic pH in the alkaline range.

In contrast to mitochondria-dependent cell death stimuli, H^+ pump inhibitors and protonophores did not inhibit Fas-induced apoptosis in Type I cell lines in which Bcl-2 does not rescue from Fas-induced apoptosis.[24] Thus, the changes in pH observed in Type I cells treated with Fas are a downstream event, which does not make major contributions to apoptosis. In some types of cells (so-called Type II), death receptors such as Fas trigger apoptosis through a pathway in which mitochondria participate, probably as a result of caspase-mediated cleavage and activation of Bid, a pro-apoptotic member of the Bcl-2 family that targets mitochondria and activates Bax.[34] In a Type II cell line where apoptosis was induced by anti-Fas antibody, it was reported that clamping pH in the neutral range partially suppressed apoptosis.[68] These differences in Type I and II cells with respect to the effects of H^+ pump inhibitors and protonophores on Fas-induced apoptosis also argue for the existence of two mechanisms of cytosol acidification and for two apoptosis pathways – one in which cytosol acidification makes measurable contributions to apoptosis (mitochondria-dependent) and another in which cytosol acidification is entirely expendable (mitochondria-independent/death receptor).

How does cytosol acidification facilitate apoptosis? The answer appears to lie in the effects of pH on some caspase activation mechanisms and perhaps on the activity of some caspase-family proteases. For example, cyt-c induces caspase activation by binding the protein Apaf-1, inducing its dATP/ATP-dependent oligomerization, resulting in recruitment of pro-caspase-9 to the complex followed by activation of this cell death protease (reviewed in[12,84,85]). pH has a profound influence on caspase activation induced in cytosolic extracts *in vitro* by addition of exogenous cyt-c and dATP. The pH optimum for this reaction was reported to be ∼pH 6.4.[24] In contrast, at pH 7.4, cyt-c-mediated caspase activation occurred at only 25% of maximal, suggesting that preventing cytosol acidification may suppress apoptosis induced by mitochondria-dependent stimuli at least in part by interfering with cyt-c-induced assembly of the Apaf-1 apoptosome or activation of caspases in this pathway (Figure 2). Indeed, kinetic analysis of the effects of pH on cyt-c-triggered caspase activation favor the hypothesis that pH affects the rate of assembly of the Apaf-1 apoptosome, though this remains to be directly tested. It is also possible that cytosolic pH could indirectly affect the measured
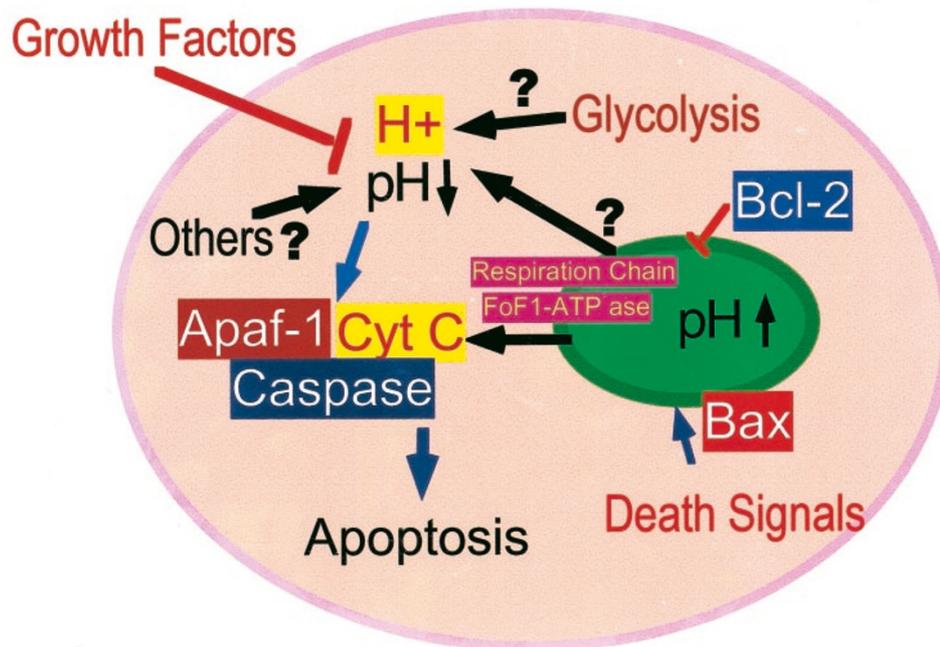
Mitochondria-Dependent Cell Death

Figure 2 pH and apoptosis regulation. Bax and related mitochondria-dependent apoptosis stimuli induce mitochondrial matrix alkalinization with concomitant cytosol acidification via a Bcl-2 suppressible mechanism. The apparent net efflux of H$^+$ from mitochondria may be regulated by the mitochondrial F$_0$F$_1$-ATPase of the inner membrane. Additional H$^+$ transporters such as the plasma-membrane Na$^+$/H$^+$ exchanger and vacuolar H$^+$/ ATPase also make contributions to regulation of cytosolic pH, as well as lactate production via glycolysis. Acidification of the cytosol promotes cyt-c/ Apaf-1-mediated activation of caspases and may also increase the activity of some processed caspases against some substrates. Growth and survival factors oppose cytosol acidification, often through effects on the Na$^+$/H$^+$ exchanger or vacuolar H$^+$/ATPase (not shown), decreasing caspase activation and apoptosis

activity of caspases by modulating the activity of endogenous caspase inhibitory proteins, particularly the IAPs, which in humans bind and suppress the apical caspase in the Apaf-1/cyt-c pathway, caspase-9, as well as the next caspase downstream, caspase-3.[86,87] In this regard, IAPs are suppressed by SMAC/Diablo, a protein having a pI of ~5.3.[88,89] In addition, caspase-9 has been reported to have a pH optimum of ≈pH 5.5 when measured against a non-ideal substrate (DEVD), in contrast to an optimal substrate (LEDH) where the pH optimal is ~pH 7.[90,91] However, those studies were performed with a recombinant version of caspase-9 missing its N-terminal prodomain (which is not usually cleaved off *in vivo*) and in the absence of Apaf-1, an important cofactor for this protease *in vivo* which is necessary to form the holoenzyme complex.[92] In contrast to cyt-c, activation of caspases in cytosolic extracts by granzyme-B or caspase-8 occurs optimally at pH 7.1–7.4 ('neutral' pH).[93]

Why was this pH-dependence of the cyt-c reaction not previously detected? One possibility is that typically investigators add not only cyt-c to their cytosolic extracts when interrogating this caspase-activation mechanism, but also 1 mM dATP (or ATP) because Apaf-1 binds nucleotides and requires them for oligomerization. However, at these concentrations, these nucleic acids can induce a 0.3–0.4 pH unit drop in the pH of extracts unless counter-measures

are taken, thereby bringing pH toward the optimum required for cyt-c-mediated activation of caspases.

These influences of pH on the activation and activity of caspases offer a possible explanation for the effects of pH on apoptosis. They also suggest a possible mechanism for the phenomenon of cyt-c resistance, where introduction of cyt-c into cells by microinjection or electroporation fails to activate caspases in some cellular context but not others.[94–96] Again, it is intriguing to speculate that cytosol alkalinization induced by growth factors or neurotrophins plays a role here. However, multiple alternative mechanisms for interfering with cyt-c-induced apoptosis have been elucidated (reviewed in[97]) and thus the relative importance of pH remains to be established.

Ischemia pre-conditioning and pH

Resistance to infarction can be induced in myocardial tissue by sublethal exposure to ischemia-reperfusion injury, apparently through a signal transduction pathway involving protein kinases. This resistance phenomenon is known as 'preconditioning' (reviewed in[98,99]). Ischemia results in cytosol acidification, probably in part from rapid conversion to anaerobic glycolysis. Preconditioning is associated with changes in pH regulation that oppose ischemia-induced

cytosolic acidification, correlating with reduced cell death due to either necrosis or apoptosis.[100–102] The mechanisms responsible for the diminished decline in cytosolic pH in preconditioned myocardial tissue are unclear, but increased activity of the vacuolar H$^+$/ATPase has been proposed as at least one contributor.[102] The role, if any, of mitochondria in the phenomenon of preconditioning remains to be explored. However, any mechanism that tends to maintain cytosolic pH toward the alkaline range seems likely to reduce apoptosis, given that acidification of the cytosol enhances cyt-c-mediated activation of caspases.[24]

A paradox of ischemia-reperfusion injury relevant to the issue of pH concerns the observation that Ca^{2+}-induced PT pore opening is more easily achieved under alkaline than acidic conditions.[14] Thus, after cytosolic acidification is induced by ischemia, subsequent reperfusion tends to restore pH toward neutral, making it easier for the PT pore to open if excess Ca^{2+} remains (reviewed in[103]). This enhancing effect of alkalinization on PT pore opening has been associated with necrosis in the context of ischemia-reperfusion injury, rather than apoptosis.

Effects of pH on Bcl-2 family proteins

In addition to pH effects on activation and activity of caspases, changes in pH may modulate the functions of Bcl-2 family proteins. For instance, many Bcl-2 family proteins interact with themselves and each other, forming homo- or heterodimers. Dimerization of these proteins *in vitro* is enhanced at lower pH, with optima probably in the pH 4–5 range.[104] Moreover, channel formation by pore-forming Bcl-2 family proteins in synthetic membranes (including anti-apoptotic Bcl-2, Bcl-X$_L$ and pro-apoptotic Bax and Bid) is also markedly enhanced by low pH, again with optima in the pH 4–5 range.[50,105–108] However, since extraordinarily low pH's of this magnitude are unlikely to be encountered *in vivo*, the significance of these observations is unclear. Nevertheless, since the channels formed by Bcl-2 family proteins in planar bilayers at neutral pH are voltage-dependent, it is conceivable that the contributions of the F$_o$F$_1$-ATPase to ΔV_m are required to create a permissive environment for either channel formation by Bcl-2 family proteins and/or for the cyt-c release mechanism.

Another way that pH may influence Bcl-2 family proteins is by affecting their intracellular targeting. In this regard, the Bax protein is often found in the cytosol in a latent state. Translocation of Bax to mitochondria is induced by various apoptotic stimuli, though the mechanism remains unclear.[45,109] Recently, it has been reported that alkaline pH (approximately pH=8.1) can induce Bax to assume a membrane-inserting ('active') conformation *in vitro*.[110] Further, evidence was presented indicating that lymphokine-deprivation in pro-lymphocytes induces cytosolic alkalinization, correlating with Bax translocation to and insertion into mitochondrial membranes. However, since growth factors treatment (not deprivation) generally promotes cytosol alkalinization in concert with protection from apoptosis,[70–72,83] the generality of these observations is unclear.

Mitochondria volume regulation, cytochrome *c* release, and the role of pH

The question of whether the release of cyt-c from mitochondria requires swelling and rupture of mitochondria remains hotly debated and will not be reviewed in detail here.[12,18,34] Rather, we will focus on some of the ways that pH might influence mitochondrial volume and the cyt-c release mechanism. Suffice it to say, however, that swelling requires a change in inner membrane permeability or ion-transport that would result in a net gain of water into the matrix space. In contrast, if outer membrane permeability to proteins such as cyt-c is selectively altered, then inner membrane function and mitochondrial volume need not change.

The opening of the PT-pore in response to Ca^{2+} is known to be affected by pH with lower pH favoring maintenance of a closed state (reviewed in[103]). Since sustained PT-pore opening leads to osmotic swelling of mitochondria, pH alterations are potentially relevant to volume regulation of mitochondria in cases where the PT-pore plays a primary role. In contrast to Ca^{2+} or oxidant-induced PT-pore opening, less is known about the role of pH when apoptosis is induced via mitochondria-dependent pathways where PT-pore opening is a later event and where apparent hyperpolarization rather than depolarization is observed (e.g Bax; staurosporine; anticancer drugs; irradiation; growth factor deprivation). Some studies support the idea of an osmotic imbalance occurring in association with hyperpolarization.[19] However, others have failed to reveal evidence of gross mitochondrial swelling despite hyperpolarization of the inner membrane, at least when caspases are prevented from circling back and damaging mitochondria.[20,23,24,111,112] Since ΔpH makes major contributions to ΔV_m across the inner membrane of mitochondria, pH may indirectly alter the transport characteristics of a variety of voltage-dependent ion-channels in the inner membrane, thus affecting volume regulation (reviewed in[7,10,113]). Among the potentially relevant molecules of the inner membrane are the K$^+$/H$^+$ antiporter, Na$^+$/H$^+$ exchanger, H$^+$/P$_i$ and H$^+$/pyruvate symotransporters, and the electrogenic K$^+$ uniporter. Hyperpolarization of mitochondria due to an increase in ΔpH would drive more anions such as pyruvate and P$_i$ into the matrix and more K$^+$ due to the electrogenic K$^+$ uniporter, consistent with models which envision swelling.[19] In contrast, increases in ΔpH and ΔV_m, would be expected to drive K$^+$ and Na$^+$ from the matrix into the IMS via K$^+$/H$^+$ and Na$^+$/H$^+$ exchangers,[6] (reviewed in[10]), which would theoretically favor osmotic shrinking rather than swelling of mitochondria. In this regard, Martinou and colleagues have noted what appears to be shrinkage of mitochondria in a model of apoptosis induced by NGF deprivation of sympathetic neurons.[23,114] However, whether this is a common occurrence in apoptosis induced by growth factor-deprivation has not been explored. Clearly, therefore, much remains to be understood about the effects of hyperpolarization on mitochondrial volume regulation.

Because the outer membrane of mitochondria is typically in very close apposition with the inner membrane, it is plausible that the influence of the proton-electrochemical gradient can be translated to outer membrane proteins as

well. In this regard, VDAC (porin) is a voltage-gated channel which represents the most abundant protein in the outer membrane of mitochondria. VDAC has been implicated in the cyt-*c* release mechanism. Evidence has been presented suggesting that both pro- and anti-apoptotic Bcl-2 family proteins directly interact with VDAC and that in collaboration, VDAC plus pro-apoptotic Bax or Bak can create channels in synthetic liposomes that are large enough for passage of cyt-*c*.[51] The formation of collaborative channels by VDAC and pro-apoptotic Bcl-2 family protein is pH-dependent, occurring more efficiently at lower pH, at least *in vitro*.[51] In addition to speculations of a direct role for VDAC in formation of the cyt-*c* release channel, VDAC has been implicated in a more indirect way in mitochondria-dependent apoptosis through its effects on ATP/ADP exchange between mitochondria and cytosol. The transfer of high energy equivalents from respiring mitochondria to the cytosol is mediated by VDAC, either by direct exchange of ATP and ADP or by transport of creatine-phosphate from the IMS across the outer membrane and into the cytosol where it can be used by creatine kinase to convert ADP to ATP.[19] By measuring the ratios of creatine phosphate: creatine in mitochondria, it has been suggested that VDAC-mediated transport may be shut-down during apoptosis. Over-expression of Bcl-X$_L$, in contrast, restores VDAC-mediated transport. How closure of the VDAC channel and impaired net ATP/ADP exchange induces apoptosis is unclear, though several hypotheses have been offered.[19,24]

Conclusions

Emerging evidence suggests that an alteration in cellular pH regulation represents an early event associated with apoptosis induction via the mitochondria-dependent pathway. Though many details remain unclear, some types of physiologically-relevant apoptotic stimuli appear to induce mitochondrial matrix alkalinization, with attendant hyperpolarization of these organelles. Further, at least under some circumstances, these changes in pH regulation and hyperpolarization can precede cyt-*c* release, caspase activation and PT pore opening. Genetic and biochemical data also suggest that a change in mitochondrial pH regulation lies close to the mechanism of action of Bcl-2 family proteins. Finally, changes in cytosolic pH in response to cell death and cell survival stimuli can influence caspase activation pathways in cells. It will be interesting in the future to explore whether pharmacological agents that modulate pH in cells through their effects on specific membrane transporters can be employed for therapeutic purposes in the modulation of apoptosis pathways *in vivo*.

Acknowledgments

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