Hypothesis

# Control of apoptosis by the cellular ATP level

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**Abstract** **Apoptosis is a physiological form of cell death. Its causes and execution mechanisms are not clearly understood. Oxidative stress, nitric oxide and its congeners, $Ca^{2+}$, proteases, nucleases, and mitochondria are considered mediators of apoptosis. At present their importance and exact role are elusive but it is clear that mitochondria are both the target and the source of oxidative stress, nitric oxide, and $Ca^{2+}$. The mitochondrial membrane potential ($\Delta\psi$), which is the driving force for mitochondrial ATP synthesis, declines during apoptosis, and maintenance of $\Delta\psi$ prevents apoptosis. Since apoptosis is highly regulated and involves the activity of hydrolytic enzymes, chromatin condensation and vesicle formation apoptosis is likely to have a high energy demand. We propose that the cellular ATP level is an important determinant for cell death. This hypothesis is supported by circumstantial evidence, is consistent with the available data, has a corrolary in aging, and is amenable to direct experimental testing particularly with flow cytometry as a promising tool.**

*Key words:* Mitochondria; Reactive oxygen; Nitric oxide; Calcium; Adenosine triphosphate; Apoptosis

## 1. Apoptosis: general considerations – activation vs. execution

Apoptosis is an evolutionarily conserved form of physiological cell death important for tissue development and homeostasis. Its hallmarks are distinct morphological alterations such as nuclear condensation, cell shrinkage, and bleb formation, and the absence of inflammatory responses of the affected tissue. Deranged apoptosis plays a major role in diseases such as cancer, acquired immune deficiency syndrome, autoimmune diseases, and neurodegeneration (reviewed in [1]).

The program for apoptotic cell death appears to be present constitutively in virtually all mammalian cells and can be activated by a variety of extra- and intracellular signals. Although the various steps and biochemical mechanisms participating in apoptosis are not completely understood it is clear from genetic studies in lower organisms and comparative investigations in mammalian systems that apoptosis generally comprises four distinct stages, namely the decision to die, the execution of death, the engulfment of dead cells or fragments thereof, and their degradation (reviewed in [2]).

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*Abbreviations:* $\Delta\psi$, mitochondrial membrane potential, negative inside; NAC, *N*-acetylcysteine; ROS, reactive oxygen species; TNF-$\alpha$, tumour necrosis factor-$\alpha$.

## 2. Mediators of apoptosis

Several conditions, molecules, or organelles such as oxidative stress, $Ca^{2+}$, proteases, nucleases, or mitochondria are considered participants of apoptosis, but at present it is not always clear whether they are required for or are the consequence of apoptosis. This is due to our incomplete knowledge of the cellular machinery and timing of the various stages of apoptosis. Also, studies with reconstituted systems have shown that characteristic apoptotic responses occur in isolated nuclei as well as in nuclei-free cell fractions. Thus, it is conceivable that some of the conditions or molecules are required to direct the cell to undergo apoptosis, whereas others may be required for its execution. There may also exist cell type-specific requirements. Our understanding of apoptosis is further limited by the fact that until recently most studies were done on cell batches, i.e. relied on bulk measurements. Unless cells are in perfect synchrony, such studies conceivably hide cause/effect relationships.

There is ample evidence that apoptosis is accompanied by oxidative stress (reviewed in [3]). A valuable tool used to elucidate the importance of oxidative stress is the protooncogen bcl-2 (see also below), which stimulates an antioxidative response in cells and prevents apoptosis [4,5]. The importance of oxidative stress as a cause for apoptosis was questioned because cells can undergo bcl-2-inhibitable apoptosis also under very low oxygen tensions [6,7], which supposedly precludes formation of reactive oxygen species (ROS). However, 10 nM molecular oxygen still reacts efficiently in vivo, as revealed in bioluminescence studies with bacteria [8], making the case against ROS involvement in apoptosis weaker.

Also the requirement of $Ca^{2+}$ for apoptosis is controversial (see [9]). Early reports suggested that a rise of the intracellular $Ca^{2+}$ leads to apoptosis via endonuclease activation, and more recent work indicated that apoptosis is accompanied by shifts of $Ca^{2+}$ between various intracellular pools. It is worth noting that cellular $Ca^{2+}$ handling and ROS production are related. Thus, increased mitochondrial $Ca^{2+}$ release followed by reuptake driven by $\Delta\psi$ ($Ca^{2+}$ 'cycling') stimulates ROS production (see below).

Presently, the role of proteases in apoptosis receives much attention (reviewed in [10]). Gene analysis in *C. elegans* identified a key pro-apoptotic gene, ced-3, which encodes a putative cysteine protease that is related to the mammalian protease interleukin-1$\beta$-converting enzyme. Related proteases cleave poly(ADPribose) polymerase, which results in the activation of a $Ca^{2+}/Mg^{2+}$-dependent endonuclease implicated in internucleosomal DNA cleavage characteristic for apoptosis.


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### 3. Bcl-2 links oxidative stress, $Ca^{2+}$, and the mitochondrial membrane potential to apoptosis

Given that bcl-2 elicits an antioxidative response in cells, what are the biochemical mechanism(s) by which bcl-2 prevents apoptosis? We showed [11,12] that one mechanism is the prevention of ROS-induced mitochondrial $Ca^{2+}$ cycling, a process which results in a collapse of $\Delta\psi$ and in cellular ATP depletion. Thus, bcl-2 prevents disturbances of the cellular $Ca^{2+}$ homeostasis and ROS production at the mitochondrial level. Based on these and other findings we suggested [13] that a prooxidant-induced $Ca^{2+}$ release from mitochondria, followed by $Ca^{2+}$ 'cycling' and ATP depletion, is a common cause of apoptosis. Accordingly, maintenance of $\Delta\psi$ stabilises mitochondria and thereby prevents apoptosis. Bcl-2 thus provides the link between the antioxidant defense system, $Ca^{2+}$, and $\Delta\psi$ (reviewed in [14]). In this context it is interesting to recall that many carcinoma cells have an increased $\Delta\psi$ [15].

Another mechanism by which bcl-2 prevents apoptosis could relate to a shift of the NADH/$NAD^+$ ratio in favour of the reduced form: bcl-2 prevents $Ca^{2+}$ release from the endoplasmic reticulum via the inositol triphosphate-insensitive pathway [16], known to be stimulated by $NAD^+$-derived cyclic ADPribose (reviewed in [17]). It is conceivable that bcl-2 prevents this release by shifting $NAD^+$ to NADH, thereby preventing the formation of cyclic ADPribose.

### 4. Nitric oxide and peroxynitrite in apoptosis

It was recently shown in several systems that nitric oxide (nitrogen monoxide, NO•) can cause apoptosis [18–21]. NO•-induced apoptosis can be the consequence of DNA damage and subsequent expression of the tumor suppressor gene p53 [21]. However, according to [22] NO• inhibits apoptosis in lymphocytes. Very recently it was found that also peroxynitrite ($ONOO^-$) induces apoptosis in a time- and concentration-dependent manner [23], and that depending on the concentration of $ONOO^-$ cells die either by apoptosis or necrosis [24].

### 5. Mitochondrial $Ca^{2+}$ in cell death

Attention has been drawn to $Ca^{2+}$-induced cell death many years ago [25]. Excessive intracellular $Ca^{2+}$ is thought to contribute to a final common pathway of cytotoxic events leading to ROS formation, necrosis or apoptosis. These events include overactivation of protein kinase C, $Ca^{2+}$/calmodulin-dependent protein kinase II, phospholipases, proteases, protein phosphatases, xanthine oxidase, endonucleases, and nitric oxide synthase.

Although the exact role of $Ca^{2+}$ in cell killing is unclear, a disturbance of mitochondrial $Ca^{2+}$ handling can be fatal. The normal $Ca^{2+}$ 'cycling' across the inner mitochondrial membrane requires little energy [26]. However, when the $Ca^{2+}$ release pathway is stimulated by prooxidants, 'cycling' may become excessive and lead to loss of $\Delta\psi$, general leakiness of the inner mitochondrial membrane, inhibition of ATP synthesis, mitochondrial damage, and cell death (reviewed in [27]). Accordant with this concept cyclosporine A (CSA), an inhibitor of prooxidant-induced $Ca^{2+}$ release from mitochondria [28], protects against loss of cell viability induced by prooxidants [29] or by NO• [30], and favourably alters liver mitochondrial functions in the postischemic phase at the organ level [31].

### 6. Nitric oxide and the regulation of cytochrome oxidase

The most-cited and best understood physiological target of NO• is guanyl cyclase. NO• stimulates it and thus controls cell functions via cGMP, cGMP-gated channels, cGMP-dependent protein kinases, and phosphodiesterases. However, NO• also binds to cytochrome oxidase and reversibly inhibits mitochondrial respiration by competing with oxygen for this enzyme [30,32]. NO• thereby deenergizes mitochondria and nonspecifically induces $Ca^{2+}$ release from mitochondria. $ONOO^-$, in contrast, induces $Ca^{2+}$ release from mitochondria by activating a $Ca^{2+}$-specific pathway (Schweizer and Richter, manuscript submitted). Concentrations of NO• measured in a range of biological systems are similar to those shown to inhibit cytochrome oxidase and mitochondrial respiration, and inhibition of NO• synthesis results in a stimulation of respiration in many systems. It was, therefore, recently proposed that NO• exerts a good part of its physiological and pathological effects on cells by inhibiting cytochrome oxidase [33].

### 7. Are mitochondria (or parameters which they control) essential for apoptosis? If yes, which mitochondrial parameters?

As mentioned above, ROS and $Ca^{2+}$, two entities presumably important for apoptosis, are linked to mitochondria. These two entities are under certain conditions even directly related: ROS stimulate a specific $Ca^{2+}$ release from mitochondria, and when mitochondria 'cycle' $Ca^{2+}$ excessively their ROS production increases [11,34,35]. Accordingly, tumour necrosis factor-$\alpha$ (TNF-$\alpha$) stimulates ROS production by mitochondria, and the TNF-$\alpha$-induced apoptotic killing of at least some cell types is due to ROS-induced mitochondrial $Ca^{2+}$ cycling since apoptosis is prevented by mitochondrial $Ca^{2+}$ uptake inhibitors [11,12].

The suspected requirement of mitochondria for apoptosis was directly investigated by two groups. In one study cells harbouring respiratory chain-deficient ($\rho^0$) mitochondria were capable of apoptosis and it was concluded that mitochondrial respiration is not required for it [36]. However, it should be remembered that such cells very likely up-regulate glycolysis and have high ATP levels, as indicated by the findings [37–39] that their mitochondria maintain a high $\Delta\psi$, presumably generated by intramitochondrial ATP hydrolysis [39]. The other study used isolated nuclei in combination with various cell fractions [40]. In this reconstituted system, which is believed to be a valid model for apoptosis, mitochondria were indeed required for apoptosis. The exact function of mitochondria was, however, not established and it was postulated that they may produce a hitherto unidentified factor necessary for apoptosis.

A clue as to which mitochondrial parameter(s) may be important for the control of apoptosis comes from the observation that the stabilisation of $\Delta\psi$ either by overexpression of bcl-2 or by the ionophor nigericin, prevents apoptosis induced by TNF-$\alpha$ [12].

### 8. Hypothesis: ATP levels as gauge, or mitochondria as the central switchboard for apoptosis

There is general agreement that apoptosis is an active,

energy-requiring process. We propose that the cellular ATP level is an important determinant for cell death, either by apoptosis or necrosis. This hypothesis is supported by circumstantial evidence, is consistent with most available data, has a corrolary in aging, and is amenable to direct experimental testing. We argue that a cell stays alive as long as a certain ATP level is maintained. When ATP falls below this level apoptosis ensues provided enough ATP is still available for energy-requiring apoptotic processes such as enzymatic hydrolysis of macromolecules, nuclear condensation and bleb formation. Only when there is a severe drop in cellular ATP controlled cell death ceases and ushers in necrosis.

A decreased cellular ATP level is characteristic for cell death, but there is no systematic investigation whether the decrease is the cause or the consequence of cell death. However, it has been shown with six human and murine leukemia/lymphoma cell lines [41] that the expression of the bcl-2 gene and the cellular energy status are highly correlated, and that both parameters are inversely related to the sensitivity for glucocorticoid-induced apoptosis. An ADP/ATP ratio of about 0.2 was the critical discriminator between survival and apoptosis in all cell types. Also, acute inhibition of the mitochondrial respiratory chain induces apoptosis in cells carrying normal mitochondria, but not in $\rho^0$ cells, which maintain their ATP levels by glycolysis [42]. This was taken as support for the argument that active mitochondria are required for apoptosis, and it was suggested that cells with impaired mitochondrial energy metabolism reach an energy threshold which triggers apoptosis. It was similarly argued that aging and age-related diseases are caused by a fall of the energy charge below a threshold (reviewed in [43]).

A critical evaluation whether mitochondria, and which mitochondrial parameter(s), are important for apoptosis could come from the analysis of apoptosis, $\Delta\psi$, mitochondrial $Ca^{2+}$ handling, ROS production, and the cellular ATP level at the single cell level and in selected cell populations.

## 9. New perspectives: single cell measurements

In the last years, flow cytometry contributed greatly to the understanding of apoptosis. In particular, it became possible to determine at the single cell level morphological, biochemical, metabolic and phenotypical changes [44] including those regarding physical modifications of the cell (forward and side scattering), DNA content [45], stainability [46], and strand breaks [47], plasma membrane fluidity [48], phospholipids [49], intracellular pH [50], the activity of the ATP-dependent lysosomal proton pump [51], and cytoskeleton [52].

A growing attention is now devoted to mitochondrial functions during apoptosis. Using the mitochondria-specific probe JC-1 we developed a cytofluorimetric technique to study $\Delta\psi$ in intact cells [53] or in single isolated mitochondria [54], which was applied to rat thymocytes undergoing dexamethasone-induced apoptosis. $\Delta\psi$ is preserved in its early phases [55], and decreases after the onset of DNA alterations, assessed by agarose gel electrophoresis and detection of the DNA content with flow cytometry. A similar, delayed decrease in $\Delta\psi$ was observed in other models of apoptosis, such as that induced by Sendai virus or Herpes virus type I [56] or by 2-deoxy-D-ribose in human lymphocytes [57,58]. In a more rapid model of apoptosis, i.e. that induced by TNF-$\alpha$ in cycloheximide-treated U937 cells [44], the percentage of apoptotic cells 4 h after the addition of TNF-$\alpha$ was similar to that of cells with depolarized mitochondria. *N*-Acetylcysteine (NAC) protected cells against apoptosis and prevented $\Delta\psi$ decrease, suggesting a direct effect of NAC on mitochondria. A protective effect of NAC on both apoptosis and $\Delta\psi$ was also observed in human lymphocytes treated with 2-deoxy-D-ribose [58]. Our data indicate that mitochondria play a complex role during the different phases of apoptosis. Indeed, their functional integrity appears to be important during the early phases, where a variety of energy-requiring intracellular processes occurs. Later on, a functional impairment of mitochondria follows, which likely contributes to the drop of ATP observed when apoptosis has progressed.

At variance with our results, $\Delta\psi$ decrease prior to DNA damage was reported by others [59,60]. These discrepancies could be due to the dye used, i.e. $DiOC_6(3)$, which binds to intracellular membranes other than mitochondria, including the endoplasmic reticulum [61], and is probably an indicator of the 'cytoplasmic membrane potential' [62].

Alterations of the cellular energy charge may play a major role in the decision of the cell to die by apoptosis or necrosis. In this context, analysis of ATP at the single cell level by sorting cells with different $\Delta\psi$ will be of crucial importance. Single cell measurements of $\Delta\psi$ and ATP, applied to the study of apoptosis induced by various stimuli, can help to understand the intriguing relationship between mitochondrial functions and apoptosis.

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