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## Mitochondrial reactive oxygen species in cell death signaling

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**Abstract** - During apoptosis, mitochondrial membranes permeability (MMP) increases and the release into the cytosol of pro-apoptotic factors (procaspases, caspase activators and caspase independent factors as AIF) leads to the apoptotic phenotype. Apart from this pivotal role of mitochondria during the execution phase of apoptosis (documented in other reviews of this issue), it appears that reactive oxygen species (ROS) produced by the mitochondria can be involved in cell death. These toxic compounds are normally detoxified by the cells, failing which oxidative stress occurs.

However, ROS are not only dangerous molecules for the cell, but they also display a physiological role, as mediators in signal transduction pathways. ROS participate in early and late steps of the regulation of apoptosis, according to different possible molecular mechanisms. In agreement with this role of ROS in apoptosis signaling, inhibition of apoptosis by antiapoptotic Bcl-2 and Bcl-x<sub>L</sub> is associated with a protection against ROS and/or a shift of the cellular redox potential to a more reduced state. Furthermore, the fact that active forms of cell death in yeast and plants also involve ROS suggest the existence of an ancestral redox-sensitive death signaling pathway that have been independent of caspases and Bcl-2.

**Key words:** mitochondria; reactive oxygen species; apoptosis; signaling

## 1. Introduction

The Bcl-2/Ced-9 family proteins are involved in positive and negative regulation of apoptotic cell death (review in [1]). Among the anti-apoptotic members, Bcl-2 and Bcl-x<sub>L</sub> are negative regulators of cell death, preventing cells from undergoing apoptosis induced by various stimuli in a wide variety of cell types [2, 3], whereas others, such as Bax and Bid promote or accelerate cell death. In mammals, most of our understanding of Bcl-2 regulation of apoptosis involves its mitochondrial membrane association. Although for a long time apoptosis was considered to be under the control of nuclear events, mitochondria appear today as the central control point of programmed cell death [4-7]. It is now clear that mitochondrial membrane-associated Bcl-2 acts by regulating the release in the cytosol of pro-apoptotic factors usually sequestered in the mitochondrial intermembrane space (reviewed in other articles of this special issue of "Biochimie"). Moreover, inhibition of apoptosis by antiapoptotic Bcl-2 and Bcl-x<sub>L</sub> is associated with a protection against oxidants and/or a shift of the cellular redox potential to a more reduced state. These results have suggested that reactive oxygen species (ROS) could be mediators of apoptosis. However, others data have shown that apoptosis can occur in very low oxygen environments [8, 9], which has cast doubt on this interpretation. A growing number of data show now that ROS, mainly produced by the mitochondria, can be involved in cell death. On the one hand, they can produce an oxidative stress leading to cell destruction, as observed during necrosis or the so-called "post-mitochondrial" phase of apoptosis. On the other hand, in several instances, ROS derived from mitochondria are also involved in the initiation phase of apoptosis contributing to cell death signaling. The present review will focus on the role of mitochondrial ROS in cell death signaling and the possible molecular mechanisms.

## 2. Mitochondria are the major site of ROS production

ROS, such as superoxide anion, radicals, hydrogen and organic peroxides are generated by all aerobic cells as by-products of a number of metabolite reactions and in response to various stimuli [10]. Mitochondria are believed to be a major site of ROS production, according to an endogenous and continuous physiological process under aerobic conditions (figure 1). However, endoplasmic reticulum and nuclear membranes also contain e<sup>-</sup> transport chains that can lose e<sup>-</sup> and generate superoxide radical. Some fatty acid metabolites, such as those derived from arachidonic acid by the lipooxygenase pathway, are also ROS.

The physiological activity of the respiratory chain leads to the production of semi quinones, a potential source of ROS [11]. Indeed, the respiratory chain produces ROS at Complex I (NADH/Ubiquinone oxidoreductase) and Complex III (ubiquinol/cytochrome c oxido-reductase [12-14]. The ubiquinone site in complex III appears as the major site of mitochondrial ROS production: this site catalyzes the conversion of molecular oxygen to superoxide anion radical (O<sub>2</sub><sup>-</sup>) by a single electron transfer to molecular oxygen (figure 1). Moreover, the inhibition of the respiratory chain, due to a lack of oxygen or to an inhibitor, such as cyanide or antimycin A, increases the ubisemiquinone free radical level in the normal catalytic mechanism of complex III [14].

In aqueous solutions, O<sub>2</sub><sup>-</sup> is moderately reactive but can generate other potent ROS such as H<sub>2</sub>O<sub>2</sub> by dismutation. About 1-5% of mitochondrial O<sub>2</sub> consumption leads to H<sub>2</sub>O<sub>2</sub> production [15]. A reductive homolytic cleavage of H<sub>2</sub>O<sub>2</sub> produces the highly oxidative and cytotoxic hydroxyl radical (OH<sup>•</sup>) according a metal-dependent breakdown. This highly reactive radical is assumed to be

directly responsible for most of the oxidative damages leading to the non-physiological necrosis [16]. Alternatively, mitochondria superoxide may react with nitric oxide to produce peroxynitrite [17], a potent oxidant, which causes irreversible inhibition of mitochondria respiration and damage to mitochondria components (complexes I, II, IV and V, creatine kinase, aconitase, membranes, DNA, SOD...). It induces cell death in several cell types [18-21]. Nitric oxide (NO) can also promote or inhibit apoptosis, according the cell type, the rate of production and the interaction with other intracellular molecules [22].

### **3. Oxidative stress**

An excess of ROS in mitochondria causes an oxidative stress, with an enhanced activity of the antioxidant defense system and mitochondrial damage. Several situations, such as dysfunctional complex I [23], chemical poisoning and ischaemia followed by reperfusion, may subject tissues and cells to oxidative stress. The main targets of ROS in mitochondria are the protein components of the membranes and the polyunsaturated fatty acids. In membranes, ROS and lipids affect cysteine residues (sulfhydryl groups), causing intramolecular cross-linkings and formation of proteins aggregates. OH<sup>•</sup> radicals can initiate lipid peroxidation and generate peroxy- and alkoxy radical intermediates. Oxidants increase the release of calcium from mitochondria, thus stimulating calcium-dependent enzymes, such as proteases, nucleases and phospholipases. Because of the absence of mitochondria DNA-protecting proteins, the low efficiency repair mechanism and the proximity of the respiration chain, mtDNA is a privileged target for ROS [24].

### **4. Protection of the cell**

To prevent oxidative damage, mammalian cells have developed a complex antioxidant defense system that includes non-enzymatic antioxidants (e.g. glutathione, thioredoxine) as well as enzymatic activities (e.g. catalase, superoxide dismutase (SOD)) [25].

Mitochondria possess an antioxidant system with the superoxide dismutase (SOD), NADH and a complete glutathione redox system, which is formed of glutathione reductase, reduced glutathione (GSH), and glutathione peroxidase (figure 1). This system allows the reduction of oxidants, such as hydroperoxide. As mitochondria are generally devoid of catalase, the hydroperoxide detoxification mainly relies on GSH peroxidase. Various chemicals are known to induce oxidative stress, either as the result of an enhanced free radical production (e.g. xenobiotics utilizing cytochrome P450 for detoxification) or by impairment of their destruction provoked by glutathione depletion. Indeed, irreversible injury may occur following a depletion of the mitochondrial pool of glutathione [26]. Mitochondrial GSH plays an important role in preserving mitochondria membrane integrity and in assuring the reduced state of the intramitochondrial protein thiol groups. Moreover, although coenzyme Q10 can also act as a pro-oxidant [27], it can prevent ROS accumulation occurring under unfavorable condition [28]. Last, macrophages of uncoupling protein-2 deficient mice generate more ROS [29], which is in agreement with the idea that a mitochondrial mild uncoupling and non-coupled oxidations could safe guard cells from high oxygen level and ROS generation [30].

### **5. Physiological role**

Cells possess multiple sites for ROS production and a few mechanisms for their degradation. ROS are also involved in different physiological processes, as mediators in signal transduction pathway, activating proteins such as tyrosine kinase, mitogen-activated protein kinase system or small Ras proteins. Current evidence indicates that different stimuli use ROS as signaling

messengers to activate transcription factors, such as AP-1 and NF- $\kappa$ B, and induce gene expression [31]. Thus, they are responsible for the inducible expression of genes associated with inflammatory and immune responses. Additionally, ROS may have divergent effects according to the cell type, as they can function as mitotic stimuli [32], cellular senescence inducers [33], inhibitors of drug-induced tumor cell death [34] or cell death mediators [35, 36]. The large diversity of their effects on cells suggests that ROS not only cause unspecific changes in macromolecules, but also modify a number of signaling pathways.

The ability of oxidative stress, which is an excessive accumulation of ROS, to provoke necrotic cell death as a result of massive cellular damages associated to lipid peroxidation and alterations of proteins and nucleic acids, is well documented for a long time [37]. When free radicals generation exceeds the cellular capacity to detoxify them, cellular protein and DNA damage may occur, leading to the cell death. In this point of view, aerobic cells appear as being under a continual "oxidative siege", their survival depending on a balance between ROS and antioxidants.

The possible implication of ROS as signaling molecules in more physiological deaths such as apoptosis is a more recent concept. First evidence suggesting the involvement of mitochondrial ROS in cell death arose from the study of the TNF- $\alpha$ -induced cytotoxicity [38, 39]. The observed inhibition of the mitochondrial respiratory chain was assumed to result in the over production of ROS which would act as mediators of the death signaling pathway [40]. There is now mounting evidence that these compounds may be central in the cell death transduction pathways: 1) the addition of ROS or the depletion of endogenous antioxidants can promote cell death [41-44]; 2) some antioxidants, such as thioredoxine or N-acetylcystein, act as intracellular ROS scavengers, and can inhibit activation of caspases and subsequent steps leading to the apoptotic cell death. In these models, ROS could be considered as major mediators, and not only the source of direct damage to DNA, proteins and lipids [45-49]; 3) increases in intracellular ROS are sometimes associated with PCD [50-55]; 4) MnSOD overexpression restores mitochondrial transmembrane potential [56] and inhibits the cell death caused by an inhibition of the respiratory chain [57]; 5) Cu/Zn SOD overexpression delays apoptosis by scavenging of O<sub>2</sub><sup>-</sup> in neuronal cells deprived of nerve growth factor [47] and prevents early release of cytochrome c in ischemic brain [58]; 6) mitochondrial phospholipid hydroperoxide glutathione peroxidase overexpression inhibits increase of ROS and apoptosis [59] and 7) although some PCD can occur in very low oxygen environments [8, 9], ROS might be produced in such condition [60]. Thus, ROS could be considered as major mediators, and not only the source of direct damage to DNA, proteins and lipids [61]

## **6. ROS participate in early and late steps of the regulation of apoptosis**

Since the discovery of ROS involvement in TNF- $\alpha$ -induced killing, their contribution to the activation of the execution machinery was extended to PCD triggered by a wide range of apoptosis inducers [62]. Notably, ROS accumulation preceded mitochondrial membrane alterations, nuclear condensation and other typical apoptotic events. This point can be illustrated by TNF- $\alpha$  induced apoptosis. Indeed, experiments using antioxidants show that ROS act upstream of mitochondrial membrane depolarization [63], Bax relocalization, Cytochrome c release, executing caspases activation and nuclear fragmentation (figure 2). Similarly, an early increase in ROS levels has been found to precede mitochondrial membranes permeabilization, and in some case to be independent of caspases, in various models including apoptosis of Retinitis pigmentosa photoreceptors [41, 54] and

apoptosis induced by Fas [64], p53 [65], ischaemia [66], DNA alkylation [67] or etoposide (Vayssière *et al.* unpublished results).

Some data raise the possibility that ROS are also required for the execution of the death program [68, 69]. This must be cautiously considered inasmuch as, in the majority of these systems, it is difficult to ascertain that the observed ROS accumulation corresponds to a causal effect and is not a side effect of the other changes accompanying the killing process [70]. For example, release of cytochrome c triggers accumulation of ROS and increases the oxidation state of the apoptotic cells. Moreover, in these cases, ROS increase most often arises during the later stage of the death program, i.e. during the destruction phase when the cell is broken down, and may be associated with a necrotic type terminal degradation of the cell. Exogenous sources of ROS such as hydrogen peroxide can induce PCD or necrosis depending upon the dose added [44]. So a burst in ROS, in response to a dramatic perturbation of the physiology of the dying cell, could convert the late PCD steps into necrotic death. Therefore, it appears that at any moment the level of intracellular ROS can determine the fate of the cell: low levels of ROS can induce PCD while accumulation of high levels promotes necrosis or can lead PCD-committed cells toward necrotic-like destruction.

## **7. Origin of apoptosis-mediated ROS**

Although fatty acid metabolites, such as those produced from arachidonic acid by the lipoxygenase pathway, may be mediators of apoptosis [71], it was established that both ROS accumulation and programmed cell death process require the presence of a functional mitochondrial respiratory chain in most ROS-dependent cell death systems [40, 51, 63, 72]. Indeed, it was shown that an upstream inhibition, with chemical compounds acting on complex I [39, 51], or an elimination of the electron transfer chain [40, 63, 72], by depletion of the mtDNA, prevent ROS accumulation and consequently protect cells against PCD. Another indirect argument is provided by the scavenger role of mitochondrial glutathione in the regulation of ROS-mediated PCD [73]. The ubiquinone site in complex III appears as the major site of mitochondrial ROS production as this site catalyzes the conversion of molecular oxygen to superoxide anion which can lead to the formation of other potent ROS such as hydrogen peroxide and hydrogen radicals (figure 1). The inhibition of the respiratory chain, due to a lack of oxygen or to an inhibitor, such as cyanide or antimycin A, increases the ubisemiquinone free radical level in the normal catalytic mechanism of complex III [14]. Such a model is supported by the observed potentiation of cell death processes in ROS-dependent PCD when electron flow was inhibited distal to the ubiquinone pool.

## **8. Mechanisms of ROS signaling**

The involvement of mitochondrial ROS in some cell death transduction pathways leads to the fundamental questions concerning, on the one hand, the causal event of the increased ROS generation and, on the other hand, the molecular mechanisms underlying the ROS signaling. Two viewpoints must first be considered to address the question of the origin of ROS accumulation, which can indeed result from an increased production or from a reduced scavenging by the cellular detoxifying systems. Much of the available data converge to the hypothesis that ROS increases are the consequence of an impairment of the mitochondrial respiratory chain [39, 50, 51, 60, 70, 74]. In agreement with the above considerations, the observed alterations are distal to the ubiquinone site of the complex III, but the origin of these electron flow disturbances are not clear. The only strong evidence comes from the study of ceramide-induced PCD, in which an increased H<sub>2</sub>O<sub>2</sub> production

was linked to mitochondrial  $\text{Ca}^{2+}$  homeostasis perturbation as inhibition of the mitochondrial  $\text{Ca}^{2+}$  uptake was shown to abolish both ROS accumulation and cell death.

Beside the question of the process of mitochondrial ROS accumulation, arises the problematic concerning the targets of these compounds or more precisely: how can they mediate PCD? Unlike fatty acid metabolites which harbor specific reactivity and are known to mediate particular signals from surface receptors, mitochondrial ROS are characterized by a lack of biological specificity or even an extreme reactivity, as for the hydroxyl radical: these are all features contrary to the requirements of a specific signaling role [62]. In this way, a direct influence of ROS on PCD process would be correlated to a general damaging effect on cellular structures resulting in necrotic cell death, or perhaps to a more limited action on mitochondria, their site of production, which in turn could activate some mitochondria-dependent downstream cascades leading to PCD. Another alternative explanation would be to consider that the major effect of an increased ROS production is the subsequent imbalance of intracellular redox status, i.e. an enhancement of the cellular oxidative tonus, and that in fact the oxidative stress is the central common effector of PCD. Hence, ROS accumulation would only be one way which leads in some PCD to an oxidative status. Anti-Fas/APO-1 antibody or IL-3 withdrawal-induced PCD represent good illustrations of this model [75, 76]. Indeed, no ROS accumulation can be measured in these two systems and anaerobic cultured cells deprived of IL-3 still undergo PCD [9, 77]. However, an oxidative stress can be shown in these models as a depletion of glutathione (GSH), a non-enzymatic cellular antioxidant, as a result of a rapid and specific efflux of glutathione, an event that takes place at the very beginning of the apoptotic process [75, 76]. Moreover, it has been shown that Bcl-2 can protect cells from PCD by shifting the cellular redox potential to a more reduced state (see below). However, the observation that oxidation of thiols other than glutathione can mediate induction of PCD suggest that the intracellular thiol redox status would be the real key factor of the cell death signaling pathways [42, 43, 78, 79]. In this model, the redox state of glutathione or other cellular antioxidants such as thioredoxine, would be in equilibrium with that of thiols resident in some redox sensitive crucial components of the execution machinery [80]. Pro-apoptotic members of the Bcl-2 family might be targets for such a regulatory mechanism. Indeed, in healthy cells these proteins are cytosolic, whereas upon a death they redistribute to mitochondria, promoting cell death [81, 82]. One could imagine that such a conformational change is induced by ROS. It has also recently been proposed that ROS could be responsible for a decrease in bcl-xL mRNA levels [55].

Where do ROS fit this thiol hypothesis? In this putative model, an increased production of mitochondrial ROS would result, either by a direct modification of the thiols or indirectly via a depletion of the intracellular antioxidant pool, in a shift of the redox state of the sensor SH groups to a more oxidized state. The nature of the ROS and the level of the intracellular antioxidant defenses would determine in which way regulatory components are activated to commit cells to PCD. In a hypothetical model, mitochondrial ROS modify the membrane permeability leading to the release of pro-apoptotic factors and/or activate directly executing caspases.

## **9. Inhibition of apoptosis by Bcl-2 and Bcl-x<sub>L</sub> is associated with a protection against ROS and/or a shift of the cellular redox potential to a more reduced state**

Several lines of evidence support the idea that Bcl-2, in addition to its role on the regulation of the permeability of the outer mitochondrial membrane, might act as an antioxidant partner to block a putative ROS-mediated step in the cascade of events required for apoptosis. For example, during

TNF- $\alpha$  induced apoptosis, which involves ROS signaling [63], Bcl-2, as antioxidants do, prevents ROS accumulation and the subsequent events (mitochondrial membrane depolarization, Bax relocalization, Cytochrome c release, executing caspases activation and nuclear fragmentation) (compare figure 2 and 3). However, the way by which Bcl-2 protects from ROS remains unclear. In some systems, Bcl-2 (or Bcl-x<sub>L</sub>) appears to influence the generation of ROS [42, 49], while in other cases it does not affect ROS production but does prevent oxidative damage to cellular constituents [83-85]. It has also been proposed that it functions as a pro-oxidant and influences the levels of ROS, inducing in this way endogenous cellular antioxidants [86]. However, bcl-2 knock-out in mice genders perturbation of oxidant metabolism and antioxidant status in some tissues [87].

Since apoptosis might be modulated by redox sensitive proteins, the effect of Bcl-2s on cellular redox potential has been studied. Activities of antioxidant enzymes and levels of glutathione and pyridine nucleotides have been measured in pheochromocytoma PC12 and the hypothalamic GnRH cell line GT1-7 cells transfected with *bcl-2* [88] Both cell lines overexpressing bcl-2 had increased total glutathione levels and reduced ratios of oxidized glutathione to total glutathione. In addition, the NAD<sup>+</sup>/NADH ratio was two- to threefold less than that of control cell lines while they had approximately the same level of catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase activities as control cells. These results indicate that the overexpression of *bcl-2* shifts the cellular redox potential to a more reduced state, without consistently affecting the major cellular antioxidant enzymes. Furthermore, depleting cellular thiols reversed the resistance to radiation in Bcl-2 expressing lymphoma cell lines [78].

In the same way Bax and Bcl-x<sub>L</sub> were shown respectively to increase and decrease GSH levels [76] without modifying the activities of superoxide dismutase, catalase, and glutathione peroxidase or glutathione reductase. These results suggest a possible role for GSH in the mechanism by which Bax and Bcl-x<sub>L</sub> control cell death.

Thus, both Bcl-2 and Bcl-x<sub>L</sub> can protect cells from apoptosis by shifting the cellular redox potential to a more reduced state. Assuming that mitochondrial thiols constitute a critical sensor of the cellular redox potential during apoptosis [79], these effects could be at the mitochondrial level.

## **10. ROS, plants PCD and Yeast pseudo-apoptosis**

In plants, ROS are also involved in some PCD pathways, such as the synchronous programmed death of petal cells [89], the hypersensitive response (HR) of plants resistant to microbial pathogens [90], or the hormonally regulated cell death pathway in barley aleurone cells [91]. More surprising is the occurrence of a ROS associated apoptosis-like process in unicellular eukaryotic cells like yeasts [92]. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* yeast species do not contain endogenous caspases or Bcl-2s gene [93-95] and their cytochrome c has been shown to be ineffective to induce nuclear apoptosis in acellular assay [96]. However, several data obtained with *S. cerevisiae* suggest the existence of yet unknown PCD pathways, independent of Bcl-2s and caspases families, in which the place and the role of mitochondria are not fully understood. The *cdc48*<sup>S565G</sup> mutant show typical markers of apoptosis, including exposure of phosphatidylserine, chromatin condensation and fragmentation, DNA fragmentation, and formation of minicells, which look like apoptotic bodies and it has been recently observed some markers of oxidative stress and apoptosis in aged mother cells of *S. cerevisiae* [97]. The heterologous expression of mammalian pro-apoptotic genes (*ced-4*, *bax*, *bak*) provokes mitochondrial dysfunction and the death of *S. cerevisiae* and *S. pombe* cells [93, 95, 98-101]. Several authors reported that this induced-cytotoxicity was characterized by typical phenotypic markers of



apoptosis [95, 101], identical to those observed in the pseudo-apoptotic mutant *cdc48<sup>S565G</sup>* [92], and other features, such as release of cytochrome c [102]. Moreover, as observed in mammals, coexpression of Bcl-2 or Bcl-x<sub>L</sub> with Bax abolishes the pseudo-apoptotic phenotype and the cell death [101-106]. Since on the one hand, Bax cytotoxicity is increased under respiratory condition [107], and, on the other hand, yeast mutants lacking superoxide dismutase were partially rescued by expression of Bcl-2 [42, 108], it is tempting to speculate that a mitochondrial ROS production is involved in the pseudo-apoptotic process. In agreement with this hypothesis, the apoptotic-like features observed in these yeast strains are accompanied with ROS accumulation [109]. Moreover, low concentration of H<sub>2</sub>O<sub>2</sub> treatment or glutathione depletion could induce the pseudo-apoptosis in yeast [109]. The induced-cell death is not due to a direct cell damage, but requires an active cooperation of the cell machinery. In all these models, ROS accumulation seems to be necessary and sufficient to lead to the pseudo-apoptotic phenotype. These observations suggest the existence, in yeasts, of an ancestral redox-sensitive apoptosis pathway, which is independent of caspases and Bcl-2.

## 11. Conclusion

In conclusion, mitochondria are involved in the decision of cells to survive or not at several levels (figure 4). First, mitochondria can activate the cell death machinery by releasing in the cytosol pro-apoptotic factors such as procaspases, caspase activators (i.e. cytochrome c and Smac/Diablo) or caspase independent factors as AIF. Furthermore, the importance of mitochondria in apoptosis has been reinforced by studies showing the contribution of reactive oxygen species in the cell-death signaling. The mechanism of ROS signaling is still unknown, but it could be speculated that they act by modifying the activity of some apoptotic key regulators, such as proteins of the Bcl-2 family. If the primer function of apoptosis was possibly to clear ROS-producing cells from tissues [110, 111], mitochondrial ROS production are used as active mediators in the regulation of apoptosis at different levels. Moreover, the fact that pseudo-apoptosis phenotype in yeast invariably involves ROS [109] points out these compounds as major evolution-conserved regulators of cell death.

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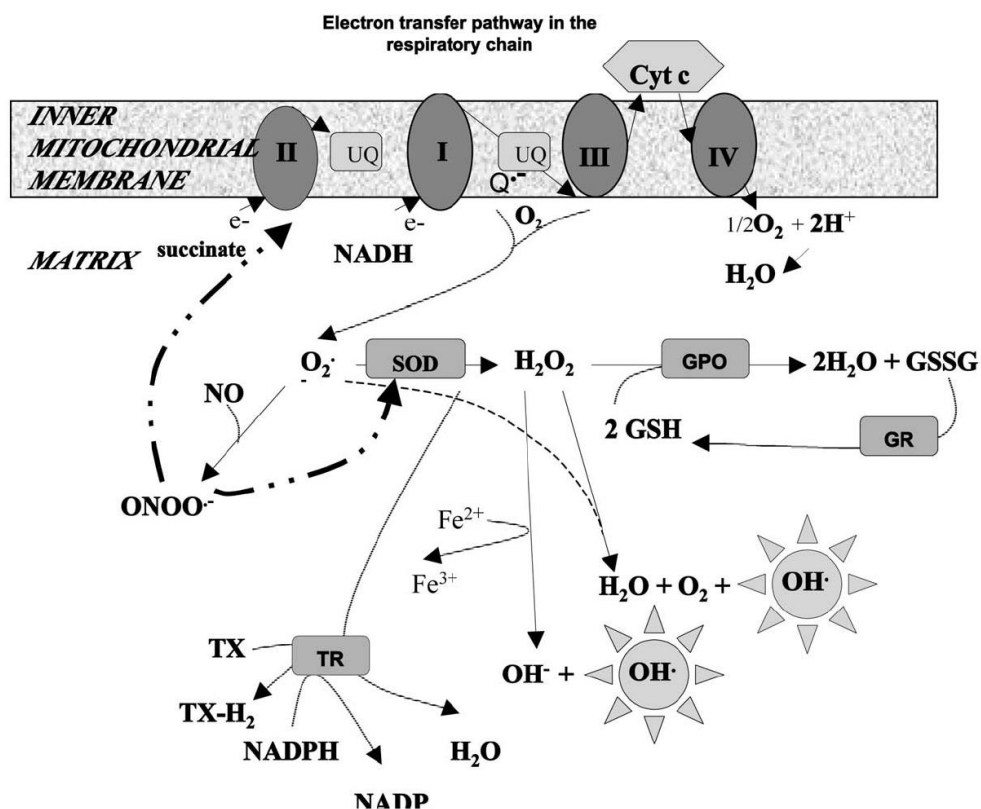
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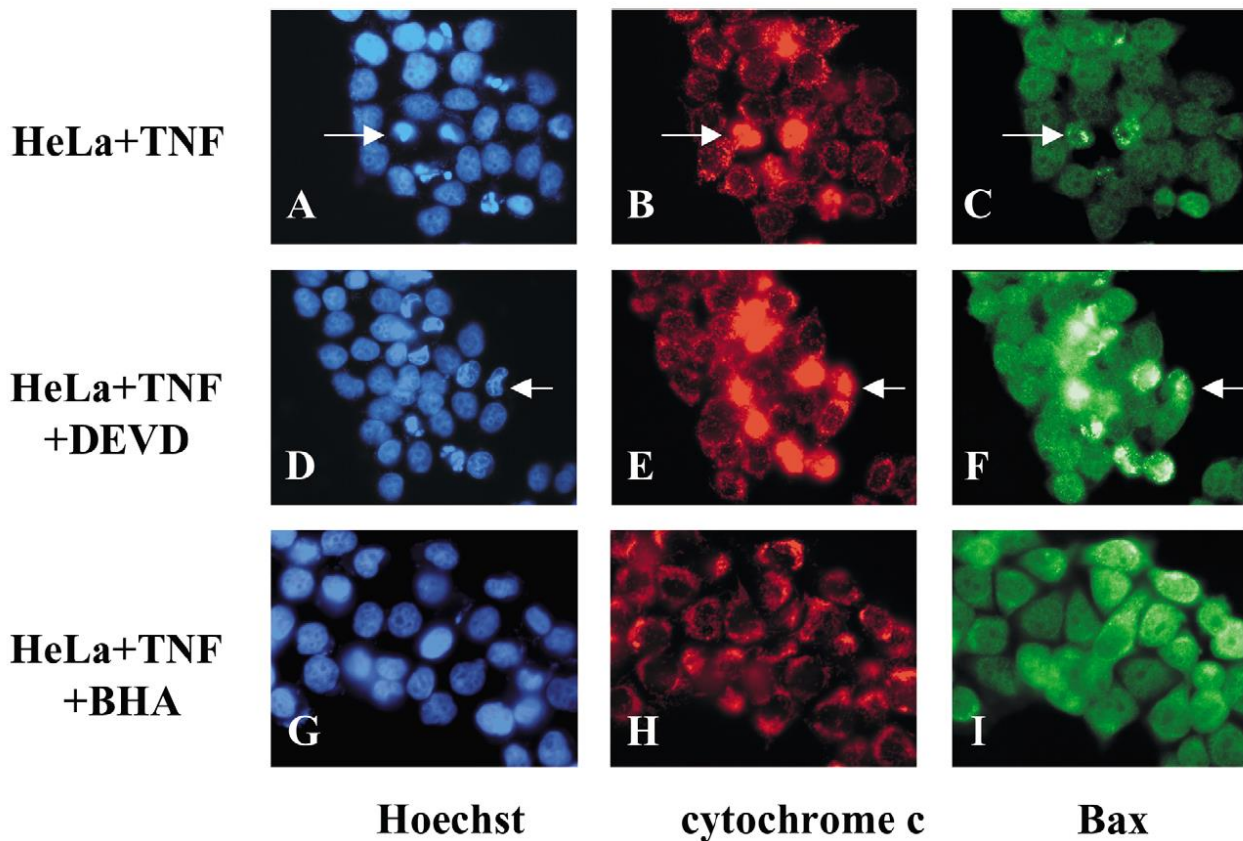
**Figure 1: Schematic representation of the formation and the fate of superoxide ions ( $O_2^{\cdot-}$ ) in mitochondria** (adapted from Fleury *et al.* [112]).

ROS can be generated by various enzymes (xanthine oxidase, lipoxygenases, NADPH oxidases,...) or during the mitochondrial oxidative phosphorylation at complex I and complex III (see text). Complex I (NADH-ubiquinone oxidoreductase) catalyzes the following reaction with electron transfer:  $NADH + Q + nH^+_{(matrix)} \rightleftharpoons NAD^+ + QH_2 + (n-1)H^+_{(intermembrane\ space)}$ , where Q is the ubiquinone Q10. The ubiquinone site (UQ) in complex III catalyzes the conversion of molecular oxygen to superoxide anion radical ( $O_2^{\cdot-}$ ) by a single electron transfer to molecular oxygen.  $O_2^{\cdot-}$  are converted to hydrogen peroxide ( $H_2O_2$ ) by the superoxide dismutase (SOD).

$H_2O_2$  detoxification normally occurs according two different reactions, either by the thioredoxine reductase (TR), which reduces thioredoxine (TX  $\rightarrow$  TX- $H_2$ ), or by reacting with glutathione (GSH) by glutathione peroxidase (GPO). The latter reaction produces water and oxidized glutathione (GSSG), and GSSG is recycled to GSH by glutathione reductase (GR). Glutathione is mainly maintained in a reduced state, via NADPH generation in the reversible non-oxidative part of the pentose phosphate pathway [113]. When GSH level is low,  $H_2O_2$  can react with  $Fe^{2+}$  to produce  $OH^-$  and the harmful hydroxyl anions ( $OH^\cdot$ ). Alternatively,  $H_2O_2$  reacts with  $O_2^{\cdot-}$  producing water, molecular oxygen and  $OH^-$ .

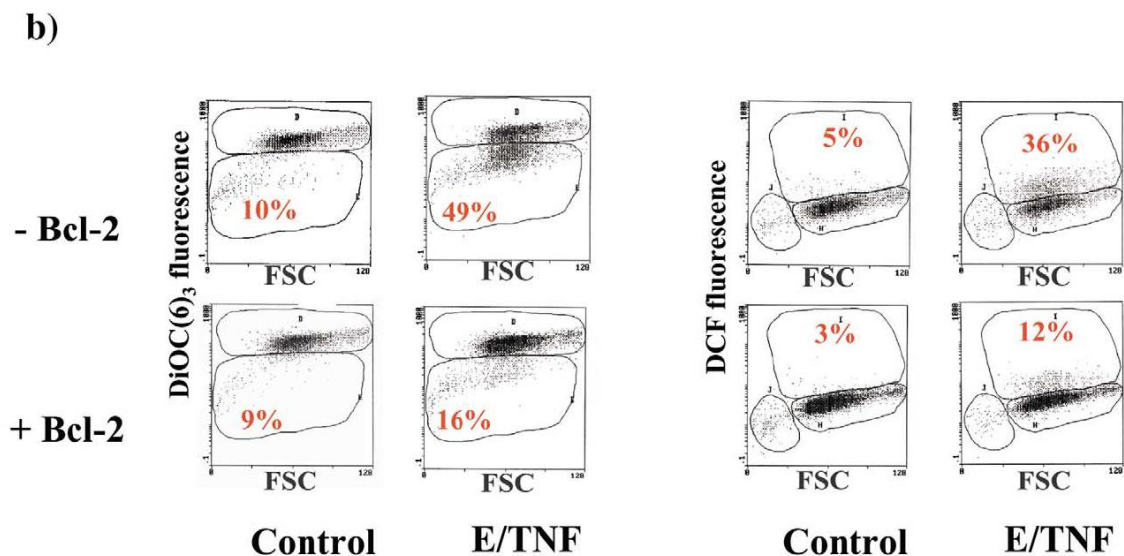
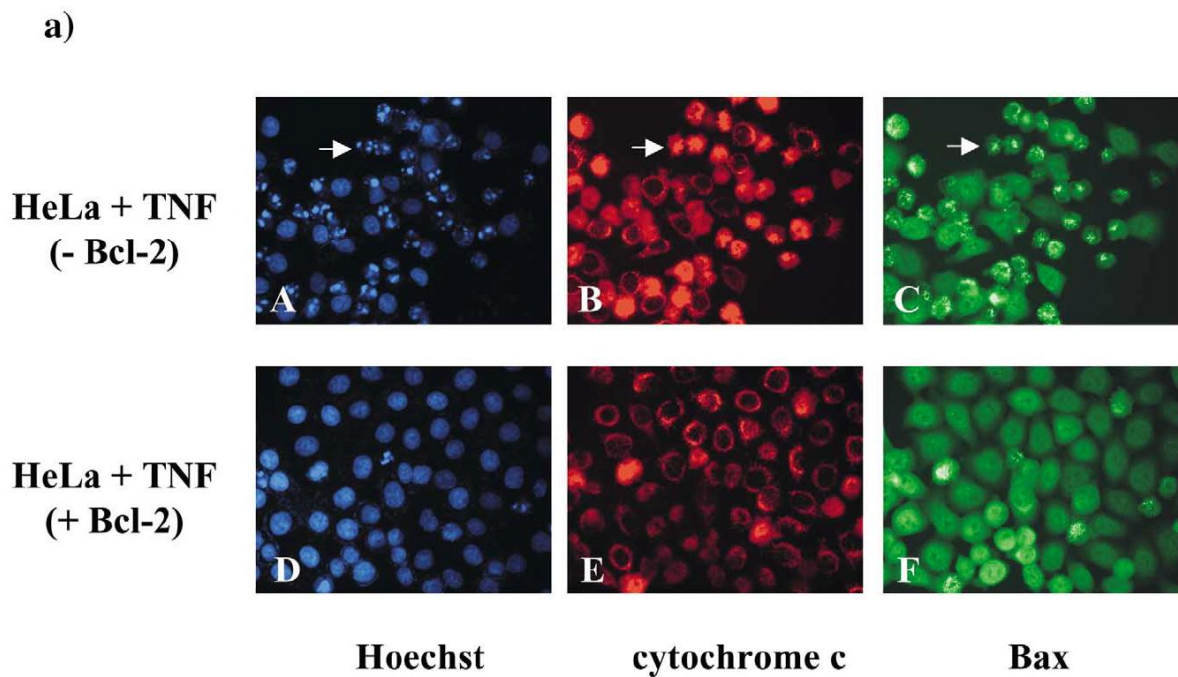
Superoxide may also react with nitric oxide (NO) to produce peroxynitrite ( $ONOO^-$ ), a potent oxidant, which causes irreversible inhibition of mitochondria respiration and damage to mitochondria components (complexes I, II, IV and V, creatine kinase, aconitase, membranes, DNA, SOD,...).

The complexes of the electron transfer chain in the inner mitochondrial membrane are numbered from I to IV. The electron ( $e^-$ ) pathway through the respiratory chain is indicated by arrows. Cyt c refers to cytochrome c.



**Figure 2: Effect of antioxidant on Bax and cytochrome c localization in HeLa cells undergoing TNF- $\alpha$ -induced apoptosis.**

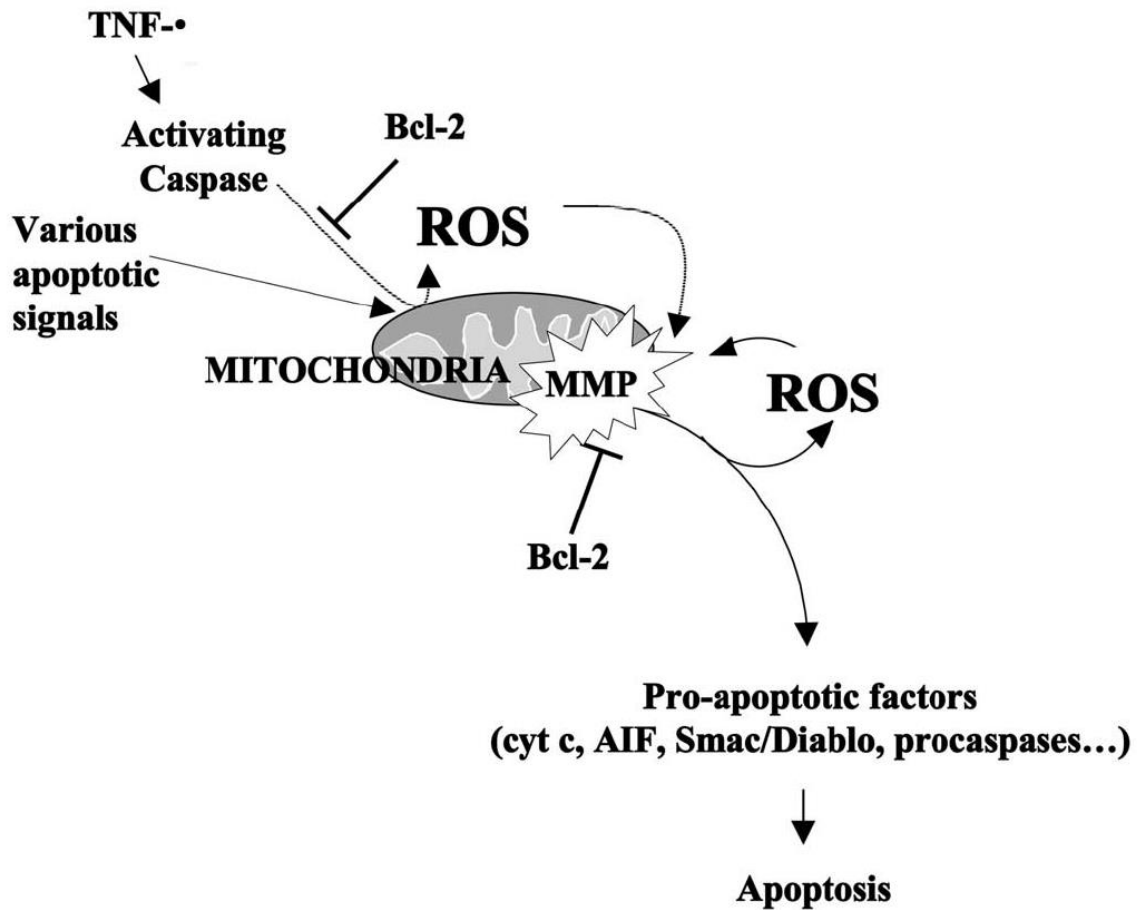
Cells treated with E/TNF (5ng/ $\mu$ l of TNF- $\alpha$  plus 1 $\mu$ g/ml of emetine) for 6 h in the absence (A-C) or presence 300  $\mu$ M DEVD-cho (D-F) or 200  $\mu$ M BHA (G-I) were double immunostained for cytochrome c (B, D and H) and Bax (C, F and I). Nuclei were visualized by Hoechst 33258. Arrows indicate apoptotic cells. DEVD-cho is an inhibitor of executing caspases. BHA is a potent antioxidant (similar results are obtained with other antioxidants, such as NDGA). The Bax N20 pAb (Santa Cruz biotechnology) was used in all cases.



**Figure 3: Effect of Bcl-2 on mitochondrial events occurring during TNF- $\alpha$ -induced apoptosis of HeLa cells.**

**Part a: Effect of Bcl-2 on the mitochondrial membrane potential and ROS accumulation.** Panels depict typical cytograms (FSC versus fluorescence) obtained with flow cytometric analysis of Control or E/TNF treated HeLa cells that overexpress (bottom) or not *bcl-2* (top). The mitochondrial membrane potential, measured by the DiOC<sub>6</sub>(3) fluorescence (left panel), and the ROS production, measured by the DCF fluorescence (right panel), are reported. Values of FSC (Forward angle Scatter) correlate to the size of the cells.

**Part b: Effect of Bcl-2 on Bax and cytochrome c localization in cells undergoing TNF- $\alpha$ -induced apoptosis.** HeLa cells overexpressing (D-F) or not (A-C) a tetracycline-regulated inducible *bcl-2* gene were treated with E/TNF for 6 h. Cells were double immunostained for cytochrome c (B, D and H) and Bax (C, F and I), and nuclei were visualized by Hoechst 33258. Arrows indicate apoptotic cells.



$\alpha$

**Figure 4: Schematic model of the roles of mitochondrial ROS in apoptosis**

Numerous signals can lead to apoptosis and the induction pathways seem to converge to events leading to an increase in mitochondrial membranes permeability (MMP) and the release of pro-apoptotic factors from the mitochondria. Before these events, in response to numerous apoptosis inducers, among which TNF- $\alpha$ , mitochondrial ROS production can contribute to apoptosis signaling during the induction phase. ROS accumulate as a result of a dysfunction in the mitochondrial respiratory chain. ROS also intervene in later steps in an amplifying loop, acting on mitochondrial membrane permeability, and contributing then to the activation of execution caspase.