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Mitochondrial Events in the Life and Death of Animal Cells: A Brief Overview

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Traditionally, mitochondria have been viewed as the "powerhouse" of the cell, i.e., the site of the oxidative phosphorylation machinery involved in ATP production. Consequently, much of the research conducted on mitochondria over the past 4 decades has focused on elucidating both those molecular events involved in ATP synthesis by oxidative phosphorylation and those involved in the biogenesis of the oxidative phosphorylation machinery. While monumental achievements have been made, and continue to be made, in the study of these remarkable but extremely complex processes essential for the life of most animal cells, it has been only in recent years that a large body of biological and biomedical scientists have come to recognize that mitochondria participate in other important processes. Two of these are cell death and aging which, not surprisingly, are related processes both involving, in part, the oxidative phosphorylation machinery. This new awareness has sparked a new and growing area of mitochondrial research, that has become of great interest to a wide variety of scientists ranging from those involved in elucidating the role of mitochondria in cell death and aging to those interested in either suppressing or facilitating these processes as it relates to identifying new therapies or drugs for human disease. It is the purpose of this brief introductory review to provide an overview of those mitochondrial events involved in the life and death of animal cells and to indicate how these events might relate to the human aging process. Much more is known, much remains controversial, and even more remains to be learned as indicated in the excellent set of minireviews that follow.

KEY WORDS: Cell death; aging; necrosis; apoptosis; mitochondria; oxidative phosphorylation; electron transport chain; ATP synthase; cytochrome *c*; mitochondrial DNA; reactive oxygen species (ROS).

INTRODUCTION

Mitochondria, the site of most ATP production of animal cells, are intracellular organelles consisting of four well-defined compartments (Fig. 1A), an inner membrane, an outer membrane, a matrix space enclosed by the inner membrane, and an intermembrane space between the inner and outer membranes (Lehninger, 1964; Schnaitman and Greenawalt, 1968; Munn, 1974). These organelles dominate the intracellular scenery of most animal cells as their total number per cell usually far exceeds that of any other organelle. Liver and heart cells, for example, contain more than a thousand mitochondria per nucleus. Today, we know that mitochondria not only play a major role in the lives of animal cells but also a major role in their death and that these events may have direct relevance to aging and other human diseases (reviewed in Wallace, 1999; Ozawa, 1997, 1999; Cortopassi and Wong, 1999). The purpose of this introductory minireview is threefold. The first is to discuss briefly those mitochondrial events that occur during cell life with emphasis on both ATP synthesis by oxidative phosphorylation and the production of reactive oxygen species. Within the context of this discussion, attention will be focused

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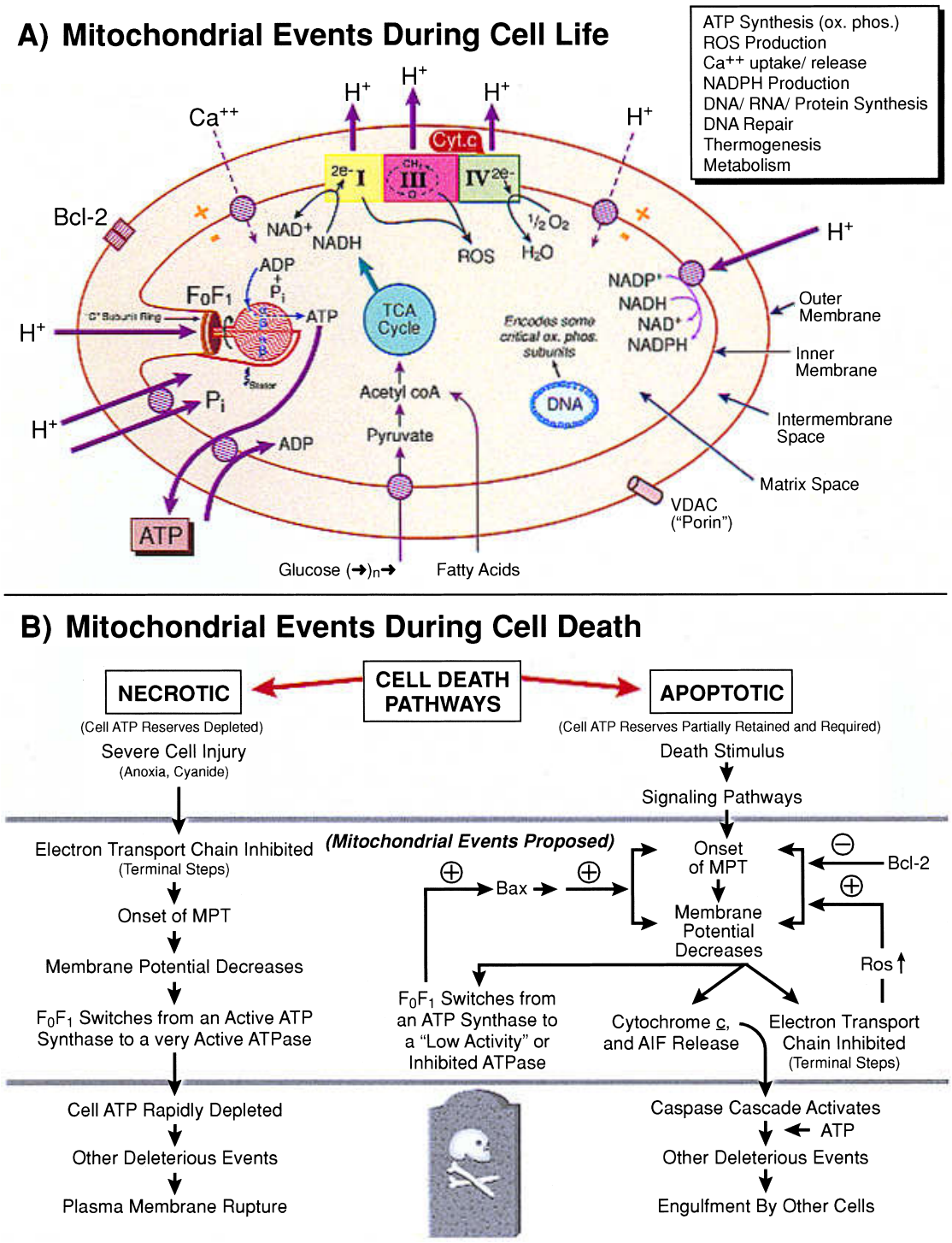


Fig. 1. (A) Mitochondrial events which take place during the life of animal cells. As indicated in the figure, there are a number of events that take place within the mitochondria of animal cells. Of these, the two that comprise the focus of this review are ATP synthesis by the process known as oxidative phosphorylation and the production of reactive oxygen species (ROS). These processes are discussed at length in the text. (B) Mitochondria events which are believed to occur during cell death. There are two types of cell death pathways that are widely discussed, one involving necrosis where the cell ATP levels become depleted and the plasma membrane ruptures. This type of cell death pathway is induced by serious cell injury such as that which occurs during a stroke, heart attack, or cyanide poisoning. The other type of cell death pathway is that involving apoptosis. Here, the ATP level of the cell is retained, at least in part, as ATP is essential for at least one of the major steps in the pathway. In the terminal steps of this pathway, the cell remnants are englufed by neighboring cells. These two types of cell death pathways are discussed at length in the text.

on what happens to these processes when mitochondria are injured, as the consequences of such are believed to have direct relevance both to pathways of cell death and to the aging process. Second, those mitochondrial events believed to be involved in pathways for cell death will be summarized and some of the controversies noted. Finally, how the above processes may relate to those events that occur in mitochondria during the aging process will be considered.

MITOCHONDRIAL EVENTS INVOLVED IN CELL LIFE

Oxidative Phosphorylation

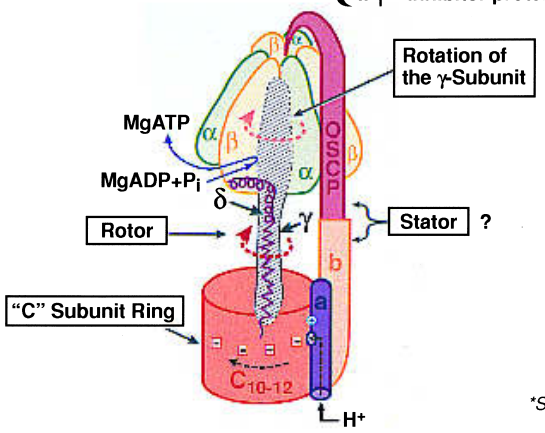
As shown in Fig. 1A, mitochondria of living cells participate in a number of different processes. Among these are ATP synthesis by oxidative phosphorylation, the production of reactive oxygen species (ROS), Ca^{2+} uptake and release, the production of NADPH, the synthesis of DNA, RNA, and protein, DNA repair, thermogenesis (in some cases), and metabolic pathways. Of these, the major mitochondrial event involved in cell life is ATP synthesis by oxidative phosphorylation (reviewed in Hatefi, 1985; Pedersen, 1997; Saraste, 1999). This unusually complex process occurs in the inner membrane and requires an electron transport chain to generate an electrochemical proton gradient, two key transport systems for the entry of ADP and P_i into the matrix space, and an ATP synthase complex, called F_0F_1, that binds the ADP and P_i and utilizes the bulk of the gradient to drive the synthesis and release of ATP. The remaining part of the gradient is used, in part, to drive the transport of P_i and ADP into the matrix space and to drive the net synthesis of NADPH via a transmembrane enzyme called transhydrogenase, which is not part of the oxidative phosphorylation apparatus. [*Note*: The energy stored within the electrochemical gradient of protons generated across the mitochondrial inner membrane during electron transport is derived partly from the chemical gradient of protons and partly from the membrane potential (negative inside and positive outside), both of which result as protons are translocated to the outside.]

Collectively, the oxidative phosphorylation process in animal cells requires more than 80 different proteins, >60 of which are associated with the electron transport chain, 16 with the ATP synthase complex, and one each with the H^+/P_i and ADP/ATP transporters. The electron transport chain consists of three mainstream transmembrane complexes referred to as Complex I (42 subunit types), Complex III (11 subunit types), and Complex IV (13 subunit types) (Fig. 1A). Cytochrome *c*, although also a mainstream electron transport chain component, is unique, consisting of only a single subunit, and is located on the outer surface of the inner membrane where it interacts with Complex IV. Complex I (NADH dehydrogenase) initiates the process of electron flow by oxidizing NADH to NAD^+. Electrons then flow sequentially through Complex III (bc_1 complex), cytochrome *c*, and finally through Complex IV (cytochrome oxidase) where bound oxygen is reduced to water. During the process of electron flow from NADH to molecular oxygen, each of the three complexes (I, III, and IV) catalyzes the translocation of protons across the mitochondrial inner membrane, resulting in the formation of an electrochemical proton gradient. The mechanism by which each of these complexes contributes to the formation of this gradient is not known, although extensive data collected over the past four decades, including recent crystal structures of Complexe III (Xia *et al.*, 1997; Zhang *et al.*, 1998; Iwata *et al.*, 1998) and Complex IV (Iwata *et al.*, 1995; Tsukihara *et al.*, 1995) implicate the involvement of quite different chemistries. For example, Complex III catalyzes a unique cycle known as the "Q cycle" in which the proton carrier is a lipid called coenzyme Q or ubiquinone (Trumpower, 1990; Matsuno-Yagi and Hatefi, 1996). In contrast, cytochrome oxidase is believed to translocate protons via one (or more) proton pathways that involve specific amino acid functional groups (Iwata *et al.*, 1995; Brzenzinski, and Adelroth, 1998).

The ATP synthase complex of animal cells is comprised of two major units called F_1 and F_0 (Fig. 2A). F_1 (371 kDa) projects into the mitochondrial matrix space, is water soluble, and consists of five subunit types in the stoichiometric ratio $\alpha_3\beta_3\delta\varepsilon$ (Catterall and Pedersen, 1971; Catterall, *et al*., 1973). Three recent crystal structures of F_1 preparations show that the α and β subunits alternate forming a hexagonal array (Abrahams *et al.*, 1994; Shirakihara, *et al.*, 1997; Bianchet *et al.*, 1998). Two of these structures also show that the center of F_1 is occupied by the γ subunit (Abrahams *et al.*, 1994; Bianchet *et al.*, 1998) (Fig. 2B). Significantly, each of the 3 $\alpha\beta$ pairs in this array forms a catalytic site, which can either synthesize or hydrolyze ATP depending on the presence or absence of an electrochemical proton gradient. In contrast to F_1, F_0 ($>$200 kDa) is located both in and out of the inner membrane, is detergent soluble, and contains at

A. ATP Synthase (F_0F_1)

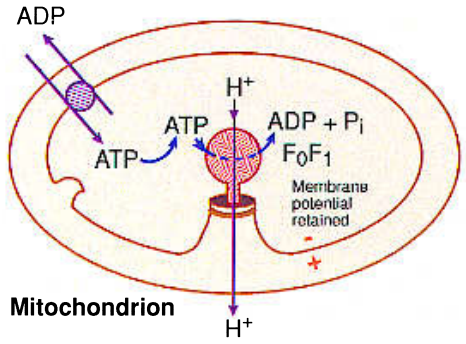
- 16 Subunit Types (at least)
- $F_1 = \alpha_3 \beta_3 \gamma \delta \varepsilon$
- *F_0 = a, b,c,d,e,f,g,F_6, A_6L,OSCP
- *IF_1 = inhibitor protein



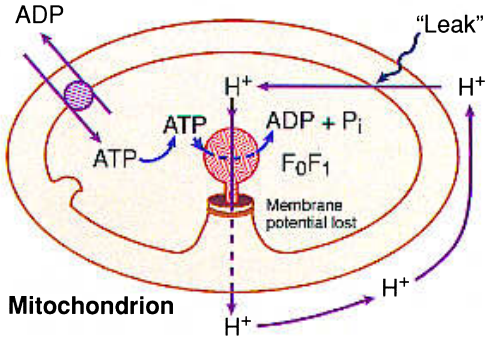
*Stoichiometries not well established

B. X-Ray structure at 2.8 Å of the F_1 moiety of Rat Liver ATP Synthase

C. <u>Limited ATP Hydrolysis by F_0F_1</u> can help maintain the electrochemical proton gradient under conditions where electron transport becomes limiting.



D. <u>Futile ATP Hydrolysis by F_0F_1</u> results under "uncoupled" conditions where the inner membrane "springs a leak" and becomes highly permeable to protons. (Note: The outer membrane is normally permeable to low molecular weight solutes.)



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least ten different subunit types (a, b, c, d, e, f, g, oscp, A6L, and factor 6) (reviewed in Pedersen, 1996). F_0 is responsible for delivering the energy contained within the electrochemical proton gradient to each of the three catalytic sites within F_1. Another subunit type called IF_1 is a small inhibitory protein, which depending on conditions, is either bound to F_0F_1 or free in the matrix space (reviewed in Schwerzmann and Pedersen, 1986; Rouslin, 1988; Walker, 1994). The role of this inhibitor protein is to minimize ATP hydrolysis by F_0F_1 when this complex is not operating as an ATP synthase. F_0F_1 also contains at least two "stalks" between its matrix-located headpiece and its membrane-located basepiece (Wilkens and Capaldi, 1998; Karrash and Walker, 1999). The central stalk is comprised, in part, of the γ and δ subunits of F_1 (Abrahams *et al.*, 1994; Bianchet *et al.*, 1998; Pan *et al.*, 1998) while the side "stalk" may be comprised, in part, of the b and oscp subunits of F_0. Other stalk components have yet to be clearly identified.

When intact mitochondria carry out oxidative phosphorylation, the F_0 basepiece transmits the energy from the electrochemical proton gradient (generated by the electron transport chain) to the F_1 unit, promoting the synthesis and release of ATP. The process is quite remarkable as a ring comprised of 10 to 12 "c" subunits within the F_0 basepiece (Dmitriev *et al.*, 1999), upon binding protons, is believed to rotate and drive the rotation of the F_1 γ subunit (Vik and Antonio, 1994; Duncan *et al.*, 1995; Engelbrecht and Junge, 1997). This rotating subunit which extends from the c subunit ring within the F_0 basepiece through the central cavity of the F_1 headpiece, transmits the energy in sequence to each of the three catalytic $\alpha\beta$ pairs, thus driving the synthesis of three ATP molecules/360° rotation ("rotational catalysis") (reviewed in Cross, 1981; Boyer, 1997 with recommended amendments in Weber and Senior, 1997; Bianchet *et al.*, 1998). Thus, during oxidative phosphorylation the F_0F_1 complex is likely to be operating as a "motor-driven" ATP synthase, where the "driving" motor is contained within the F_0 basepiece and the side "stalk" (b + oscp subunits) acts as a stator to hold the F_1 headpiece in place.

Significantly, in mitochondria, ATP synthesis catalyzed by F_0F_1 can be reversed quiet easily (Nicholls, 1982). This can happen under conditions following the uptake of a cation, which utilizes the electrochemical proton gradient to support its uptake. Assuming the availability of substrate (e.g., NADH) to supply the electron transport chain is limiting, F_0F_1 cannot synthesize ATP as the gradient has been utilized for cation transport. Rather, F_0F_1 will hydrolyze ATP until the gradient is reestablished (Fig. 2C). The mitochondria, and the cell in which they are housed, will not be damaged and F_0F_1 will be able to participate in ATP synthesis again when substrate becomes more available to the electron transport chain. The same could occur if an agent binds selectively to a pore protein or channel localized in the inner membrane, induces its opening and then upon debinding induces its closing. In a different scenario, the mitochondria may incur injury such that their inner membranes are rendered permeable to protons (Fig. 2D) and other ions. In this case, a stable proton gradient cannot be formed (uncoupling) and F_0F_1 will continue to hydrolyze ATP in a futile attempt to reestablish the gradient. Such a scenario is not compatible with cell life and cell death will occur. [In the latter case, the inhibitor protein (IF_1) is likely to function to help suppress the rising rate of ATP hydrolysis. However, IF_1 does not inhibit F_0F_1-ATPase activity completely (Lebowitz and Pedersen, 1996) and, therefore, can only slow the mitochondrial and cell ATP depletion process, rather than prevent it altogether.] In the examples just noted, F_0F_1 is functioning as an ATP-driven motor involving ATP hydrolysis, where the driving motor is contained within the F_1 headpiece. Here, ATP hydrolysis occurring sequentially on each of the three $\alpha\beta$ pairs of F_1 drives rotation of the $\gamma\delta$ subunit pair (Noji *et al.*, 1997; Kato-Yamada *et al.*, 1998), which is believed to then drive

Fig. 2. (A) The mitochondrial ATP synthase complex. This complex which is also called F_0F_1 consists of at least 16 different polypeptides, as indicated in the figure. In intact mitochondria, it carries out the synthesis of ATP from ADP and P_i by a rotary process that is driven by an electrochemical proton gradient across the inner membrane. This is described in detail in the text. (B) The three-dimensional structure of the F_1 part of the ATP synthase from rat liver. The central γ subunit forms part of the rotor, which rotates during catalysis and transmits the energy from the electrochemical proton gradient, in turn, to each of the three surrounding $\alpha\beta$ pairs. Here ATP is made from ADP and P_i at catalytic sites that are predominantly on the β subunits.(From Bianchet *et al.*, 1998 with permission). This process is discussed in detail in the text. (C) Limited ATP hydrolysis by F_0F_1. In addition to catalyzing ATP synthesis, F_0F_1 also catalyzes ATP hydrolysis. If the membrane is not damaged, this hydrolysis can drive the formation of an electrochemical proton gradient across it. (D) Futile ATP hydrolysis by F_0F_1. As indicated in the figure, this occurs when the membrane is leaky to protons preventing a stable electrochemical gradient of protons from being formed.

rotation of the ring within F_0 comprised of proton containing c subunits. This induces release of the protons in an attempt to restore the electrochemical proton gradient. [Note: F_1 subunits that have actually been shown to rotate, are the γ and ε subunits of the bacterial enzyme, where ε is the equivalent of the δ subunit in the mitochondrial enzyme.]

From the above discussion, it should be clear that the mitochondrial F_0F_1 complex can be regarded as a cellular life/death switch. When functioning as a motor-driven ATP synthase, cell ATP reserves and cell life will be favored. In addition, when functioning as an ATP-driven motor in the absence of uncoupling conditions, ATP hydrolysis will occur only until the electrochemical proton gradient is reestablished (Fig. 2C). Here, depletion of cell ATP reserves will be very small and cell life will still be favored. However, when functioning as an ATP-driven motor under uncoupling conditions (Fig. 2D), cell death will be favored as cell ATP reserves will be rapidly depleted. Only a few cell types might be expected to survive damage to the mitochondria that induces uncoupling as the catalytic turnover number ($> 300\ s^{-1}$) of the F_1 unit (Cross *et al.*, 1982; Reynafarje and Pedersen, 1996) is the highest of any known ATPase. The most likely survival candidates are those malignant cells exhibiting the high glycolytic phenotype. Here, where as much as 60% of the total ATP may be produced via glycolysis (Nakashima *et al.*, 1984), cells may survive until new mitochondria have been produced to replace damaged mitochondria eliminated by turnover.

Production of Reactive Oxygen Species (ROS)

It has been known for over two decades that mitochondria are involved in ROS production (Boveris and Chance, 1973). Although the overproduction of ROS is usually considered to be unhealthy for life, it may also have beneficial effects, e.g., by facilitating the death of cells where injury to the mitochondria has occurred (Skulachev, 1996). In mitochondria, most ROS formation normally occurs as a byproduct when electrons flow through Complexes I and III (reviewed in Lenaz, 1998). Thus, a small fraction ($< 5\%$) of the total electrons that flow from NADH to molecular escape and react directly with oxygen (Chance *et al.*, 1979). This results in the superoxide anion $O_2^{\cdot -}$ and, subsequently, other ROS species, which can oxidatively damage lipids, proteins, and nucleic acids. Considering that in most animal cells, the mitochondria account for as much as 90% or more of the total oxygen consumption, their contribution to total cellular ROS production is expected to be very high.

Normally superoxide anion production of mitochondria is expected to be controlled, at least in part, by the sequential action of two other enzymes. One of these is mitochondrial superoxide dismutase (SOD) (Autor, 1982; Wispe *et al.*, 1989), which, in the presence of protons, converts the superoxide anion to hydrogen peroxide (H_2O_2) and oxygen. The uncharged H_2O_2 can diffuse out of the mitochondria where the peroxisomal enzyme catalase converts it to molecular oxygen and water. However, when the terminal steps of electron transport (i.e., cytochrome *c* through oxygen) are impaired or inhibited, superoxide anion production may rise to levels that cannot be contolled by the normal protective enzymes. In such cases, the oxidative effects of this anion, together with those of its spontaneously formed ROS products, i.e., the hydroperoxyl radical $HO_2^{\cdot}$ (formed when the superoxide anion reacts with protons), and hydrogen peroxide (formed when $HO_2^{\cdot}$ reacts with itself), can severely damage normal cellular processes. The superoxide anion and the hydroperoxyl radical may have more localized damaging effects on mitochondria than the uncharged hydrogen peroxide molecule, which can diffuse across the mitochondrial membranes and, when in excess of the capacity of the peroxisomal catalase to neutralize its effect, exert damage in the cytoplasm. Here, via the action of glutathione peroxidase, hydrogen peroxide is expected to convert reduced glutathione (GSH) to oxidized glutathione (GSSG), thus changing the reducing potential of the cytoplasm.

Recently, a mechanism has been proposed for the exit from the mitochondria of the hydroperoxyl radical (Liu, 1999). Therefore, this potent mitochondrially derived ROS species may also contribute in part to redox-related damage in the cytoplasm. In addition, it has been reported that, in addition to the cytoplasm, mitochondria contain a glutathione peroxidase (Oshino and Chance, 1977). Therefore, the action of this enzyme in converting GSH to GSSG in the presence of excess hydrogen peroxide may also have deleterious effects on the mitochondrial redox state.

From the discussion above, it should be clear why current views about cell death and aging, discussed briefly below and in more detail in the subsequent reviews, frequently emphasize possible roles for mitochondrially derived ROS. Unfortunately, as these species are reactive with so many different types of biological molecules, it is difficult to discern which

of the experimentally demonstrated effects of ROS are directly involved. Research on this topic is likely to become one of the hottest during the next decade.

MITOCHONDRIAL EVENTS INVOLVED IN CELL DEATH

Types of Cell Death

The study of cell death pathways is currently a very active area of investigation in numerous laboratories, and it seems fair to say that this field of research is in its infancy with many important contributions still to come. A major focus in recent years has been to elucidate the roles that mitochondria play in these pathways. Although much remains sketchy and controversial, sufficient information is now available both in the recent literature and in the reviews that follow to begin to synthesize some reasonably coherent views. However, prior to summarizing such views, it is important to note first that there are two major types of cell death pathways, *necrotic* and *apoptotic*. Necrotic cell death is the type that occurs following severe cellular injury such as stroke or heart failure (reviewed in Lemasters *et al.*, 1998, 1999; Di Lisa *et al.*, 1998). It can be induced by anoxic conditions (defined here as the absence of oxygen), metabolic poisons, and high doses of oxidants. Cell death induced in this way is characterized morphologically by the formation of plasma membrane blebs (protrusions), followed by the eventual rupture of some of these blebs with the release of cytosolic components. In contrast, apoptotic cell death is programmed. Thus, following an initial signal (to die), cells proceed through a series of steps that result in their death without release of cellular contents (Kerr *et al.*, 1972; Wyllie, 1980; Nunez et al., 1998). It is believed that this form of cell death is employed to rid a population of cells from those that may be damaged or potentially harmful (e.g., virally infected), and also important in regulating developmental and differentiation programs. Apoptotic cell death is accompanied by cell shrinkage and "blebbing" of the plasma membrane and terminated when the blebs of cells completing the death program are engulfed by neighboring healthy cells. Simply put, the healthy cells within a population cannibalize their unhealthy neighbors as they complete the apoptotic program.

Another major difference between necrotic and apoptotic pathways for cell death resides at the level of bioenergetics. Thus, ATP levels are rapidly depleted in cells undergoing necrosis and this loss of energy reserves is believed to be a primary cause of cell death (Lemasters *et al.*, 1998, 1999; Di Lisa *et al.*, 1998). In fact, cells undergoing necrosis can be rescued from cell death by partially restoring or conserving their energy reserves by metabolic intervention. In sharp contrast, cells undergoing apoptosis not only maintain, at least in part, their ATP reserves but require ATP to complete the cell death program (Liu *et al.*, 1996).

With the above in mind, the mitochondrial events involved in necrotic and apoptotic cell death pathways can be considered. At the outset, however, it is important to note that many, but not all, apoptotic pathways appear to require mitochondrial involvement. It is important to note also that the sequence of events outlined in Fig. 1B for necrotic and apoptotic cell death pathways may not be agreeable to all. These pathways have been constructed on the basis of the author's impressions from reading the recent literature and his personal experiences working for many years with both mitochondria and several of its key oxidative phosphorylation components. It is presented here only to provide the reader with a general framework for the excellent reviews that follow.

Cell Death Involving Necrosis (Fig. 1B)

Mitochondrial events thought to be involved in necrotic cell death induced by anoxia and cyanide are perhaps the most frequently discussed (reviewed in Lemasters, 1998, 1999). Here, the initial event most likely originates at the terminal step of the electron transport chain, namely Complex IV (cytochrome oxidase). This is because both anoxia and cyanide prevent Complex IV from catalyzing the reduction of molecular oxygen to water. Initially, ATP hydrolysis by F_0F_1 is expected to maintain the electrochemical proton gradient across the mitochondrial inner membrane (Fig. 2A). However, blockage of Complex IV must result in structural changes, certainly in the oxidase and perhaps in one or more other membrane components, as the mitochondria become permeable to low molecular weight solutes. This process is referred to as the mitochondrial permeability transition (MPT) (Haworth and Hunter, 1979; Lemasters *et al.*, 1998) and the pore involved as the permeability transition pore (PTP) (Fontaine and Bernardi, 1999). Accompanying the anoxia or cyanide-induced MPT is swelling of the mitochondria and loss of their capacity to maintain a

stable membrane potential and proton gradient (i.e., uncoupling, or very loose coupling has occurred). In this case, the onset of the MPT is evidently such that it is either poorly reversible or irreversible altogether with opening of the PTP favored more than its closing. As indicated earlier in this review, loss of the electrochemical proton gradient because of uncoupling results in F_0F_1 carrying out ATP hydrolysis unabated until the cell's ATP reserves are exhausted (Fig. 2C). Without its ATP reserves, any cell is expected to experience a quick and unceremonious death. Although cells undergoing necrotic cell death can be rescued temporarily either by metabolic intervention (e.g., adding a glycolytic substrate, which increases the cell's ATP levels), or by inhibiting F_0F_1-ATPase activity with oligomycin, neither rescue approach can restore a stable electrochemical proton gradient across the mitochondrial inner membrane. In summary, it would appear that an early critical event leading to necrotic cell death induced by anoxia or cyanide is onset and persistence of the MPT, i.e., a constant "leak," such that F_0F_1 is unable to maintain a stable electrochemical proton gradient by hydrolyzing mitochondrial ATP reserves (Fig. 2C). Rather, continued ATP hydrolysis by F_0F_1 leading to cell death occurs under these uncoupled conditions (Fig. 2D).

From the above, one can make certain predictions about the role that ROS might play in necrotic cell death pathways. First, as necrotic cell death can be initiated by lack of oxygen, it is difficult to argue that ROS is directly involved in all necrotic cell death pathways, although oxidants have been shown to activate the MPT (Zoratti and Szabo, 1995). However, when necrotic cell death is induced in the presence of oxygen by an inhibitor of Complex IV, e.g., cyanide, one would expect ROS to accumulate. In this case it may help facilitate the MPT. Also, see Jiang *et al.* (1999) for more recent insights into the role of ROS.

Cell Death Involving Apoptosis (Fig. 1B)

Mitochondrial events occurring during apoptotic cell death pathways, although bearing some similarities to those occurring during necrotic cell death pathways, are quite different (reviewed in Susin *et al.*, 1998; Cai *et al.*, 1998; Montal, 1998). Apoptotic cell death pathways are programmed via an initial "death signal" and frequently lead to factors that initiate mitochondrial involvement. As there are many such pathways, there are many such factors, each of which force the mitochondria to redirect their role from that of powering cell life to facilitating cell death. Among these factors (proposed or demonstrated) are Ca^{2+}, ceramide, nitric oxide, upstream caspases (cysteine proteases acting at aspartic acid), ROS, and complexes of Bcl-2 family members (Sussin *et al.*, 1998). The net effect of many of these factors is to release from the mitochondrial outer compartment the two proteins, cytochrome *c* and apoptosis-inducing factor (AIF). These two proteins then participate in postmitochondrial events that are necessary to complete the cell death program.

The manner in which those factors derived from programmed cell death-signaling pathways force mitochondria to participate in cell death programs is not entirely clear and many pieces of the puzzle remain to be established. However, from the extensive literature on this subject, it seems clear that one of the most widely discussed hypothesis involves an important role for the permeability transition pore (PTP) responsible for the MPT. The popularity of this view most likely stems from the findings that several factors that induce the MPT are complexes of Bcl-2 family members, which include Bcl-2, Bcl-X_L, Bcl-W, Bax, Bak, Box, Bid, and Bad (reviewed in Reed *et al.*, 1998). Significantly, Bcl-2 is a novel type of oncogene that binds to the outer mitochondrial membrane and increases cell survival by acting as an antiapoptotic factor rather than promoting cell proliferation (reviewed in Cory *et al.*, 1999; Korsmeyer, 1999). It has been shown to inhibit the MPT, while one of its family members called Bax can form a complex with Bcl-2, overriding its inhibitory effect on MPT and, when in sufficient concentration, promote the MPT. Significantly, the relative levels of Bcl-2 and Bax, or their respective homologs, may serve as a checkpoint in many apoptotic pathways, which determines whether or not the cell death program will proceed (Korsmeyer, 1999). If the original death stimulus results in a greater production of Bax (or its homologs) than Bcl-2 (or its homologs), then it is envisioned that some event (unknown) will be conveyed to the PTP in the inner membrane. This will result in the induction of MPT followed by loss of membrane potential (at least temporarily), swelling of the mitochondria, and finally rupture of the outer membrane with release of cytochrome *c* and AIF, a protease. Cytochrome *c* has an activating effect on one of the downstream caspases that helps perform the dirty work in the final execution events of the cell.

As it regards the energetics of the apoptotic cell death programs involving the mitochondria, it is

important to note that at least one of the postmitochondrial caspases is dependent on ATP for its function (Liu *et al.*, 1996). This emphasizes that in many apoptotic pathways for cell death, in sharp contrast to necrotic cell death pathways, ATP depletion is not a causative factor. It also emphasizes that those mitochondrial events discussed above, upstream from the ATP-dependent caspase, do not result in irreversible uncoupling of the mitochondria, as observed in necrotic cell death pathways. Thus, during apoptotic cell death, the ATPase function of mitochondrial F_0F_1 must be turned down or turned off altogether. A likely possibility is that following cytochrome *c* release from the mitochondria, which inhibits the electron transport chain and, therefore, electrochemical proton gradient formation, the pore associated with the MPT reseals. Then, F_0F_1, by hydrolyzing mitochondrial ATP reserves, immediately reestablishes the electrochemical proton gradient and upon reaching electrochemical equilibrium ceases further hydrolysis (Fig. 2C). Interestingly, recent studies implicate a role for F_0F_1 in Bax-induced apoptosis (Matsuyama *et al.*, 1998; Shaham *et al.*, 1998), a role that may involve reestablishing the mitochondrial membrane potential following its Bax-induced loss necessary for cytochrome *c* release.

There are reports that during apoptosis cytochrome *c* release from the mitochondria is not accompanied by a change in the membrane potential across the inner membrane, implicating that the MPT is not required (Yang *et al.*, 1997; Kluck *et al.*, 1997). This suggests that there may be intracellular mechanisms other than those operating through the MPT for releasing both cytochrome *c* and AIF from the mitochondria. Alternatively, it could be that before cytochrome *c* release is detected by the methods employed, that the MPT has already resealed and the F_0F_1 has reestablished the membrane potential, as suggested above (also see Fig. 2C). Recently this controversial issue has been addressed directly in an elegant experiment involving monitoring cytochrome *c* release and membrane potential changes in single cells subjected to staurosporine-induced apoptosis (Heiskanen *et al.*, 1999). These studies showed that mitochondrial depolarization (loss of membrane potential) accompanies cytochrome *c* release. However, these studies do not rule out the possibility that cytochrome *c* release during apoptotic pathways may occur by more than one mechanism, i.e., MPT-dependent and MPT-independent. It should be noted also that in the recent literature some studies, e.g., those by Holinger *et al.* (1999) show that apoptosis can occur through a cytochrome *c*-independent mechanism, implicating more than one downstream pathway for completing the cell death program.

In those apoptotic cell death pathways where cytochrome *c* release from the mitochondria is a necessary requirement, the molecular details involved in inducing its release remain unclear. However, new insights are being obtained as it concerns those pathways involving participation of Bcl-2 and its family members. Thus, a caspase cleavage product of one proapoptotic member of this family (Bid) and a caspase cleavage product of an antiapoptotic member (Bcl-2) have both been shown to induce release of cytochrome *c* from mitochondria (Srinivasula *et al.*, 1996; Luo *et al.*, 1998; Li *et al.*, 1998; Kirsch *et al.*, 1999). These finding suggest that the mitochondrial roles of the Bcl-2 family members in apopototic cell death programs are quite complex and that peptide fragments of some of these members may be critical signals for switching mitochondria from their normal role in facilitating cell life to a role in facilitating cell death.

As it concerns the role of ROS in apoptotic cell death pathways, there has been a great deal of debate (reviewed in Cai and Jones, 1999; Jiang *et al.*, 1999). Specifically, as it concerns those pathways involving cytochrome *c* release from the mitochondria, an event which inhibits electron transport, ROS production is expected to increase. This rise in ROS may have a positive feedback in sustaining the opening of the PTP for that time necessary to promote the release of cytochrome *c* and AIF, as ROS is known to promote opening of the PTP. However, there must be some opposing regulatory events within the apoptotic cell as constant opening of the PTP would result in uncoupling followed by unabated ATP hydrolysis by F_0F_1 (Fig. 2D). This, in turn, would deplete the cell ATP reserves sending it into a necrotic rather than apoptotic death pathway.

Finally, it should be noted that despite the recent attention the PTP involved in the MPT has been given as it relates to a predicted key role in many apoptotic cell death pathways, its molecular identity remains unknown (reviewed in Fontaine and Bernardi, 1999). It is known to have an exclusion limit of 1.5 kDa, to exist in at least two forms, to be modulated by over 40 classes of unrelated compounds, and to be inhibited by cyclosporin A. Current wisdom, based on many experiments, suggests that the PTP is a known membrane component of the mitochondria. One of the most attractive candidates at the moment is Complex I of the electron transport chain, which Fontaine and Bernardi (1999) believe can account for many of the effects on

the MPT, although they do not rule out other possible candidates, such as the adenine nucleotide carrier and complexes involving both inner and outer membrane components.

MITOCHONDRIAL EVENTS INVOLVED IN AGING

There is great interest today in research directed at the cellular, molecular, and chemical basis of aging in order to discover clues as to how we might improve the quality of life as we age and hopefully extend our lives with good quality beyond that expected. Mitochondria are a major focus of these research efforts as these prolific intracellular organelles are now believed by many scientists not only to be involved in powering our lives but to be also major participating instruments in our death. How mitochondria came to be viewed by many as major facilitators of the aging process is considered briefly below, together with current views about chemical, genetic, and bioenergetic events involved. The possible relationship of mitochondrial events that occur during aging and those that occur during programmed "apoptotic" cell death programs is also considered. A more detailed discussion of this topic is found in three minireviews in this series (Ozawa, 1999; Barja, 1999; Bohr and Anson, 1999), in reviews by Lenaz (1998) and Cortopassi and Wong (1999), and in other reviews referenced below.

Theories of Aging

There are several related theories of aging that led to the view that mitochondria may be major players in the aging process. Among these theories are five, which are perhaps best categorized: (1) The *Free Radical Theory* (Harman, 1956, 1983), (2) The *Oxygen Radical-Mitochondrial Injury Theory* (Miquel *et al.*, 1980; Miquel and Fleming, 1984), (3) The *Integration of Mitochondrial DNA into Nuclear DNA Theory* (Richter, 1988), (4) The *Somatic Mutation Theory* (Linnane *et al.*, 1989; Ozawa, 1995; Wallace, 1992, 1994, 1999), and (5) The *Redox Theory* (Ozawa *et al.*, 1995; Ozawa, 1997, 1999). Although there are some differences among these various theories, there are two common denominators. They all either include or implicate a role for reactive oxygen species (ROS) and a role for mitochondria. Arising from these theories is the simplistic view that mitochondria, as major producers of ROS, are also primary targets of ROS damage, and that, with age, this results in a decline of the bioenergetic capacity of the mitochondria and, therefore, of the whole organism. Consequently, those whose mitochondrial ROS production is high might be predicted to have lower life-spans than those whose mitochondrial ROS production is low.

Sites of Mitochondrial ROS Production

There is an extensive literature on this subject (reviewed in Lenaz, 1998) which indicates that the two major sites of ROS production are Complexes I and III of the electron transport chain. Recently, Barja and his colleagues (1998,1999) have extended this earlier work. They find in a study of mitochondria from two mammals and three birds that the sites of ROS production, and the respiratory states in which this production occurs, are tissue dependent. Thus, they show in heart and nonsynaptic brain mitochondria from these animals that Complex I generates ROS in both respiratory State 4 (without ADP) and in respiratory State 3 (with ADP), whereas Complex III generates ROS only in heart mitochondria and only in State 4. Taken at face value, this suggests that heart tissue may be more susceptible to free radical damage than that of brain. The precise electron carrying components within these complexes, which make one electron transfers to molecular oxygen to give the superoxide anion, is not known. Although in Complex III, this role is frequently ascribed to a coenzyme Q intermediate, Lenaz (1998) presents cogent arguments why this is not the case. In addition, he emphasizes that the physiological CoQ_{10} is an antioxidant, not a prooxidant. Therefore, it would appear that more work is needed to fully elucidate the initial chemical events involved in ROS production in the mitochondria.

Targets of Mitochondrial ROS Production

As indicated earlier in this review, ROS can oxidatively damage proteins, lipids, and nucleic acids, i.e., RNA, and DNA. Of these, the major focus in recent years has been placed on the role that ROS damage to mitochondrial DNA may play in the aging process (Linnane *et al.*, 1999; Ozawa *et al.*, 1995; Ozawa, 1995, 1997, 1999; Wallace, 1992, 1994, 1999). The reasons for this are severalfold. First, the mitochondrial genome is present in many cells in several thousand copies relative to only one copy of the nuclear genome. Second, mitochondrial DNA lacks introns,

histones, and other DNA-associated proteins making it readily accessible to oxidative damage. Third, mitochondrial DNA encodes for thirteen polypeptides of which two are critical for the function of the F_0 component of the ATP synthase, and seven, one, and three are critical, respectively, for the function of electron transport chain Complexes I, III, and IV (Wallace, 1994). Fourth, mitochondrial DNA is near the electron transport chain, where ROS production occurs. Finally, it has been generally believed, on the basis of an earlier study (Clayton *et al.*, 1974), that mitochondria lack the enzymatic machinery to repair their DNA, a view that is now no longer valid (Anson *et al.*, 1998; Bohr and Anson, 1999). Significantly, the view that mitochondrial DNA is a target for ROS damage has been demonstrated directly with cultured cells (Yoneda *et al.*, 1995). Moreover, it has been demonstrated that 8-hydroxy-D-guanosine, a marker for oxidative DNA damage, is increased in both aging and age-associated diseases (Dizdaroglu, 1991; Hayakawa *et al.*, 1991; Shigenaga and Ames, 1991; Mecocci *et al.*, 1993, Mecocci and Massarvey 1994; Shigenaga *et al.*, 1994).

Current Hypothesis of Aging: Mitochondrial ROS View

All that has been said in this brief review article thus far leads to an extended view of previous hypotheses for aging and incorporates the ideas and work of numerous investigators working on free radical biochemistry, mitochondrial function, cell death pathways, and degenerative diseases. This extended view combines what some investigators have referred to as the *mitochondrial theory of aging* (Miquel *et al.*, 1980; Miquel and Fleming, 1984; Linnane *et al.*, 1989) with the *redox mechanism of aging* (Ozawa et al., 1995; Ozawa, 1997, 1999), which now includes a role for apoptotic cell death. Briefly stated, this current view envisions that somatic cell mutations introduced by electron transport chain-induced ROS damage to mitochondrial DNA accumulate during our lifetime. Some of these mutations will be in the coding regions for one or more of the 13 polypeptides encoded by mitochondrial DNA, while others are likely to be in the tRNAs (22) and ribosomal RNAs (12 s and 16 s) also encoded by this genome. The predicted consequences of these mutations being carried through numerous mitochondrial divisions and cell divisions is a decline in respiratory capacity, which will not only impact negatively on the efficiency of ATP production, but will lead to increased ROS production, thus resulting in what has been coined a viscious circle of mitochondrial DNA mutations and oxidative stress. In such cases, the increased ROS production via its action on the PTP is envisioned to initiate those events described earlier in this review, that result in cytochrome *c* release from the mitochondria and activation of the apoptotic cell death pathway. Presumably, this mechanism would be activated to remove those cells containing a preponderance of damaged mitochondria from the total cell population.

It might be argued that if the above series of events do take place as we age, that initially (in our youth) cells bearing ROS-damaged mitochondria will be recognized by their neighboring cells or by immunosurveillance cells and eliminated, either by the suggested apoptotic scheme above or by cell death pathways, yet to be discovered. Alternatively, as mitochondria turnover every few days (Geller-Lipsky and Pedersen, 1981, 1982), much faster than most host cells, it is possible that the damaged mitochondria within the total mitochondrial population will be eliminated in this process. However, it could be further argued that as we get older and are subjected to numerous other stresses that we were shielded from in our youth, the rate at which cells bearing damaged mitochondria may increase. In addition, the rate at which damaged mitochondria are produced within these cells may also increase. Thus, it can be finally argued that the combined effect of these two events may be so great as to overwhelm our extracellular and intracelluar defense systems' capacity for dealing with the problem. Therefore, our slow decline may be facilitated by this complicated process ("aging") and, when coupled to other risk factors, may help facilitate a variety of degenerative diseases, including Alzheimer' disease, Parkinson's disease, and Huntington's disease (reviewed in Lenaz, 1998; Ozawa, 1997, 1999; Wallace, 1992, 1994, 1999).

Despite the attractiveness of the mitochondrially based redox/ROS hypothesis for aging, it is compromised by recent reports that indicate that in some tissues of human subjects there is no significant decline in the activity of the electron transport chain complexes with age (Barrientos *et al.*, 1996; Brierley *et al.*, 1997).

SUMMARY

In this review, the author has attempted to provide a brief synopsis of those events that occur within mitochondria during the life and death of animal cells, and to relate these events to the aging process. Figure 3

summarizes some of the salient features of this review by illustrating similarities and differences between mitochondrial events believed to occur in cell death pathways resulting in necrosis and those resulting in apoptosis. In necrotic cell death pathways and in many apoptotic cell death pathways, the elusive, unidentified permeability transition pore (PTP) is believed to play an important role. During cell death resulting in necrosis (e.g., that following severe cell injury induced by Complex IV poisons), the PTP appears to open and remain open inducing a mitochondrial permeability transition (MPT), such that uncoupling occurs with the irreversible loss of the electrochemical proton gradient. In a futile attempt to reestablish this gradient, F_0F_1 continues to hydrolyze ATP until the cell's ATP stores are depleted. During cell death resulting in many types of apoptosis, the PTP is also believed to open and to induce an MPT, an event under the control of signal transduction events originating, via death stimuli, outside the mitochondria. In this case, the induction of the MPT is believed by many, but not all, to be essential for the release of cytochrome *c* and apoptosis inducible factor (AIF), which are required for the downstream cell death events, also occurring outside the mitochondria. In cell death pathways culminating in apoptosis, it is important to note that ATP is required, thus implicating the presence of ample ATP stores in the cell. This suggest that either the PTP after inducing the MPT must close and remain closed, or that F_0F_1 is not operating (or both). Therefore, it is predicted that in the case of cell death resulting in apoptosis, in contrast to cell death resulting in necrosis, that either the PTP or F_0F_1 (or both) are down regulated. Finally, apoptotic cell death pathways, unlike necrotic cell death pathways, are highly regulated, with members of the Bcl-2 family playing major roles.

Figure 3 also emphasizes that reactive oxygen species (ROS) may play a role in cell death pathways involving both necrosis and apoptosis, most likely by positively affecting the PTP involved in the MPT. From the author's point of view, however, it seems unlikely that ROS is essential in either case for inducing the MPT. Thus, necrotic cell death pathways can be induced in the absence of oxygen, making it unlikely, if not impossible, to generate ROS. However, when induced by poisons like cyanide, which inhibit Complex IV of the electron transport chain, ROS is expected to rise and to positively affect the MPT. In apoptotic

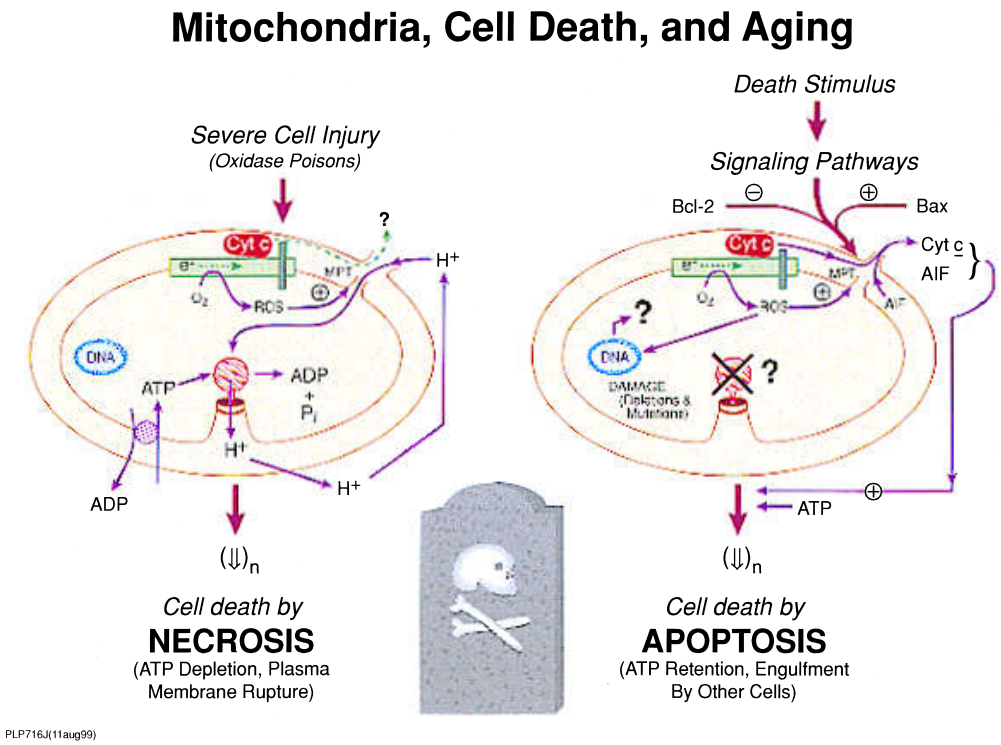

Fig. 3. Summary of the topics of this review. See text under ™Summary∫ for a description of this figure and a synopsis of this review article.

cell death pathways, the rise in ROS cannot occur until after the release of the cytochrome *c* essential for continuation of the cell death program. Thus, cytochrome *c* has already activated the terminal stages of the apoptotic program, leaving only a regulatory role for ROS.

During aging, the mitochondrial events involving ROS are more complex and still not well understood. Nevertheless, there seems to be an emerging redox/ROS view of aging, which implies that even during normal function of the electron transport chain sufficient ROS may be produced, albeit low, to bring about somatic mutations in the nearby mitochondrial DNA. As this DNA encodes one or more subunits in each of the electron transport chain complexes, these complexes may become impaired resulting in a decline in respiratory activity. This could result in more ROS, more somatic mutations in the mitochondrial DNA, and so on, as a viscous cycle leading to a further decline of mitochondrial function sets in. In our youth, it could be argued that any build up of ROS may act as a primary signal for activating PTP opening, thus facilitating the MPT and release of the cytochrome *c* necessary for activating the apoptotic cascade. Presumably, this would eliminate cells with damaged mitochondria. Alternatively, it could be argued that ROS, by eventually irreversibly opening the PTP, may force the damaged mitochondria into intracellular degradation pathways. Via such defenses, the aging process may be delayed. However, as we grow older and experience many more stressful insults, it could be argued that the increased rate at which damaged mitochondria may appear may be such as to overwhelm our natural defenses for dealing with the problem. Thus, we age more rapidly and become susceptible to a number of degenerative and other types of disease. Despite the attractiveness of this redox/ROS based view for aging, it is compromised by recent reports that there is no decrease in mitochondrial respiratory activity with age.

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