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# Role of mitochondria in oxidative stress and ageing

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**Abstract**

Mitochondria are deeply involved in the production of reactive oxygen species through one-electron carriers in the respiratory chain; mitochondrial structures are also very susceptible to oxidative stress as evidenced by massive information on lipid peroxidation, protein oxidation, and mitochondrial DNA (mtDNA) mutations. Oxidative stress can induce apoptotic death, and mitochondria have a central role in this and other types of apoptosis, since cytochrome *c* release in the cytoplasm and opening of the permeability transition pore are important events in the apoptotic cascade. The discovery that mtDNA mutations are at the basis of a number of human pathologies has profound implications: maternal inheritance of mtDNA is the basis of hereditary mitochondrial cytopathies; accumulation of somatic mutations of mtDNA with age has represented the basis of the mitochondrial theory of ageing, by which a vicious circle is established of mtDNA damage, altered oxidative phosphorylation and overproduction of reactive oxygen species. Experimental evidence of respiratory chain defects and of accumulation of multiple mtDNA deletions with ageing is in accordance with the mitochondrial theory, although some other experimental findings are not directly ascribable to its postulates. 

*Keywords:* Mitochondrion; Oxidative stress; Mitochondrial DNA; Aging

## 1. Introduction

After the elucidation of the major aspects of energy conservation in oxidative phosphorylation, the declined interest in mitochondria received a novel impetus by discoveries of cell biology and pathology. Two major developments opened breakthroughs in mitochondrial pathology: first, the discovery that mitochondrial DNA (mtDNA) mutations are at the basis of diseases [1], and second, the unexpected role of mitochondria in the mechanisms of cell death [2]. A common denominator of these aspects is the role of reactive oxygen species (ROS). This article attempts to provide a rationale for the role of oxidative stress by ROS on different aspects of pathology where mitochondria seem to have a major role. There is almost no area of human pathology where oxidative stress has not been implicated [3,4]; this review, therefore, will be restricted to few selected topics and will be centered on the role of mitochondria in ageing.

Abbreviations: $CoQ_n$, coenzyme Q with *n* isoprenoid units; LHON, Leber's hereditary optic neuropathy; MELAS, mitochondrial enkephalomyopathy with lactic acidosis and stroke-like episodes; MERRF, myoclonal epilepsy with ragged-red fibers; mtDNA, mitochondrial DNA; NARP, neurogenic muscle weakness, ataxia and retinitis pigmentosa; NMDA, *N*-methyl-D-aspartate; PCR, polymerase chain reaction; PEO, paralysis of the extraocular muscles; ROS, reactive oxygen species

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## 2. Mitochondria as sources of ROS

Reactive oxygen species [5,6] include oxygen free radicals (the superoxide radical anion, as the primary product of one-electron dioxygen reduction [7,8], and the extremely aggressive hydroxyl radical deriving from subsequent chemical reactions [9]), singlet oxygen and strong non-radical oxidants such as hydrogen peroxide; furthermore nitric oxide and the derived peroxynitrite radical [10] can be included in this category.

Sources of ROS in living cells are represented by physiological enzymatic mechanisms [11]; oxidative stress [11] may ensue when ROS production is excessive, due either to a particular metabolic situation, or to the presence of xenobiotic compounds [12], or also to damage-mediated liberation of non-enzymatic catalysts such as free metals [13,14], or when the cellular defences are lowered by the depletion of physiological antioxidants.

Among the cellular sources of ROS, besides specific enzyme systems involved in phagocytosis [8], eicosanoid metabolism [15] and nitric oxide production [16], are cytoplasmic systems such as xanthine oxidase [4], that can be formed from xanthine dehydrogenase by oxidation of thiol groups [17], microsomal $P_{450}$ oxygenases and quinone reductases [18], and the mitochondrial respiratory chain [19].

The respiratory chain is a powerful source of ROS, primarily the superoxide radical and consequently hydrogen peroxide, either as a product of superoxide dismutase [7] or by spontaneous disproportionation. It is calculated that 1–4% of oxygen reacting with the respiratory chain is incompletely reduced to ROS [20,21]. Their production may increase in state 4 with respect to state 3 [22], because $O_2$ concentration increases and the level of reduced one-electron donors in the respiratory chain is concomitantly increased [23]. A similar result is obtained when cytochrome oxidase activity is lowered, since this leads to the concomitant raise of oxygen concentration and of the reduced state of one-electron donors in the respiratory chain [24]. According to Skulachev [25], uncoupled electron transfer chains, by enhancing oxygen consumption, represent a device for preventing or decreasing ROS production by mitochondria.

There are two major respiratory chain regions where ROS are produced, one being complex I (NADH coenzyme Q reductase) [8,20,26–28] and the other complex III (ubiquinol cytochrome *c* reductase) [8,28–30].

In complex III, antimycin is known not to completely inhibit electron flow from ubiquinol to cytochrome *c*: the antimycin-insensitive reduction of cytochrome *c* is mediated by superoxide radicals; the source of superoxide in the enzyme may be either cytochrome $b_{566}$, [31] or ubisemiquinone [32] or Rieske's iron-sulfur center [33]. Ubisemiquinone is relatively stable only when protein bound [34], therefore the coenzyme Q (CoQ) pool in the lipid bilayer is no source of ROS.

The role of ubiquinone within ROS production deserves some comments (cf. [18]), since it has been described both as a prooxidant [22,28,32] and as a powerful antioxidant [35–37]; the former action has been ascribed to either oxidized or reduced quinone, whereas the latter exclusively to ubiquinol.

In some instances, a prooxidant effect may be ascribed only apparently to CoQ: for example the enhanced ROS production when CoQ-depleted mitochondria oxidizing succinate are reconstituted with CoQ [22] is a consequence of the increased rate of electron feeding to complex III via the quinone, and presumably complex III itself is the source of ROS generation.

Early experiments proved the involvement of complex I in ROS production [38]; addition of either NADH at low concentration or of NADPH, which feeds the electrons at decreased rate into the complex, led to copious ROS production detected by lipid peroxidation; on the other hand, addition of NADH at high concentration, but in the presence of rotenone, also induced peroxidation. In another study [28] water-soluble CoQ homologs used as electron acceptors from isolated complex I stimulated $H_2O_2$ production in the order $CoQ_1 > CoQ_0 > CoQ_2$, whereas $CoQ_6$ and $CoQ_{10}$ were inactive; the rate of $H_2O_2$ production was partly inhibited by rotenone, indicating that water-soluble quinones may react with oxygen when reduced at sites both prior and subsequent to the rotenone block. There is evidence that the one-electron donor to oxygen in complex I is a non-physiological quinone reduction site different from the physiological site(s) [39–41]; the former, hydrophilic, site reduces several quinones to the corresponding semiqui-

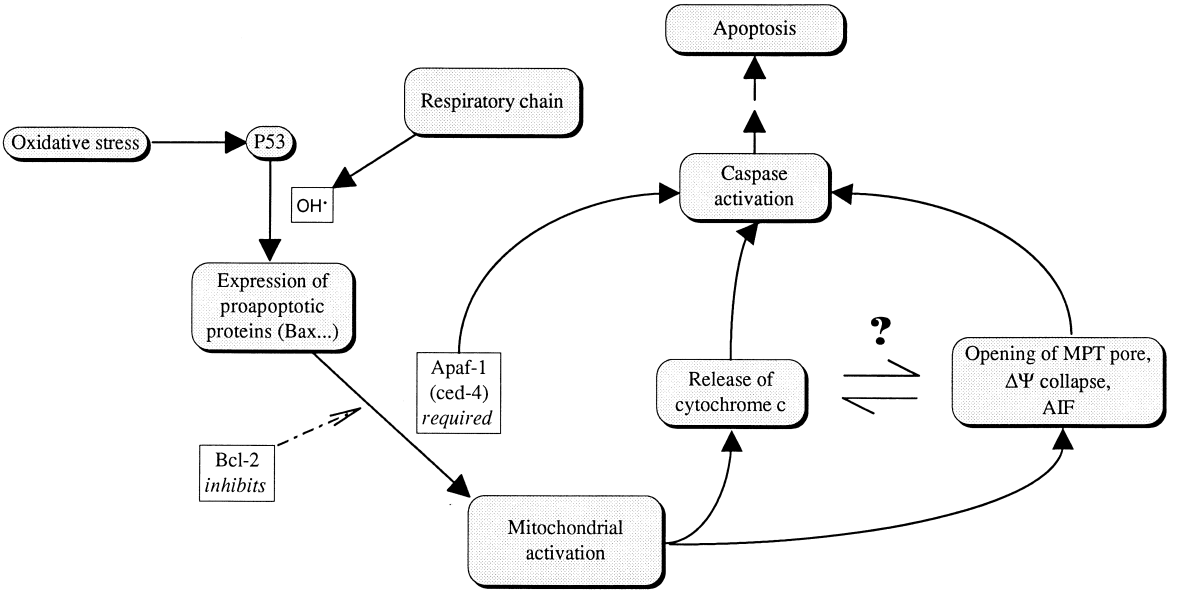


Fig. 1. A scheme of oxidative stress-induced apoptosis (cf. [113,159]). See text for explanations. MPT, mitochondrial permeability transition; AIF, apoptosis inducing factor. Mitochondrial activation refers to an early unknown step (steps) within the bulk of phenomena occurring at the mitochondrial level in the apoptotic cascade.

none forms, which are unstable and can reduce oxygen to superoxide. This mechanism is shared by several quinones, including such drugs as anthracyclines [42] and the clinically employed CoQ analog, idebenone [43]. However, auto-oxidation of fully reduced quinones [18], such as those formed by NADH CoQ reductase past the rotenone inhibition site, is also a source of ROS, but this effect exclusively pertains to hydrophilic quinones, and not to the physiological hydrophobic ubiquinol. Finally, in view of the experiments of Takeshige et al. [38], the hydrophilic, rotenone-insensitive, site can apparently reduce oxygen to superoxide in the absence of intermediate acceptors.

To conclude about the role of CoQ, it is clear that hydrophobic ubiquinones, such as the physiological $CoQ_{10}$ for man, do not behave as prooxidants in mitochondria; on the contrary, all evidence points out that they behave as antioxidants in their reduced form [35–37]; possibly, their deep membrane insertion prevents contact with non-physiological reduction sites, and they are not auto-oxidizable. The mechanism by which reduced CoQ functions as an antioxidant in vivo is out of the scope of this review and is discussed at length elsewhere [35–37].

ROS production by the respiratory chain is increased after a period of anoxia, when the oxygen concentration is reestablished by reperfusion [44–46]; one reason, besides those pointed out above (sudden high oxygen concentration in the presence of the respiratory chain in the reduced state), may be that the anoxic period, by depleting ATP, has induced damage to cellular structures and released catalytic metals [47], such as iron and copper, which are abundant in the inner mitochondrial membrane.

Mitochondria contain antioxidant enzymes, including superoxide dismutase (Mn form) [48,49] and glutathione peroxidase [50], and lipid-soluble antioxidants such as vitamin E [51] and reduced CoQ [52]. Ubiquinol may exert its antioxidant function indirectly by reducing α-tocopheroxyl radical back to vitamin E [53] or directly as a quencher of oxygen and lipid peroxyl radicals [54,55].

## 3. Mitochondria as targets of ROS

Being major producers of ROS, mitochondrial structures are exposed to high concentrations thereof and may therefore be particularly susceptible to their attack. Evidence exists, however, that even ROS produced outside the mitochondrion may damage mito-

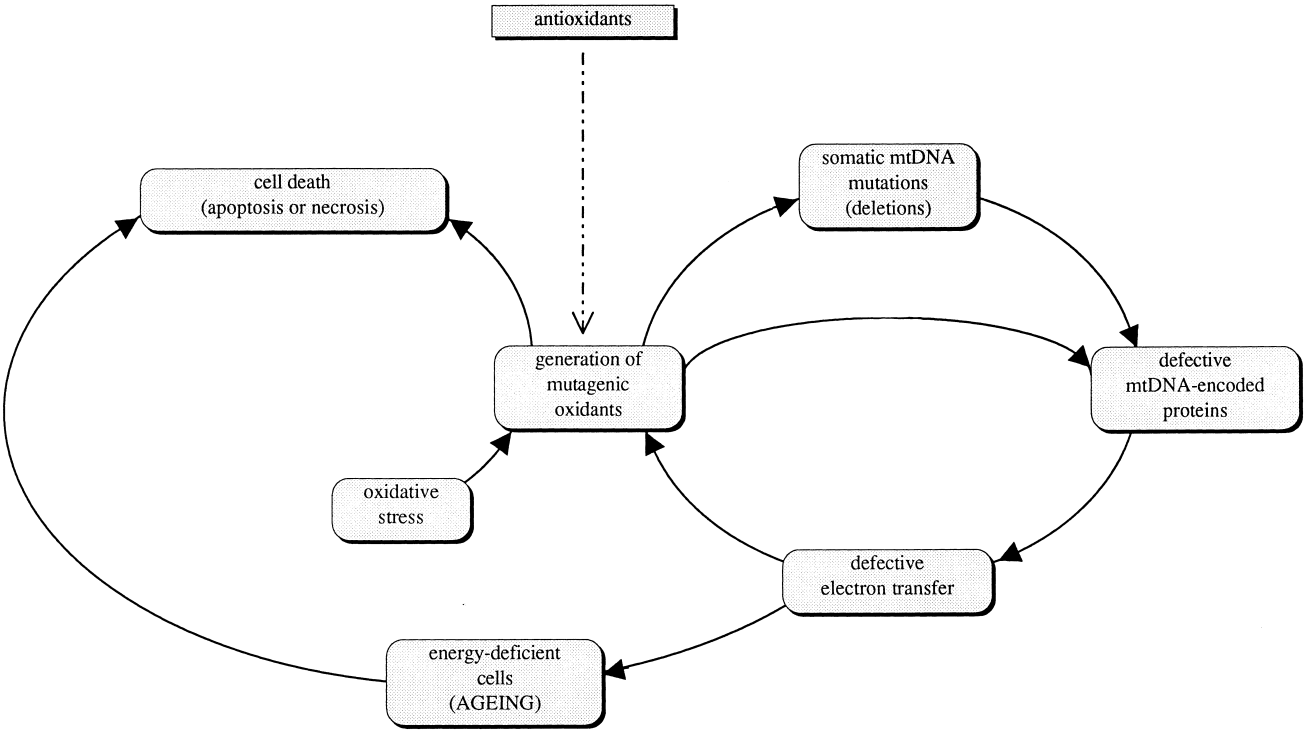


Fig. 2. A vicious circle of oxidative stress and mtDNA mutations in cell death and ageing (freely redrawn from [159,218]).

chondrial components: decreases of respiratory enzyme activities and of mitochondrial membrane potential, induced by adriamycin in perfused rat liver [56] or isolated hepatocytes [57], are prevented by incubation with $CoQ_{10}$; the exogenous quinone is taken up by the intracellular compartment but is not incorporated in the mitochondria under the conditions of the experiments [58], suggesting that the radicals quenched by CoQ are also produced in the extramitochondrial compartment.

Damage by oxidative stress to mitochondrial components includes lipid peroxidation, protein oxidation and mtDNA mutations.

Lipid peroxidation [14,59–61] might be particularly harmful in mitochondria, that contain cardiolipin as a major component of the inner mitochondrial membrane [62], since this lipid is required for the activity of cytochrome oxidase [63] and of other mitochondrial proteins [64]. Oxidative stress decreases cardiolipin to a larger extent than other lipids [65,66], perhaps as a consequence of its high unsaturation [67]; cardiolipin decrease appears to be directly related to reversible decrease of cytochrome oxidase activity [68,69].

Protein oxidation as a result of oxidative stress may occur either directly or as a consequence of lipid peroxidation [70–73]; it has been described to affect respiratory chain enzymes [74], ATPase [73–76], the adenine nucleotide translocator [77] and transhydrogenase [78] and to determine opening of the permeability transition pore [79]. To this respect, it is worth emphasizing the striking susceptibility of the adenine nucleotide translocator to oxidative stress [77], since this protein is considered part of the permeability transition pore [80] (cf. [81] for review). Complex I is particularly susceptible to oxidative damage [82], and its decreased activity in Parkinson's disease may be linked to an enhanced production of ROS and to the consequent damage of the complex [83]. Modification of the redox state of vital sulfhydryl groups may be at the basis also in mitochondria of important regulatory mechanisms, similar to those suggested to modulate signal transduction cascades [84]. Inactivation of Mn-superoxide dismutase in transgenic mice [85] enhances ROS production and results in animal death by dilated cardiomyopathy, with partial inactivation of mitochondrial enzymes containing iron-sulfur centers.

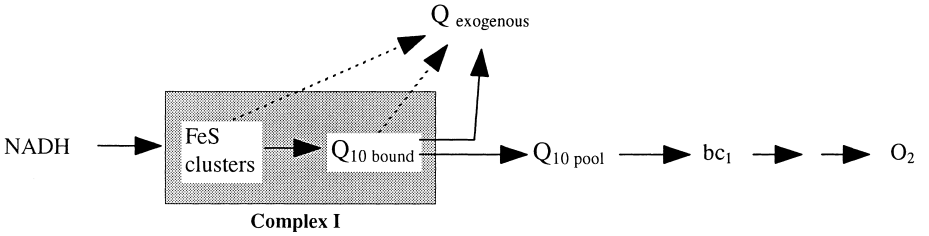


Fig. 3. A decreased electron transfer to oxygen via the CoQ pool may be masked, when assaying complex I activity, by concomitant reduction of the exogenous quinone acceptor by electrons flowing through both the physiological site and a non-physiological site (cf. [40]). A decreased electron flow to oxygen might depend on a decreased $V_{max}$ of NADH-$CoQ_{10}$ reductase activity of complex I, but also on its increased $K_m$ for $CoQ_{10}$ in the pool. In fact such a higher $K_m$ may be responsible for decreased electron flow to oxygen, since the physiological $CoQ_{10}$ concentration in the inner mitochondrial membrane is not saturating for NADH oxidation [176].

Of special interest is damage to mtDNA [86–89], since this small molecule is extremely susceptible to oxidative damage: being located in the matrix, it is close to the major source of ROS; moreover, lacking introns and being devoid of histones and other DNA-associated proteins, the probability of oxidative modification of a coding region of mtDNA is very high. After an oxidative stress to cultured cells, the damage to mtDNA is higher and persists longer than that to nuclear DNA [90], indicating that the repair devices of the former, even if present, are largely insufficient to overcome extensive DNA damage [91].

ROS were shown to induce extensive fragmentation and deletions in mtDNA; up to 187 different deletions were listed by Ozawa after the third day of an oxidative stress to a transformed human fibroblast cell line [92]. The 8-hydroxy-D-guanosine content is considered a marker of oxidative damage to DNA [93]; its increased content in mtDNA has been reported in ageing [93–95] and age-associated diseases [96–98].

## 4. Mitochondria, ROS, and cell death

Cell death can occur by either necrosis or apoptosis [99] as a result of exogenous and endogenous insults. There seems to be no net border between these phenomena [100], depending mainly on the extent of stress [101] and on the ATP levels [102]; however, the mechanisms are rather different, since apoptosis involves a well defined chain of enzymatic events which are genetically programmed [103]. Apoptosis induced by oxidative stress has been well documented [92,101,104–106] and appears to involve the same steps in the commitment and execution stages as in the other causes of apoptosis [105,106]. Actually, apoptosis may be a mechanism to eliminate ROS-producing cells [107].

A recent exciting development has been the discovery that there is an early mitochondrial involvement in the apoptotic cascade [103,106] (Fig. 1). The release of cytochrome *c* from the intermembrane space has been reported as an early event activating the product of a gene (corresponding to *ced-3* in the nomenclature of the activation sequence of the nematode *Caenorhabditis elegans*), which is *caspase-3* (a cysteine-aspartate protease) [108–111]; this activation requires the product of the *ced-4* gene [112] and is inhibited by *ced-9* or Bcl-2 and similar proteins [110,111,113–115]. The proteins of the Bcl-2 family are localized in the outer mitochondrial membrane [116], where pro-apoptotic proteins such as Bax and Bad, structurally related to the former, are also present [117]. Since opening of the permeability transition pore, with fall of the mitochondrial membrane potential, also represents a fundamental step in apoptosis [103,106,118], and Bcl-2 may also prevent pore opening [119], the relation between cytochrome *c* release and pore opening is still controversial: whether the latter phenomenon, with consequent swelling and rupture of the outer membrane, is the cause of cytochrome *c* release, as it happens in isolated mitochondria [120,121], whether cytochrome *c* loss and consequent alteration of the respiratory chain induce pore opening, or whether they are two independent phenomena. The fact that Bcl-2 and related proteins can polymerize [122] to form large channels in the membrane [123,124] has suggested

that such channels in the outer membrane may allow the release of cytochrome *c* in the cytosol [117,123].

Oxidative stress may not just be *one* out of several signals committing the cell to apoptosis, but represent an early intrinsic component of any apoptotic cascade, since an oxidative stress component was shown in apoptosis induced, among others, by p-53 expression [125], by dexamethasone [126], and by ceramide [127]; in the latter, the oxidative step was identified in complex I of the respiratory chain [127].

Although oxidative stress is one of the causes of pore opening [79], the commitment to apoptosis seems to be an earlier event, at least in some forms of apoptosis [128]: induction of pro-apoptotic proteins and cytochrome *c* release seem to occur before any decrease of the mitochondrial transmembrane potential can be shown [110,111].

## 5. Mitochondrial cytopathies as a model for ageing

The discovery that mtDNA mutations are at the basis of a number of human pathologies [129] has opened a new and extremely active chapter of mitochondrial research [130–132]. Not all mitochondrial cytopathies are due to mtDNA mutations, but many are due to mutations in nuclear genes encoding for mitochondrial proteins [133,134]. The diseases due to mtDNA mutations, contrary to those of nuclear genes, are genetically well characterized. There are diseases due to point mutations of a structural gene and others due to mutations in tRNA genes; in addition sporadic or familiar forms characterized by extensive deletions of mtDNA are also described [129–134].

Mitochondrial genetics is peculiar for many respects when compared with mendelian genetics [132,134]: besides the maternal type of transmission, the presence of multiple mtDNA copies in the same cell (polyplasmy) and the possibility for a mutation to experience different degrees of heteroplasmy are at the basis of the threshold effect, by which a phenotypic lesion becomes evident only when over 80% of the mtDNA in a cell is mutated, and of mitotic segregation, by which the proportion of mutant mitochondrial genomes may shift in daughter cells during cell division; surprisingly, the process appears to be non-random, so that different genotypes segregate to different extents in different tissues during embryogenesis [135,136], a finding that can have strong relevance to ageing (Section 6).

Mitochondrial structural genes encode for 13 polypeptide chains belonging to those complexes of the inner mitochondrial membrane which are involved in the transmembrane movement of protons. Thus, the phenotypic consequence of a mtDNA mutation must be a defect in the oxidative phosphorylation machinery of mitochondria [129].

A lesion in a structural gene, such as due to a point mutation, would interfere with the function of the polypeptide encoded by that gene, leading to enzyme activity decrease. Indeed this is often found in such kinds of cytopathies, such as a form of Leber's hereditary optic neuropathy (LHON), characterized by a mutation in the genes for the ND1 subunit of complex I at position 3460 of mtDNA [137], but not in the form harboring the 11 778 ND4 mutation, where complex I activity is unchanged [138]; in spite of normal complex I activity, the flux over the entire respiratory chain is significantly decreased [138].

The finding that in the 11 778 ND4 mutation there is a decreased sensitivity to rotenone [139] and a decreased affinity for a quinone homolog used as the acceptor [139,140] agrees with the notion that the seven ND subunits of complex I are hydrophobic polypeptides belonging to the sector of the enzyme where a large series of inhibitors and the electron acceptor CoQ are bound [141]. Since the quinone-binding sites are also involved in the proton translocation activity of the complex [141,142], it is predictable that a defective energy conservation may be present in this form of the disease.

The decreased respiratory rate in spite of normal complex I activity in subjects harboring the 11 778 ND4 mutation is puzzling; possible explanations are either in a decreased affinity of complex I for endogenous ubiquinone ($CoQ_{10}$), as indeed found for an exogenous short CoQ homolog, $CoQ_2$ [139,140], or in overestimation of the physiological complex I activity due to electron withdrawal, by the acceptor used, from a non-physiological site in the enzyme [40,43], made available by an incorrect assembly caused by the mutation [139].

Another cytopathy characterized by a mtDNA point mutation, the NARP or maternally inherited Leigh syndrome, associated with an 8993 mutation in

the ATPase-6 gene [143], is characterized by decreased ATP synthesis [144], although the biochemical defect has not yet been identified; in the light of the above considerations, it would be speculated that the primary defect involves proton translocation through the $F_0$ proton channel, as found by site-directed mutagenesis to the homologous region of subunit a of *Escherichia coli* [145].

On the other hand, a lesion in a gene encoding for a mitochondrial tRNA, as in the MELAS and MERRF syndromes [131], would interfere with mitochondrial protein synthesis of all the subunits encoded by mtDNA.

Some forms of mitochondrial disease are caused by mtDNA deletions [131]; being extended to large portions of the mitochondrial genome, as the 4977 bp common deletion found in patients affected by the Kearns-Sayre syndrome [146] together with many other deletions, and removing many genes including essential tRNA genes [147], they strongly interfere with mitochondrial function.

It was found that some mtDNA alterations, particularly deletions, are also present in normal individuals, though to very small extents, and accumulate with age [148]. In particular, the common deletion of the Kearns-Sayre syndrome has often been found by PCR to accumulate in postmitotic cells in old individuals.

## 6. Mitochondria and ageing

The concept that mitochondria are primarily involved in ageing derives from the theory of Harman [149], linking senescence to the injurious effect of free radicals arising from the one-electron reduction of oxygen during metabolism. In accordance with the free radical theory of ageing is the inverse relation existing between auto-oxidation rate in different animal species and life expectancy of the same species [150,151]; the auto-oxidation rate on its hand is directly proportional to metabolic rate, so that the duration of life seems to be inversely related to the rate of oxygen consumption [152]. The increased longevity obtained by caloric restriction in rodents [153,154], which is accompanied by decreased state 4 respiration and decreased superoxide production [154], and the relation of lifespan in *Drosophila* with the simultaneous expression of the antioxidant enzymes superoxide dismutase and catalase [155] are corollaries of this proposal.

### 6.1. Mitochondrial theories of ageing

As pointed out in Sections 2 and 3, mitochondria are the major sources of oxygen radicals through the respiratory chain and are also deeply affected by ROS, resulting in serious risks to their function. Indeed, a decreased mitochondrial performance has been generally observed in senescence (cf. [156]) and, in principle, this decline could be due to alteration of each one of the macromolecular components above. The mitochondrial theory of ageing [157,158] has attempted to define in precise molecular terms the energetic changes accompanying senescence, and can be represented as a refinement of the free radical theory.

Briefly, it was proposed that accumulation of somatic mutations of mtDNA, induced by continued exposure to free radical attack, leads to errors in the mtDNA-encoded polypeptide chains; these errors are stochastic and randomly transmitted during mitochondrial division and cell division. The consequence of these alterations, which affect exclusively the four mitochondrial enzymatic complexes involved in energy conservation, would be defective electron transfer and oxidative phosphorylation. In addition, respiratory chain defects may become associated with increased production of ROS, thus establishing a vicious circle of mtDNA mutations and oxidative stress [159]; the redox mechanism of ageing [159,160] unifies both the ideas of the mitochondrial theory [157,158] and of the free radical theory of ageing [149] (Fig. 2). It is also established that oxidative stress is a powerful cause of apoptotic death (Section 4); therefore oxidative stress, ageing and apoptosis are strictly linked events. It is conceivable that an acute stress would trigger a mechanism inducing cell death directly, whereas a milder stress may slowly lead to impaired cell function as in ageing.

The mtDNA mutations are expected to accumulate and to lead to damage mainly in postmitotic cells [161], where oxidative metabolism is very elevated as in neurons or is subjected to high bursts as in skeletal muscle; moreover, in postmitotic cells, the lesions

could be conserved at difference with mitotic cells where division leads to selection with 'washing away' of the defective cells [162].

### 6.2. Mitochondrial bioenergetics in ageing

The subject of age-dependent changes in mitochondrial bioenergetics abounds in conflicting data, e.g. reporting declines of respiratory enzymes or ATPase activity or unable to find significant differences (cf. [157,163]). There may be several reasons for such conflicting data.

First, mitochondria when isolated are obtained from tissues containing both differentiated non-dividing cells and relatively non-ageing dividing cells; thus small changes only in one population may become undetectable [161]. Furthermore, energy-defective cells may undergo elimination by apoptosis [164]; the continuous cell elimination when mitochondria become deficient would prevent observing important energetic changes in the remaining population.

If the energetic impairment derives from a stochastic damage to the mitochondrial genes, then it is important to select the mitochondrial activity which is most likely to be affected. Since seven out of the 13 structural genes in mtDNA encode for polypeptides in complex I (NADH CoQ reductase), then it is complex I that is most likely to undergo functional alterations [165]. Unfortunately the assay of complex I activity suffers from serious problems due to the choice of the best quinone to be used as electron acceptor [166]; in our laboratory it was found useful, when possible, to assay this activity indirectly [167] by exploiting the pool equation [168]

$$V_{obs} = (V_o \times V_r)/(V_o + V_r)$$

whereby the rate of CoQ reduction ($V_r$) is related to total rate of NADH oxidation by oxygen ($V_{obs}$) and to rate of ubiquinol oxidation ($V_o$). Using this method, significant decreases of NADH CoQ reductase activity, undetected by the direct assay, were revealed in liver and heart mitochondria from 24-month-old rats [167], presumably by providing more accurate values of NADH CoQ reductase activity.

Another approach employed in our laboratory for recognition of possible early changes not only in postmitotic cells but also in short-living cells, such as blood platelets, has been looking for specific changes linked to subunits encoded for by mtDNA; in analogy with previous findings in LHON [139], it was found that rotenone sensitivity of NADH CoQ reductase was significantly decreased in platelets from old individuals [169]. The same change was exhibited by non-synaptic mitochondria from rat brain cortex [170].

A decrease of an individual enzyme activity in a metabolic pathway is meaningful only if it is able to affect the rate of the whole pathway, and this will depend on the degree of flux control exerted by the individual step [171]: in the respiratory chain, complex I is present in lowest amounts [62], then it is presumably the rate-limiting step of aerobic NADH oxidation [172]; however, this is not true for the oxidation of NAD-linked substrates in phosphorylating mitochondria [173,174]. In mitochondrial diseases, the flux control coefficient at site I in permeabilized cells was found to dramatically increase [175]; unfortunately this point has not been addressed in studies on ageing.

The opposite phenomenon, i.e. the mentioned decreased respiratory rate accompanied by unchanged complex I activity, detected in livers and hearts of aged rats [167], was ascribed to inadequacy of commonly used acceptors; in analogy with a similar behavior in the LHON 11 778 ND4 mutation, an incorrect assembly of the complex, witnessed by a decreased rotenone sensitivity of its activity [169,170], and leading to artifactual pathways of electron transfer to short chain quinones [40,43], might explain the observation in molecular terms (Fig. 3). Alternatively, a decreased affinity for endogenous CoQ might explain this apparent paradox, since CoQ concentration is not saturating for NADH oxidation [176]. The latter possibility has not been explored in ageing; in view of the analogous finding of decreased rotenone sensitivity in ageing as well as in LHON mutations, where the $K_m$ for a CoQ homolog is also increased [139], this point is obviously worth investigation.

Additional evidence on a respiratory impairment in senescence concerns histochemical detection of a loss of cytochrome *c* oxidase activity (but not of succinate dehydrogenase) in muscle mitochondria from old individuals [177]. The mosaic distribution of the cytochrome oxidase lesions is well in agreement with the stochastic distribution of mitochon-

drial damage predicted by the mitochondrial theory [164].

### 6.3. Mutations and deletions of mtDNA

Point mutations in mtDNA are present in normal individuals and were claimed to increase with age [178,179]; a careful study by quantitative PCR of the frequency in skeletal muscle of two point mutations observed in mitochondrial diseases has revealed that the mutations are present at very low percentage, but there is no correlation with age, at least up to 70 years [180].

The matter is different with mtDNA deletions. The so-called common deletion, observed to high extents in some mitochondrial disorders, was also found to increase exponentially with ageing, particularly in muscle and brain tissues [148]. Careful quantitation by a competitive PCR method revealed that this single deleted species was present in aged muscle at the level of only 0.1% of total mtDNA. Thus, it may be questioned whether detection of deleted mtDNA in the elderly only represents an epiphenomenon of the primary pathogenetic event, since very high levels of damage are necessary to elicit activity changes, in accordance with the threshold effect. It has to be borne in mind, however, that other deletions have been found to increase in ageing [148]. Thus, even though any single species of deleted mtDNA is present at low levels, the total amount of deleted mtDNA may reach levels that could be significant in terms of oxidative phosphorylation decline. Ozawa, by systematic use of PCR primers over the entire mDNA, found a progressive age-related fragmentation of mtDNA into various size deleted molecules up to 358 types, with a strong correlation with 8-hydroxy-D-guanosine accumulation [92,96,181]; mtDNA fragmentation also occurred in premature ageing [182]. Deleted forms of mtDNA lacking replication origins accumulated up to 280 types out of 358. Concomitantly, wild type mtDNA decreased down to 11% of total. Similar fragmentation of mtDNA [183,184] and extensive oxygen damage to tissues [185] was demonstrated in patients with mitochondrial cardiomyopathy, harboring hazardous germ-line point mutations; therefore the mitochondrial diseases seemed to be derived from premature ageing of the tissues.

A proof of the existence of a vicious circle of oxidation and mtDNA damage [159] requires demonstration that ROS production and their effects *increase* in ageing. It was indeed shown that hydrogen peroxide generation increases with age, e.g. in different species of flies [151] and in isolated rat hepatocytes ([186,187]; Cavazzoni and Lenaz, unpublished observation), although peroxide overproduction in ageing was not found by others (cf. [188]). Increased production of ROS is also inferred from the increase of oxidized cellular components with age [3,4,72,160], although this accumulation may also result from impaired disposal of the damaged species (cf. [189]).

According to the above view, the accumulation of mtDNA lesions with ageing derives from an unbalance in the rate of generation of ROS and their removal [159,160]; an alternative or additional explanation is that there is a relatively constant rate of generation of the mutations, leading to synthesis of aberrant proteins, but that in young animals cells exhibiting those mutations are eliminated by immunosurveillance by cytotoxic T-lymphocytes; since the protective immune response declines with age, leading to an altered repertoire of antigens recognized [190], one would expect an accumulation of cells containing altered mtDNA (H. Baum, personal communication). In the light of the above hypothesis, the decline in immunosurveillance with ageing may also well reflect somatic mutations of lymphocyte mtDNA (cf. [191]).

The significance of mtDNA deletions in ageing is emphasized by the species specificity of their time of arousal: mtDNA deletions accumulate in organisms with life spans significantly shorter than those of humans, at ages when there is no detectable deletion in humans [192]. To this purpose, when using rodents as experimental models for human ageing, the appropriate tissues should be considered, since not all tissues of rats accumulate mtDNA deletions in the same manner as those of humans [193].

### 6.4. Mitochondria-nucleus interplay in ageing

The available evidence suggests that mtDNA deletions accumulate to sufficient extents to be able to represent the cause for the respiratory chain and oxidative phosphorylation defects observed in ageing

[148]; the proof that this is indeed the case, however, is still lacking. The reason why deletions but, perhaps, not point mutations accumulate in ageing is not clear, although it may depend on the DNA repair capabilities of mitochondria [194]. Oxidative stress can lead to mtDNA deletions [92,94,95], although these can also arise from altered nuclear-cytoplasmic interactions. In fact an autosomal dominant form of PEO (paralysis of extraocular muscles), characterized by multiple mtDNA deletions, indicates that it is a nuclear gene product that affects the proclivity of mtDNA to suffer deletions [195]. Whether this is also the case for mtDNA deletions occurring in ageing is not known. The observation that oxidative phosphorylation defects in cultured cells from aged donors could be reversed by constructing cybrids where the nucleus from the old donor was substituted by a nucleus from an immortalized cell suggested that a nuclear mutation might be responsible for the mitochondrial deficit [196]; a subsequent extended study [197], however, demonstrated the formation of several respiratory-deficient clones by fusing mitochondria from fibroblasts of aged donors with a mtDNA-less $\rho^0$ cell line, as well as a significant decrease of respiration rate and of mtDNA content with the age of the donors. The results of the previous study [196] were ascribed to selection of respiratory competent clones during fusion.

Some additional observations on mitochondrial changes in ageing appear difficult to be directly explained by the mitochondrial theory. The decreased mtDNA transcription in heart and brain of aged rats [198] and the decreased rate of mitochondrial protein synthesis in human muscle [199] have suggested the existence of some more subtle defect in the interplay between nucleus and mitochondria. However, the findings that deleted mtDNA often lacks replication origins [159] and that the deletions usually encompass several tRNA genes may offer explanations in line with the mitochondrial theory. The suggestion that mitochondria in aged cells may increase in size [161,187,200,201] would also be in line with this explanation, as a sign of impaired mitochondrial division [160,161].

The dramatic changes in lipid composition observed in ageing, particularly the fall in cardiolipin content [202] and its relation with cytochrome oxidase activity [68,69], suggest that other factors, related to oxidative stress but not involving mtDNA, may be important pathogenetic events in the ageing process. The finding that a decreased rotenone sensitivity of NADH CoQ reductase is observed in platelets from old individuals [169], but that the 'common deletion' is not observed in mtDNA from the same cells [203], in contrast with a previous study [204], throws some uncertainty over the mtDNA damage as the *only* source of bioenergetic defect. Clearly, a mitochondrial involvement in ageing is present, though a unique role of mtDNA has yet to be established.

## 7. Mitochondria and age-related diseases

The possibility that some of the most common and devastating degenerative diseases of old age have a mitochondrial involvement has been seriously considered in recent years [205–207]. Evidence pertaining to this point will be only briefly summarized here. In particular an impairment to energy metabolism due to progressive failure of the mitochondria has been invoked in the pathogenesis of Alzheimer's disease, Huntington's disease, Parkinson's disease, and cerebellar degenerations. A common denominator of these diseases, affecting different specific brain areas with cellular atrophy, may be a reduced oxidative metabolism and ATP synthesis, leading to membrane depolarization with consequent activation of the glutamate NMDA channels and excitotoxic cell death [206], similar to the acute death produced by glutamate release in ischemia-reperfusion injury [208]. The observation that mitochondrial dysfunction in nervous tissue induces excitotoxic responses agrees with this hypothesis [209].

In Alzheimer's disease, the affected brain areas exhibit an increased production of 8-hydroxy-2-D-guanosine from mtDNA with respect to age-paired controls [96–98], an indication of enhanced oxidative stress in the diseased brain; the mtDNA deletions, however, were found to be decreased with respect to age-matched controls, shedding doubt on the significance of the deletions, at least in concern with the disease [210].

A severe compromission of complex I has been found in the substantia nigra of subjects with Parkinson's disease [211], and also, though less evident,

in muscle and in platelets and lymphocytes of diseased individuals [207,212]. The lowered levels of reduced glutathione in the substantia nigra of asymptomatic Parkinson's carriers with Lewy bodies [213] militates for an early involvement of oxidative stress in the pathogenesis of the disease [83]. The involvement of mtDNA deletions or mutations is not proven with certainty [214,215]. It was suggested that certain point mutations occurring as polymorphisms in mtDNA may serve as risk factors for neurodegenerative diseases [216]; the concomitant lesions due to ageing and to environmental stress would gradually impair mitochondrial function leading to excitotoxic cell death and progressive atrophy of the interested area. The complex I defect present in platelets from Parkinson patients was transferred to fusion cybrids, indicating a possible mtDNA defect [217]. The fact that some brain areas seem to be more susceptible to mtDNA damage in comparison with others may be related to the specific localization of neurodegenerative diseases [83]. The picture is emerging that most degenerative diseases originate from a concomitance of risk factors enhancing the danger of oxidative stress and energy failure in specific tissues.

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