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Forum Review Article

**THE INTERPLAY BETWEEN RESPIRATORY SUPERCOMPLEXES AND ROS  
IN AGING**

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## ABSTRACT

**SIGNIFICANCE:** The molecular mechanism of aging is still vigorously debated, although a general consensus exists that mitochondria are significantly involved in this process. However, the previously postulated role of mitochondria-derived reactive oxygen species (ROS) as the damaging agents inducing functional loss in aging has fallen out of favour in the recent past. In this review, we critically examine the role of ROS in aging in the light of recent advances on the relationship between mitochondrial structure and function.

**RECENT ADVANCES:** The functional mitochondrial respiratory chain is now recognized as a reflection of the dynamic association of respiratory complexes in the form of supercomplexes. Besides providing kinetic advantage (channelling), supercomplexes control ROS generation by the respiratory chain, thus providing a means to regulate ROS levels in the cell. Depending upon their concentration, these ROS are either physiological signals essential for the life of the cell or toxic species that damage cell structure and functions.

**CRITICAL ISSUES:** We propose that under physiological conditions the dynamic nature of supercomplexes reversibly controls the generation of ROS as signals involved in mitochondrial-nuclear communication. During aging there is a progressive loss of control of ROS generation so that their production is irreversibly enhanced, inducing a vicious circle in which signalling is altered and structural damage takes place.

**FUTURE DIRECTIONS:** A better understanding on the forces affecting supercomplex association would allow the manipulation of ROS generation, directing these species to their physiological signalling role.

## 1. INTRODUCTION

The molecular mechanism by which aging induces a functional loss of the cells is a pleiotropic process involving several steps; mitochondria, however, undoubtedly play a central role in the mechanism of aging. Somatic mutations in mitochondrial DNA (mtDNA), originating from progressive ROS-induced damage, have been considered the major determinants of energy loss accompanying aging, as a consequence of alterations in the subunits of OXPHOS complexes encoded by that mtDNA. Since the generation of ROS in the cell is largely a task of the mitochondrial respiratory chain, the “mitochondrial theory of aging” (268) is a direct derivation of the “free radical theory of aging” (156).

The perspective of the role of ROS in the aging process has undergone an important change after the realization that ROS are physiological messengers acting through redox modifications in signalling proteins. For this reason it is believed that aging may be, at least in part, the result of alterations of signalling pathways such as those involved in mitochondrial biogenesis and apoptosis, induced by an increasing ROS production. However, the domination of the free radical theory has faltered in recent years, being challenged by a number of studies that seem to contradict it. For example, it was shown by a meta-analysis that antioxidant vitamin supplements such as vitamin A and E, singly or combined, significantly increase mortality in the general population (32, 33). In addition, other findings seem to demonstrate that ROS increase the life span of several organisms, rather than decreasing it (119, 332, 211). The significance of these findings has been, however, challenged (14, 239) and will be discussed in this review.

Indeed mitochondria might also affect longevity by ROS-independent mechanisms. MtDNA mutations and other damages may derive from errors or infidelity in metabolic mechanisms (transcription, translation etc.) (131). Of particular impact has been the observation of genetic manipulations that impair mitochondrial function and accelerate aging

but do not increase ROS (i.e. 384). Nevertheless, though important, this observation does not necessarily contradict the mitochondrial theory (14).

In this respect, understanding the mechanisms by which damaged molecules and structures are repaired or removed as a means to keep mitochondria functional over time may be of fundamental importance for the aging process.

The relative importance and weight that each of these factors exerts in the aging process is at the moment not fully clarified. For this reason it is important to understand the mechanism underlying ROS generation from mitochondria and other sources and their interplay. In particular, it is necessary to define (i) which are the precise sources of ROS involved, (ii) which factors control ROS generation and (iii) what are the immediate and long-term responses within the mitochondria in terms of redox chemistry.

In this review, we discuss evidence that the supramolecular structure of the mitochondrial respiratory chain is a major factor controlling the generation of ROS and is therefore largely involved in the aging process.

## 2. MITOCHONDRIAL ROS IN PHYSIOLOGY AND PATHOLOGY

Reactive oxygen species (ROS) are recognised today both as important factors physiologically involved in cell signalling, by affecting the oxidoreductive (redox) state of signalling proteins, and as among the major determinants of toxicity in cells and organisms.

ROS is a collective term including oxygen derivatives, either radical or non-radical, that are oxidizing agents and/or are easily converted into radicals. Diatomic oxygen  $O_2$  is a radical because it has two unpaired electrons each located in a different  $\pi^*$  antibonding orbital, but both with the same spin quantum number: this parallel spin is the reason for its low reactivity with non-radical molecules. However, inverting the spin of one of the unpaired

electrons by an energy input converts  $O_2$  into the much more reactive singlet oxygen  $^1O_2$ ; in the excited state, the two electrons may either remain in two different orbitals or form a pair in the same  $\pi^*$  orbital (115). Spin restriction can also be overcome by adding electrons to oxygen one at a time (115).

Transition metals such as iron and copper, when in a free state, have a strong capacity to reduce  $O_2$ , thus generating radical species. If a single electron is supplied to  $O_2$ , it enters one of the  $\pi^*$  orbitals to form an electron pair there, thus leaving only one unpaired electron in the superoxide radical anion  $O_2^{\cdot-}$ . Addition of another electron to  $O_2^{\cdot-}$  gives the peroxide ion, which is a weaker acid and is protonated to hydrogen peroxide  $H_2O_2$ . Addition of two more electrons splits the molecule producing water  $H_2O$ . If one single electron is added to  $H_2O_2$  by a reduced metal ion (e.g.  $Fe^{2+}$ ), the hydroxyl radical  $OH\cdot$  is produced by the *Fenton reaction*. The hydroxyl radical is extremely reactive with a half-life of less than 1 ns, thus it reacts close to its site of formation.

Consequently it must be borne in mind that ROS are widely different molecules, each having specific chemical properties that must necessarily reflect in their different biological effects. Thus, efforts should be made to clarify the individual effects of each ROS species in physiological and pathological processes.

The only technique that can detect free radicals directly is ESR (151). However, most ROS persist for very short times *in vivo* and cannot be measured directly. In this case, one solution is to add probes that act as “traps” and intercept ROS to form stable radicals detectable by ESR. A wide range of traps, mostly nitroxides, has been used in animals and cells in culture (151). Another approach to detect transient ROS is based on the use of *probes*, mainly fluorescence probes (151). Dichlorofluorescein diacetate (DCFDA) is widely used for this. It can enter cells where it is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF) upon cleavage of the acetate groups by intracellular esterases and oxidation by

endogenous ROS. Although DCF is usually considered a probe for peroxides, it reacts slowly with  $\text{H}_2\text{O}_2$ , and also with superoxide, while reacting mostly with  $\text{OH}\cdot$  and peroxynitrite. There are several limitations and artefacts associated with the DCF assay for intracellular  $\text{H}_2\text{O}_2$  measurement and it is essential to keep these limitations in mind for proper interpretation of data (190).

Measuring the amount of oxidative damage caused by ROS or the antioxidant capacity of body fluids and cells is often taken as an indirect index of oxidative stress. It has to be borne in mind, however, that different biomarkers correlate differently with oxidative stress and that the studies employing fluorescence probes are amenable to artefacts and should be interpreted with caution (151).

## 2.1. Mitochondrial sources of ROS

ROS arise in cells from exogenous and endogenous sources. Exogenous sources of ROS include UV and visible light, ionizing radiation, drugs and environmental toxins. Among endogenous sources there are: xanthine oxidase, cytochrome P-450 enzymes in the endoplasmic reticulum, peroxisomal flavin oxidases and plasma membrane NADPH oxidases (150). Nevertheless, the mitochondrial respiratory chain in the inner mitochondrial membrane (IMM) is usually considered one of the major sources of ROS, although other enzyme systems in mitochondria can be important contributors to ROS generation (229). Among these, we mention here dihydrolipoamide dehydrogenase (a subunit of the  $\alpha$ -ketoglutarate and pyruvate dehydrogenase complexes) (369, 382, 375, 8, 311, 192, 193), monoamine oxidase (46, 256, 85), and mitochondrial nitric oxide synthase (418). The last becomes a direct source of superoxide by an uncoupling mechanism in which it reacts with oxygen and generates  $\text{O}_2\cdot^-$  instead of  $\text{NO}\cdot$  (320), particularly in the absence of the cofactor tetrahydrobiopterin  $\text{BH}_4$ .

It is worth noting that mitochondria from different tissues may vary conspicuously in their capacity to produce ROS using different substrates (207), and this capacity is also related to animal species and age.

Murphy (282) has carefully analysed the thermodynamics of mitochondrial superoxide production *in vivo*, concluding that the one-electron reduction of  $O_2$  to  $O_2^{\cdot -}$  ( $E_m = -160$  mV) is thermodynamically favoured by the existing steady-state concentrations of  $O_2$  and  $O_2^{\cdot -}$  so that the actual reduction potential  $E_h$  may approach 200 mV and over. This high potential would allow  $O_2$  to be theoretically reducible even by redox couples having high  $E_m$ . A wide range of electron donors within mitochondria could potentially carry out this reaction. However, only a small proportion of mitochondrial electron carriers is effective. The reason is that electron transfer to oxygen requires the protein-linked potential donors to be at a distance compatible with electron tunnelling, according to the Marcus theory (275).

### 2.1.1 The respiratory chain

According to Quinlan et al. (323), at least ten different sites of superoxide/ $H_2O_2$  production in the electron transport chain and associated enzymes (Krebs cycle,  $\beta$ -oxidation etc.) have been identified in mammalian mitochondria. The FMN and CoQ-binding sites of Complex I and the  $Q_o$  site (at the outer or positive side) of Complex III are often invoked as the most important mitochondrial superoxide producers, but other sites have also been defined, including glycerol phosphate dehydrogenase, ETF-CoQ reductase, and pyruvate and  $\alpha$ -ketoglutarate dehydrogenases. The absolute and relative contribution of each site differs greatly with different substrates (Fig. 1).

**INSERT FIGURE 1**

The topology of the sites is important because it establishes whether the ROS are produced on the matrix side of the IMM, where they may damage mtDNA, or directly released out of the mitochondria. In the latter case, the ROS can damage other systems within the cell or they may constitute a signalling pathway from mitochondria to the cytoplasm and nucleus.

Most of superoxide is generated at the matrix side of the IMM, as appears from the observation that superoxide is detected in submitochondrial particles (SMP) which have an opposite orientation with respect to mitochondria. A study with suitable spin traps, however, demonstrated the formation of superoxide radical in mitoplasts, indicating that a significant aliquot of this species is released at the outer face of the IMM (370). It is likely that Complex I releases ROS in the matrix while Complex III mostly in the intermembrane space (IMS). The superoxide anion released at the IMS may be directly exported to the cytoplasm through an anion channel related to the Voltage-Dependent Anion Channel, VDAC (153).

Complex I is a major source of superoxide production in several types of mitochondria.

The identification of the oxygen reducing site has been the subject of extensive investigation, and several prosthetic groups in Complex I have been suggested to be the direct reductants of oxygen. These include FMN (118, 206, 100), ubisemiquinone (208, 209), and iron sulphur cluster N2 (125, 228, 104).

Grivennikova and Vinogradov (142) investigated ROS generation by Complex I either isolated or membrane-bound at different  $\text{NAD}^+/\text{NADH}$  levels. At the optimal NADH concentration of 50  $\mu\text{M}$ , Complex I produced both superoxide and hydrogen peroxide at a 0.7 ratio  $\text{O}_2^-/\text{H}_2\text{O}_2$ . The production of superoxide was attributed presumably to FeS cluster N2, whereas hydrogen peroxide was interpreted as deriving from 2-electron oxidation of fully

reduced FMN. Rotenone, a specific inhibitor of Complex I, enhances ROS formation during forward electron transfer in the respiratory chain (164, 125).

The formation of superoxide in Complex III depends on the peculiar mechanism of electron transfer, the so-called Q-cycle (74, 75). This mechanism involves the biphasic oxidation of ubiquinol: one electron is given to the Rieske iron sulphur cluster and then to cytochromes  $c_1$  and  $c$ , while the other electron reduces low potential cytochrome  $b$  ( $b_L$ ). Since the subsequent electron transfer from cytochrome  $b_L$  to the high potential  $b_H$  occurs against the electrical gradient (from the positive to the negative side), this reaction is strongly retarded when the electrochemical potential is high, as in the controlled state (*State 4*). This retardation, prolonging the lifetime of the semiquinone ( $Q\cdot$ ) at the outer  $Q_o$  site, has been interpreted as allowing reaction of  $Q\cdot$  with  $O_2$ , thus forming superoxide (276, 184).

Antimycin A (AA), an inhibitor acting at the inner or negative side of the membrane ( $Q_i$  site), is known not to completely inhibit electron flow from ubiquinol to cytochrome  $c$ . The AA-insensitive reduction of cytochrome  $c$  is mediated by superoxide radicals. According to the Q-cycle, AA blocks ubiquinone reduction by cytochrome  $b_H$  at  $Q_i$ . The antimycin-stimulated production of ROS is inhibited by the inhibitors acting at  $Q_o$  site, where ubiquinol reduces both the Rieske iron-sulphur cluster and cytochrome  $b_L$ . Thus, we may locate the site of one-electron reduction of oxygen in presence of AA at a component located at  $Q_o$ , presumably ubisemiquinone (56).

It has been assumed that superoxide is formed during the physiological operation of the Q-cycle (in which the semiquinone is formed when ubiquinol is oxidized by the Rieske FeS cluster). More recent evidence, however, suggests that the source of the electron to reduce oxygen is the semiquinone formed in the so-called semi-reverse reaction in which cytochrome  $b_L$  reduces the fully oxidized quinone. In fact, superoxide formation is stimulated by the presence of oxidized quinone (95) and by mutations preventing the proximity of the

FeS cluster of the Rieske protein to the ubiquinone site (344). This latter condition does not allow interaction of ubisemiquinone with the Rieske cluster and thus favours its reaction with oxygen.

Several observations suggest that also Complex II may be a significant source of ROS (176, 177, 178, 420, 217, 323). Indeed, the highest rate of ROS production in isolated mitochondria occurs with succinate as substrate (327), but this phenomenon is sensitive to rotenone (208, 288, 396) therefore ROS generation is commonly attributed to the contribution of Complex I by the energy-dependent reverse electron transfer from succinate to  $\text{NAD}^+$  via the CoQ pool (202, 184, 205, 368). Nevertheless, this view has been challenged by a series of experiments that seems to exclude a significant ROS production catalysed by reverse electron flow through Complex I, thus allowing ROS production by Complex II as the only plausible explanation (273).

It is also worth noting that mitochondria in intact cells oxidize predominantly NAD-linked substrates so that reverse electron transfer cannot be a very important contributor to superoxide formation (273).

The simple passive diffusion of hydrophilic ROS across lipid membranes is limited due to permeability restrictions, but evidence exists that the superoxide produced in the mitochondrial matrix may also be exported in the cytoplasm. A new function of uncoupling proteins UCP2 and UCP3 was suggested (409) by observing that guanine nucleotides decrease the release of superoxide anion, but not of hydrogen peroxide, from respiring heart and muscle mitochondria. Since guanine nucleotides are known inhibitors of UCPs, the authors inferred that these proteins act as anion channels to export dangerous superoxide from the mitochondrial matrix. Accordingly, guanine nucleotides enhanced the damaging effect of ROS on matrix aconitase.

In addition, it has been proved that selected aquaporin homologues (e.g. AQP8) have the capacity to channel  $\text{H}_2\text{O}_2$  across the IMM (30), thus somewhat challenging the importance of ROS compartmentation with respect to the IMM. These observations, however, clearly point out that ROS export is largely a protective device, potentially allowing ROS detoxification elsewhere in the cell without consuming mitochondrial reduction equivalents (29).

An extensive review of other sites in the respiratory chain which are responsible for ROS generation (e.g. glycerol phosphate dehydrogenase, dihydroorotate dehydrogenase, electron transfer flavoprotein (ETF) and ETF dehydrogenase) can be found in (222).

### 2.1.2 Redox cycling

Mitochondrial generation of ROS can be implemented by exogenous compounds that interact with the respiratory chain by virtue of their redox properties. Among these are organic compounds and metal ions, in particular heavy metal ions. The toxicity of many of these compounds is best explained by their interaction with mitochondrial respiration and induction of oxidative stress (221).

Being so significantly present in living organisms, Fe is the metal most commonly involved in oxidative stress (103). Iron can exist in different oxidation states, varying from  $-2$  to  $+6$ . However, within biological systems, Fe is bound to specific metalloproteins and is found in the  $+2$  or  $+3$  oxidation states. Such change in its redox state is crucial to a large number of reactions in oxidative metabolism and determines Fe participation in potentially cytotoxic reactions. In fact,  $\text{Fe}^{2+}$  can catalyse the Fenton reaction by which  $\text{H}_2\text{O}_2$  is reduced to the aggressive  $\text{OH}\cdot$  radical.  $\text{Fe}^{3+}$  can also be reduced to  $\text{Fe}^{2+}$  after reacting with superoxide anion by the Haber-Weiss reaction; this reaction, however, may not have physiological

significance, due to the presence in the cells of reducers of  $\text{Fe}^{3+}$  which are more powerful than superoxide (240).

Many physiologically active substances and xenobiotics have electron transfer functionalities, either *per se*, or more usually in their metabolites. These main groups include quinones (or phenolic precursors), metal complexes (or complexors), aromatic nitro compounds (or reduced derivatives), and conjugated imines or iminium species (203). Many of these compounds generate ROS exploiting a mechanism of *redox cycling*.

*In vivo* redox cycling with oxygen can occur in a catalytic fashion giving rise to a futile cycle that regenerates the parent compound and releases ROS. Redox cycling has been suggested for several drugs, like cocaine, other abused drugs, catecholamines, and several other compounds, besides a large number of organic components of fine-particulate air pollution that initiate a series of cellular reactions and ultimately lead to cell injury (129).

Electron transfer with redox cycling occurs through interference with physiological electron transfer reactions such as microsomal cytochrome P450, xanthine oxidase, and the mitochondrial respiratory chain. For example, adrenaline may undergo oxidation and cyclisation to adrenochrome in a multi-step process in which the main oxidant under physiological conditions is the superoxide anion. On the other hand adrenochrome can be reduced to the corresponding semiquinone by mitochondrial Complex I, thus establishing a redox cycle in which the semiquinone reacts with  $\text{O}_2$  producing superoxide and regenerating adrenochrome (123).

### 2.1.3 The adaptor protein $p66^{\text{Shc}}$ .

Special mention must be made of the *adaptor protein  $p66^{\text{Shc}}$* , a 66-kDa Src collagen homologue (Shc) protein that is one of three main isoforms encoded by the SHC1 gene ( $p46^{\text{Shc}}$ ,  $p52^{\text{Shc}}$ ,  $p66^{\text{Shc}}$ ) (266). While  $p46^{\text{Shc}}$  and  $p52^{\text{Shc}}$  isoforms link activated receptor

tyrosine kinases to the Ras pathway, by recruitment of the GRB2/SOS complex,  $p66^{\text{Shc}}$  mediates an inhibitory signalling effect on the extracellular signal-regulated kinase (ERK) pathway that is required for actin cytoskeleton polymerization and normal glucose transport control.  $p66^{\text{Shc}}$  has been also identified as a sensor of oxidative stress-induced apoptosis. This action requires Ser36 phosphorylation of the protein. Pathways that regulate the cellular response to oxidative stress and life-span by leading to increase of  $p66^{\text{Shc}}$  expression involve p53 (385, 299) and serine-threonine kinases, including the PKC- $\beta$  isoform (319).

Expression of  $p66^{\text{Shc}}$  is required for mitochondrial depolarization and release of cytochrome *c* after a variety of pro-apoptotic signals (385).  $P66^{\text{Shc}/-}$  cells are resistant to apoptosis (265) and  $p66^{\text{Shc}}$  deletion in mice decreases the incidence of aging-associated diseases (285, 261, 336) and prolongs lifespan of animals (265), due to their resistance to oxidative stress.

A fraction of  $p66^{\text{Shc}}$  has a mitochondrial localization in the IMS, where it is bound as an inactive form in a high molecular weight complex, including the TIM/TOM protein import system (130). Under stress conditions, however, antioxidant systems are not able to retain  $p66^{\text{Shc}}$  in their reduced dimeric state. Pro-apoptotic signals dissociate  $p66^{\text{Shc}}$  from the complex and activate it to a tetrameric form (126) that triggers the permeability transition by opening a high conductance channel in the IMM, the *permeability transition pore* (26), which is involved in the events leading to apoptosis. This effect is due to the intrinsic property of  $p66^{\text{Shc}}$  to act as a redox protein accepting electrons from cytochrome *c* and directly producing hydrogen peroxide (130). A cytochrome *c*-binding domain near the N-terminus is responsible for this activity (127).

Thus the  $p66^{\text{Shc}}$  protein may be not only a redox enzyme but also a ROS sensor and possibly a ROS amplifier in the mitochondrial IMS through an inherent ROS-producing activity.

Since the reaction equilibrium of cytochrome *c* oxidation by  $p66^{\text{Shc}}$  is low ( $K_{\text{eq}} = 0.1$ ), the reaction is thermodynamically favoured when the level of cytochrome *c* reduction is high (130). This means that  $\text{H}_2\text{O}_2$  production by this mechanism should be enhanced when cytochrome *c* oxidase (the enzyme catalysing cytochrome *c* reoxidation by oxygen) is inhibited. The  $K_m$  of cytochrome oxidase for oxygen is very low ( $< 1\mu\text{M}$ ), thus allowing its activity even at low oxygen tensions. However, low oxygen tensions promote the activation of hypoxia-inducible factor (HIF-1 $\alpha$ ) by a still controversial mechanism (378); HIF-1 $\alpha$  triggers a series of metabolic changes among which is alteration of cytochrome oxidase subunits and activity. Thus the activation of the  $p66^{\text{Shc}}$  pathway may in part explain the paradoxical enhancement of ROS production during hypoxia (145, 28, 55).

## 2.2. Upstream control of ROS production

The steady-state level of ROS is determined by the rate and site of their generation, by their nature and lifetime, by their diffusion constants, by the interconversions occurring between different ROS, and finally by the rate of removal by different antioxidant systems.

The generation of ROS by isolated mitochondria accounts for 0.1-0.2% of oxygen consumed and may reach up to 2-3% under particular conditions that may not be found physiologically. The rate and extent of ROS generation greatly varies in different tissues and specifically depends upon the substrate employed: for example, succinate is important for ROS production in brain, heart, kidney, and skeletal muscle, while fatty acids are major generators in kidney and liver (301, 375).

There is increasing evidence that mitochondrial ROS level is physiologically regulated; this is particularly important for their action as cellular signalling molecules (cf. section 2.3.1).

### 2.2.1 Role of Mitochondrial Membrane Potential

Mitochondrial ROS production is enhanced in State 4 and when the rate of electron transfer is lowered (364). The rationale is in a more reduced state of the respiratory carriers capable of donating electrons to oxygen. Hence, uncoupling and release of excessive membrane proton potential may protect mitochondria from damage induced by excessive free radical production. In rat hepatocytes the futile cycle of proton pumping and proton leak may be responsible for 20-25% of respiration (37); in perfused rat muscle the value is even greater. Uncoupling may be achieved by activating proton leak through *uncoupling proteins* (56) (see above, however, for a different role postulated for uncoupling proteins, cf. 409). In such a way, whilst a tissue may dissipate a conspicuous part of the energy conserved by its mitochondria, it also keeps the mitochondrial respiratory chain under more oxidized conditions preventing the formation of free radicals.

The notion that mild uncoupling may be a protective mechanism that lowers mitochondrial  $\Delta\Psi$  and thus alleviates oxidative stress has been challenged on the theoretical basis that the mitochondrial membrane potential within living cells is much lower (100-130 mV) than that measured in isolated mitochondria. Accordingly, no significant change in matrix superoxide occurred after treatment of cerebellar neurons with the uncoupler FCCP (185).

It is worth mentioning that matrix pH *per se* is also an essential factor defining ROS production by the respiratory chain, even in the absence of pH gradient, and that pH increase in the matrix induces the increase in ROS generation (356). This observation is in agreement with a previous demonstration that the rate of superoxide generation increased in conjunction with an increase of medium pH from 7 to 9.2, in the presence of NADH or succinate as substrates and of rotenone or antimycin A as specific enzyme inhibitors (388). Moreover, the decrease of matrix pH induced by the addition of  $P_i$  and nigericin is accompanied by an

increase of  $\Delta\Psi$ , which is expected to stimulate ROS production (356). However, the ROS generation rate decreased, thus indicating that in these circumstances the effect of pH dominates over the opposite effect of  $\Delta\Psi$ .

### 2.2.2. Role of Post-Translational Modifications on ROS Production

Events leading to decrease of the rate of electron flow in Complex I lead to overproduction of ROS. Physiological states, such as subunit phosphorylation that inhibits Complex I activity, may modify its ROS generating capacity (326, 248, 346). It is therefore tempting to speculate that endocrine alterations may affect the capacity of ROS formation by hyper- or hypo-phosphorylation of the Complex.

Kadenbach et al. (189) have proposed a different mechanism, centred on cytochrome *c* oxidase. The cAMP-dependent phosphorylation of subunit I of the oxidase greatly increases its sensitivity to allosteric inhibition by ATP. The result of this inhibition is decrease of the  $H^+/e^-$  stoichiometry of the enzyme from 1 to 0.5, with resulting decrease of  $\Delta\Psi_m$  and, consequently, of ROS generation by the respiratory chain. It was also proposed that stress conditions would induce dephosphorylation of Complex IV with transient increase of membrane potential and a burst of ROS generation by mitochondria. The discovery of a mitochondrial  $CO_2$ -adenylyl cyclase-cAMP-PKA signalosome, conserved from the yeast *S. cerevisiae* to human cells (165) and modulating the allosteric inhibition of Complex IV by ATP, underlies a universal mechanism for metabolic regulation in eukaryotes exposed to environmental fluctuations in temperature, oxygen, and nutrient availability. However, the hormonal signals inducing phosphorylation and dephosphorylation remain to be fully established.

Other protein modifications in the respiratory complexes can modulate ROS production (154), such as thiol oxidation or S-nitrosation or S-glutathiolation of Complex I, or Complex I and II acetylation that is regulated by sirtuins (NAD<sup>+</sup>-dependent deacetylases).

In addition, ROS production by mitochondria is under signalling control through the pathways leading to mitochondrial uptake and activation of *p66<sup>Shc</sup>* under conditions of oxidative stress (cf. section 2.1.3).

### 2.2.3 Hypoxia and ROS production

The presence of a pseudo-hypoxic state in aged tissues (cf. Section 3.2) requires a brief discussion of ROS in hypoxia.

A major point of controversy regarding the role of ROS as hypoxic signalling molecules is that it is seemingly paradoxical that a decrease in a required substrate, O<sub>2</sub>, would result in an increase in ROS production. However, using new ratiometric fluorescent probes, it proved possible to demonstrate an increased ROS production during hypoxia (23). Furthermore, both DNA and lipid oxidation products accumulate during hypoxia (141).

The first breakthrough suggesting a mitochondrial oxygen sensor of the HIF pathway came with the discovery that  $\rho^0$  Hep3B cells, which contain no mtDNA and thus no electron transport, are incapable of HIF-1 DNA-binding activity following hypoxia (58). This finding implies that mitochondria are responsible for propagation of the hypoxic signal. Correspondingly, treatment of cells with exogenous H<sub>2</sub>O<sub>2</sub> or induction of cellular H<sub>2</sub>O<sub>2</sub> production are sufficient to stabilize HIF-1 $\alpha$  during normoxia (59). Nevertheless, the activating effect of ROS on HIF-1 $\alpha$  (22, 152, 308) is still a controversial issue (cf. section 2.3).

The content of oxidized cytochrome *c* in the IMS seems to be an essential factor in controlling mitochondria-derived ROS (47, 307); in fact, cytochrome *c* in the IMS oxidizes

superoxide produced by Complex III to  $O_2$ , thus preventing generation of  $H_2O_2$ . The loss of cytochrome *c* due to outer membrane permeabilization during hypoxia and ischemia (62) was considered a major factor of ROS generation under pathological conditions (307).

Inhibition of cytochrome *c* oxidase by hypoxia (57) enhances ROS formation by the respiratory chain; this result may be due to the increased membrane potential (423) or by inducing a more reduced state of cytochrome *c*. The reduction of cytochrome *c* facilitates its interaction with  $p66^{Shc}$  and further amplification of ROS production (cf. section 2.1.3) while preventing its superoxide scavenging activity. Evidence also exists that ROS generation at mitochondrial Complex III is critical for hypoxia signalling (59, 144, 201, 250) (Fig. 2). More recently, the role of Complex III was confirmed in a transgenic mouse model where deletion of the Rieske protein of Complex III (402) abolishes the hypoxia-induced increase in ROS signalling.

## INSERT FIGURE 2

In addition, it has to be pointed out that the enhanced ROS production during hypoxia has not been ascribed to mitochondria only, but also to the plasma membrane NADPH oxidase (cf. 271).

### 2.3. ROS as signals

It is clear nowadays that, at moderate concentrations, ROS act as second messengers (182, 11, 331, 138, 154, 400, 283, 69) by interfering with the expression of a number of signal transduction pathways and genes. The effects related to ROS signals are double-edged. For example, ROS may be oncogenic and promote proliferation, invasiveness, angiogenesis and metastasis (411), but they may also be anti-oncogenic and promote cell

cycle stasis, senescence and apoptosis (149). Many of the ROS-mediated responses actually protect the cell against oxidative stress and re-establish redox homeostasis. However, there is a threshold above which ROS become harmful and induce oxidative stress (331). The concentration range of ROS can vary conspicuously; moreover it must be kept in mind that the composite term ROS includes several species that have widely different properties. Thus, not all ROS are equally suitable for signal transduction; for instance, the OH· radical is too unspecific to participate in catalysed reactions, while O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub> and lipid peroxidation derivatives like 4-hydroxy-2-nonenal (HNE) are likely to be employed as signalling molecules.

Intracellular signalling achieved by ROS molecular recognition occurs at the atomic level.

Because some ROS such as H<sub>2</sub>O<sub>2</sub> are oxidants, they influence the redox state of signalling proteins through reactions with specific sulfhydryl groups (81) but enzymatic catalysis is usually required to allow the modification to occur under physiological conditions.

A number of transcription factors contain redox-sensitive cysteine residues at their DNA-binding sites (147, 381). Thiols exposed on the surface of mitochondrial proteins (283) are very reactive since the relatively high matrix pH favours the dissociation to thiolate anions; moreover, the high proportion of vicinal thiols facilitates the formation of disulphide bonds acting as redox switches to transduce the response of the protein to redox signalling (282, 283). Such clustering of thiols groups is not casual and is favoured by evolution (255).

The ROS-mediated oxidative modification of proteins is usually followed by other post-translational changes (e.g. phosphorylation, acetylation, ubiquitination, and SUMOylation among others (400)) in the same protein and in other proteins of the signalling cascade.

One notable example of mitochondrial signalling leading to changes in nuclear gene expression (406) is Nrf2 that, in the presence of ROS, is translocated from the cytoplasm to the nucleus. There, Nrf2 binds the antioxidant response element of cytoprotective genes involved in the antioxidant response (e.g. heme oxygenase, NRF-1 and other inducers of mitochondrial biogenesis) (180). Nrf2 dysfunction in ageing likely exacerbates age-related cellular oxidative stress and increases sensitivity to oxidative stress-induced cellular damage.

Signalling proteins modified by ROS include phosphoprotein phosphatases (PTPs), Ras, large G-proteins, serine/threonine kinases of the MAPK families, transcription factors as AP-1, NF $\kappa$ B, p53 and others. The effect is different with different proteins: PTPs are inhibited, nuclear transcription factors are activated (390). Thus, for example, activation of ERK1/2, Akt and NF $\kappa$ B promotes cell survival; whereas activation of c-jun N-terminal kinases, p38 kinase and p53 would lead to arrest of the cell cycle and apoptosis.

In addition, ROS appear to activate the hypoxia-inducible factor HIF-1 $\alpha$  (59, 196) by inhibiting the prolyl-4-hydroxylase that addresses the factor to proteolytic digestion (Fig. 2). HIF-1 $\alpha$  becomes stabilized and binds to hypoxia responsive elements in the DNA, stimulating a large array of genes (357, 191) and decreasing the input of reducing equivalents to the respiratory chain (199). These events mimic what happens in anoxia through lack of O<sub>2</sub> required for HIF hydroxylation.

Mitochondrial ROS production is the determinant of preconditioning, by which small doses of a noxious stimulus are required to induce a protective response against future injury. Stimulation of the hypoxia-signalling pathway by hypoxia or by cyanide treatment (71) is a major factor responsible for preconditioning in brain (361, 70), heart (284, 358) and kidney (61).

Mitochondrial ROS can also act as important signals to regulate the inflammatory response by activating the inflammasome (107, 310) and to regulate autophagic processes including mitophagy (350).

### 2.3.1 Regulation of Mitochondrial ROS in Cell Signalling

Mitochondrial ROS levels reflect the balance between their rate of generation and of removal.

The ROS generation by rat liver mitochondria was investigated under different substrate and inhibitor conditions and different oxygen tensions, with the conclusion that only Complex I may be a significant source of ROS at physiological O<sub>2</sub> concentration (167). In this scenario, the factors directly associated with respiratory activity (e.g. the redox potential of the NAD<sup>+</sup>/NADH couple and the proton-motive force) are powerful regulators of electron leaking (331). These factors in turn are regulated by the redox supply to the chain, by the degree of coupling and by physio-pathological constraints to electron transfer (enzyme phosphorylation, cytochrome *c* removal, Complex IV inhibition, oxygen concentration etc.).

In addition, mitochondria enhance ROS generation in response to external stimuli, such as TNF $\alpha$  (198), hypoxia (59), serum deprivation (215) and oxidative stress itself (ROS-induced ROS release, 422). Several proteins, such as p53, p66<sup>Shc</sup>, the Bcl-2 family and Romo-1 (11) can control ROS generation and release from mitochondria.

In the last years, interplay between mitochondria and other ROS generating systems has been discovered. This interplay is particularly puzzling between mitochondria and the NADPH oxidases (NOX) family (89). There is accumulating evidence showing that stress of the endoplasmic reticulum mediates the cross-talk between mitochondrial- and NOX-derived ROS by activating the Unfolded Protein Response (UPR) signalling pathway and regulating prosurvival and proapoptotic components (339, 340).

ROS removal by enzymatic systems may be directly involved in regulation of ROS signalling (111). Various isoforms of superoxide dismutase, including mitochondrial Mn-SOD, catalyse the dismutation of superoxide to H<sub>2</sub>O<sub>2</sub> and oxygen; whereas catalase, glutathione peroxidase and peroxiredoxins are capable of removing H<sub>2</sub>O<sub>2</sub>. In addition ferric cytochrome *c*, by virtue of its capacity to be reduced by superoxide, participates in antioxidant defence in the respiratory chain (307), while an integrated set of thiol systems within the mitochondrial matrix prevents much of the oxidative damage (283) and may transduce redox signals within and through mitochondria. Peroxiredoxins (Prx) act on small dithiol proteins, of which thioredoxin-2 is localized in the mitochondrial matrix (329, 235). Prx3 is the target of most H<sub>2</sub>O<sub>2</sub> produced in the mitochondrial matrix (72) whereas Prx5 is less effective in reducing H<sub>2</sub>O<sub>2</sub> but has a higher reactivity towards peroxynitrite (387).

Mitochondrial glutathione (mGSH) is a dominant factor in the maintenance of the appropriate mitochondrial redox environment, mainly because of its abundance and versatility to counteract hydrogen peroxide, lipid hydroperoxides, or xenobiotics, by acting as a cofactor of enzymes such as glutathione peroxidase or glutathione-S-transferase (254). The mitochondrial GSH:GSSG ratio is generally greater than 100:1 and is widely used as an indicator of the redox status (186).

Protein glutathionylation may occur through thiol disulphide exchange by protein disulphide isomerase, as a regulatory device for proteins involved in energy metabolism, redox signalling, and apoptosis (415, 77). Glutaredoxin is the primary enzyme responsible for catalysis of de-glutathionylation of protein-mixed disulphides with glutathione. This reversible post-translational modification alters the activity and function of many proteins important in regulation of critical cellular processes (5).

## 2.4. ROS and mitochondrial quality control

Damaged molecules are recognized by degrading systems; in particular, oxidatively modified proteins are extensively ubiquitinated and directed to the proteasome, a member of the ATP-dependent AAA<sup>+</sup> proteases, where their complete digestion takes place (345, 380). Inability of the proteasome to digest some proteins and the accumulation of insoluble protein aggregates may lead to profound alterations in cells.

Since mitochondria are a major source of reactive oxygen species, mitochondrial proteins are especially exposed to oxidative modification, so that their elimination is crucial for maintaining the integrity of this organelle (246, 379). Internal mitochondrial proteins may be retrotranslocated to the outer mitochondrial membrane where multiple E3 ubiquitin ligases are present. Cdc48/p97 is recruited to stressed mitochondria, extracts ubiquitinated proteins from the outer membrane and presents them to the proteasome for degradation.

Within the mitochondrial matrix, the ATP-stimulated Lon protease (Pim-1 in yeast) is devoted to the regulatory mechanism of selective degradation of oxidized proteins. For example, during hypoxia, Lon participates in remodelling cytochrome oxidase by selectively degrading the Cox4-1 subunit (116, 392). Failure of protein degradation has been implicated in the age-related accumulation of oxidized proteins (42, 389).

Cellular structures and organelles undergo turnover and after a suitable life time are directed to autophagy by lysosomal digestion (232, 25, 63).

Non-selective autophagy and mitophagy are triggered by ROS in response to nerve growth factor deprivation, rapamycin, or starvation (214, 350). In mouse and human cells, mitophagy is triggered in a mitochondrial fission dependent manner by mild oxidative stress comparable to that occurring during physical activity (113). These conditions increase ROS levels only slightly and are insufficient to trigger non-selective autophagy, thus suggesting the presence of a very specific and selective signalling cascade initiated by ROS. On the other

hand, when non-selective autophagy is induced by starvation or other means, mitochondria hyperfuse and are protected from mitophagy (134, 328).

### 3. A ROLE OF MITOCHONDRIA AND ROS IN AGING

Most research on the role of mitochondria in aging has been formerly performed using different tissues from aged animals (cf. 220) including domestic mammals and primates (108, 162, 296). The necessity of employing simpler systems that allow the investigation of a number of genetic mutations and/or to exert metabolic manipulations during a short life span (120, 200) has focussed attention more and more on invertebrates. *Drosophila* (306, 236, 238, 289), the housefly *Musca domestica* (67), the nematode *Caenorhabditis elegans* (407, 45, 78), the yeast *S. cerevisiae* (183, 39, 297, 257) and the filamentous fungus *Podospora anserina* (245, 290) have all been usefully employed in research on ageing.

Planarian flatworms (*Tricladia*, *Platyhelminthes*) share a very rare characteristic with human mitochondria, a strong control of oxidative phosphorylation by the phosphorylation system, and for this reason they have been proposed as useful models of mitochondrial disturbances (218). Other species, such as the bivalve *Arctica islandica*, have been proposed as aging models in consideration of their exceptional longevity (281).

Also, naturally occurring mouse mutants, such as the senescence-accelerated mouse (170, 179) and transgenic mice (270), have provided important clues. The “mutator mouse”, having a defect of the proof-reading function of mitochondrial DNA polymerase  $\gamma$  (Poly), is a remarkable model of premature aging due to mtDNA mutations (383, 384, 97, 166, 38).

Finally, although the use of aging *in vitro* of cell cultures as a model for senescence is controversial (160, 99, 83), primary cell cultures from senescent animals represent a useful tool for studies of mitochondria in relation to aging (148, 397, 160). An unprecedented avenue is the use of human induced pluripotent stem cell-based models of aging (241). These

cells can be transplanted into model animals and advance our knowledge of molecular mechanisms of aging and help to develop strategies for treating aging-associated human diseases.

It is also expected that the development of novel and powerful technical approaches will enable investigators to get deeper insights into structure and function of mitochondria and their interactions within the cell, as well as their alterations in disease and aging. The proteomic approach (294, 401), including redox proteomics (94), will become an indispensable tool in unravelling the unknown functions of these organelles. MitoCarta (293), an extensive study by mass spectrometry, GFP tagging, and machine learning has been used to create a mitochondrial compendium of 1098 genes and their protein expression across 14 mouse tissues.

### **3.1. A CRITICAL ANALYSIS OF THE MITOCHONDRIAL THEORY OF AGING**

The mitochondrial theory of aging is based on the following basic assumptions that are linked by a causal relationship (220):

1. Mitochondrial ROS produced during the life time are the major cause of aging
2. Mitochondrial ROS induce structural damage to mitochondrial biomolecules, in particular mtDNA somatic mutations, mostly in post-mitotic tissues.
3. The mtDNA mutations induce damage to the mtDNA-encoded proteins, and consequent decrease of oxidative phosphorylation, culminating in energy failure, metabolic derangement and cell death.

A corollary to the mitochondrial theory is that the decreased electron transfer may cause further ROS generation, establishing a vicious circle of mtDNA damage and ROS production (292).

Although experimental evidence exists, pertaining to each of these points, there are still several controversial aspects and the causal relationships among them are often lacking. The major points to be clarified are the following:

- a) What is the precise role of ROS in aging? In other words, can we determine whether, and how much, are ROS involved in the aging process?
- b) What can we learn from mtDNA genetics in relation to aging and longevity?
- c) What is the relevance for aging of mtDNA deletions and mutations?
- d) Can mtDNA damage be causally correlated with mitochondrial functional defects?
- e) Are mitochondria self-sufficient for the increase and propagation of the damage or is the expression of nuclear genes also necessary for the aging phenotype? In other words, do mitochondria elaborate signals (ROS or others) able to induce the aging process? And how are these signals regulated during the lifespan of an individual?
- f) Finally, can we predict whether, and how much, we can control the aging process by interfering with one or more of the above items?

### 3.1.1. ROS generation and ROS-induced damage

The continuous generation of ROS and other toxic species induces damage to all the biomolecules; thus it is not necessary that an increase of ROS generation occurs in aging, since it is the damage that would accumulate even at a steady ROS generation with time (14). Nevertheless, a vicious circle (292, 91) can be established if the accumulated damage to the respiratory chain would enhance ROS generation. This event is theoretically expected when electron transfer within a respiratory complex competent in ROS generation is inhibited (164, 125, 35). Exposure of mitochondria *in vitro* to different concentrations of hydrogen peroxide or cumene-hydroperoxide induces a transient increase in ROS production, followed by a steady state without further increase (342). In this case, ROS originate mainly from Complex

I as a consequence of the inhibition of the enzyme, supporting the vicious circle hypothesis. It is clear, however, that these *in vitro* conditions are not fully comparable with the situation *in vivo*.

Many reports demonstrated that the rate of production of ROS from mitochondria increases with age in mammalian tissues (366, 330, 54, 188, 17, 21) as well as in cultured cells from old individuals (174, 216, 373) and during replicative cell senescence (160, 110). An additional factor possibly eliciting an increased ROS production in aging is the postulated spreading of damage from one cell to the surroundings by means of the plasma membrane oxidoreductase (274, 82), thus triggering a chain reaction of oxidative damage.

A few reports have indicated a lack of increase of the rate of ROS generation with aging (155, 244, 343). It has to be noted that such results may be a consequence of the decrease of content and activity of respiratory complexes, so that there is a decreased total number of respiratory units producing ROS, even if each unit has an enhanced ROS generation. For this reason the rate of ROS generation should be related to the content of the enzymes that are responsible for it.

A different aspect that unambiguously relates aging to ROS generation is the strong negative correlation of animal longevity with the rate of mitochondrial ROS generation and with the degree of fatty acid unsaturation of cellular membranes (reviewed by 14). According to Barja (14) only the mitochondrial free-radical theory of aging can explain these correlations and, in particular, the latter correlation is explained by the higher oxidative damage (peroxidizability) that may be induced in unsaturated membrane fatty acids. In tissues of aged animals where there is an increase of oxidized lipids (416, 410, 377, 7), mitochondria exhibit peroxidation of their signature lipid, cardiolipin (CL), with consequent CL depletion and loss of respiratory activity (305, 304, 303, 359, 315).

Complex I is considered to be a major source of ROS involved in aging (cf. section 2.1), since ROS generated by Complex I are released in the matrix and may damage mitochondrial proteins and mtDNA. However, ROS produced by Complex III must also be taken in serious consideration, since they are released in the intramembrane space and may be exported in the cytosol, where they can activate, or interfere with, proteins involved in signal transduction (cf. section 2.3.1).

In high eukaryotes, damaged proteins, protein aggregates and damaged organelles tend to accumulate in aging, in the form of lipofuscin deposits in post-mitotic tissues such as muscle, heart, liver and brain (374, 353, 60, 338); these aggregates are removed by autophagy (272) (cf. section 2.4).

The mitochondrial theory of aging considers mtDNA as the major target of mitochondrial ROS because it is close to the site of mitochondrial ROS production and is less protected than nuclear DNA. Indeed, the damage by an oxidative stress is higher and persists longer in mtDNA than in nuclear DNA (412, 249) and the level of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) in mtDNA (but not in nuclear DNA) negatively correlates with the longevity of several animal species, including humans (16, 159, 163). A recent review (194) summarizes the evidence on mtDNA copy number, deletions and point mutations during aging in humans and mice.

Moreover, the oxidation of mitochondrial proteins and lipids also increases with age (366, 4). Genotoxic intermediates of lipid peroxidation may also have a role in causing age-associated DNA mutations (172). This idea is supported by *in vitro* experiments which show that mtDNA is damaged when mitochondria undergo lipid peroxidation.

In mutant mice deficient in SOD2, mitochondrial oxidative stress specifically promotes glycation of mtDNA and does not affect nuclear DNA or cytosolic proteins (40).

Because DNA glycation can change DNA integrity and gene functions, glycation of mtDNA may play an important role in the decline of mitochondrial functions.

A generally negative correlation has been consistently found between endogenous tissue antioxidants and longevity in animals (313, 295). The general result of these comparative studies agrees with the observation that antioxidants do not increase maximum longevity and lack the capacity to slow down aging, independently of the way in which the antioxidants are manipulated: dietary, or genetically. This disappointing lack of effect has contributed to the criticism of the basic postulates of the mitochondrial theory of aging (cf. 239, 312, 41, 341).

Contrary to this view, Barja (14) correctly reasons that these results are expected, since it is the rate of production of ROS and not their elimination that determines the life expectancy. Moreover, ROS concentration in particular compartments such as mitochondria is much more dependent on the rate of ROS generation than on antioxidants, as the free radical generation source, especially Complex I, is approached at the micro level (15). This is especially important because the main target for aging, mtDNA, is located very close to, or perhaps even in contact with the free radical generation source. This can help explain why lowering the rate of ROS generation instead of increasing levels of antioxidants occurred during the evolution of long-lived species.

An additional factor explaining why chemical antioxidants are inactive against aging is that upon scavenging ROS they may be converted into prooxidant byproducts as semiquinones. Accordingly, we suggest that the fact that the novel potent artificial hydroxylamine scavenger (IAC) can prolong the lifespan of the freshwater annelid *Aeolosoma viride* by 170% arises because, upon scavenging ROS, the molecule is converted into an even more potent nitroxide antioxidant (53).

According to Barja (14), the negative correlation between levels of antioxidants and life span of different species only shows that the level of antioxidant enzymes and of repair enzymes is adjusted to compensate for the proportion of ROS generation; thus, if a species produces lower ROS, it is expected that it has less need for antioxidants and DNA repair enzymes.

In a basic study, Trifunovic et al. (384) showed that expression of a proof-reading deficient *Poly* in a homozygous knock-in mouse strain leads to increased levels of somatic mtDNA mutations causing progressive respiratory chain deficiency; the mice develop symptoms strikingly reminiscent of aging. This is the most striking demonstration that mtDNA mutations can cause aging. The rate at which mtDNA mutations reach phenotypic expression differs markedly among tissues, which may be an important factor in determining the tolerance of a tissue to random mitochondrial mutagenesis (394, 395).

In apparent contrast with the mitochondrial theory, the mutator mice were not shown to have an enhanced ROS production (383). It is likely, however, that the lack of enhancement of ROS production results from the exceedingly severe extent of mutation in this mouse strain. Nevertheless we cannot exclude the possibility that a ROS attack is the natural way to induce mutations in other organisms. Indeed, a recent study (242) showed that the level of hydrogen peroxide was the same in the young mutator and control mice, but that hydrogen peroxide increased as the mutator mouse aged. Thus mtDNA mutations increase ROS generation contributing, also in the mutator mice, to the accelerated aging phenotype.

In agreement with the ROS origin of mtDNA defects, hydrogen peroxide induces large scale deletions of mtDNA through formation of double-strand DNA breaks (367). Moreover, some lines of evidence suggest that the frequency of mtDNA deletions is significantly decreased in mice that express the human catalase gene targeted at mitochondria (354). These results demonstrate the importance of mitochondria as a source, and possible

target, of ROS and are consistent with a role of H<sub>2</sub>O<sub>2</sub> in the limitation of mouse lifespan. Nevertheless, the effect of increased mitochondrial peroxide detoxification capacity in model animals, where the endogenous H<sub>2</sub>O<sub>2</sub> removing systems are likely to be already abundant, is not clearly explained at a molecular level and cannot be taken as a proof that preventing peroxide-mediated mitochondrial damage alone is sufficient to delay the aging phenotype.

Contrary to most evidence, a recent study (181) proposes that oxidative stress is not a major cause of somatic mtDNA mutations. The authors found that many of the features associated with mtDNA mutations in vertebrates are conserved in *Drosophila*, including a comparable somatic mtDNA mutation frequency, an increased frequency of mtDNA mutations with age, and a prevalence of transition mutations. Only a small fraction of the mtDNA mutations detected in young or old animals were G-C to T-A transversions, a signature of oxidative damage (cf. section 3.3). Although these observations are puzzling, they cannot be taken as disproving the overwhelming evidence of a contribution of ROS to the aging process.

The evidence existing on the accumulation of oxidative damage in cells and tissues of aging individuals supports the mitochondrial theory of aging in a straightforward way. Nevertheless, the effects of ROS are not limited to structural damage to cell components. In fact, some ROS are now recognized as inducing redox changes in molecules involved in signalling pathways, and ROS themselves are seen today as possible physiological signals (cf. section 2.3). We may therefore ask whether the structural damage induced by ROS, particularly to mtDNA, is sufficient to explain the aging phenotype, or whether more complex factors, possibly involving the derangement of signalling pathways, are the major driving force for the metabolic failure characterizing aging (cf. section 3.2).

### 3.1.2. Functional impact of respiratory chain damage

There is overwhelming evidence that the bioenergetic function of mitochondria declines with aging, especially in post-mitotic tissues (386, 66, 372, 227, 404, 93, 139, 363, 362, 298) and this decline may be induced by ROS, as demonstrated by the decrease of respiratory activities in MnSOD-deficient mice (260). It is also well documented that exposure of mitochondria to ROS can affect the respiratory activity via oxidative damage of CL, which is required for the optimal functioning of the enzyme complexes (302). Relevant to this observation is the decline of the content of mitochondrial CL and the increase of oxidized CL with aging (316, 317).

A mosaic pattern of cytochrome oxidase alterations in muscular tissues from elderly individuals has been found by histochemical investigations (277-280, 399). The absent or rare decrease of activity of individual respiratory complexes observed in many studies (155, 6, 273) may reflect the existence of threshold effects (335); moreover, electron transfer activity alone does not reflect the whole bioenergetic capacity of the mitochondria, since also membrane potential and ATP synthesis should be taken in account. In this regard a study (139) exploiting fibroblasts from donors of different ages, carefully exploring the P:O ratios is very informative in detecting a significant fall of phosphorylation efficiency in the elder. However, tissue differences also exist in the susceptibility of mitochondria to ROS-induced damage. For example the ATP content and production declined to 50% with age in the skeletal muscle (gastrocnemius) but underwent no change in the heart of Fischer-344 rats (93).

The altered mitochondrial function is accompanied by decreased mtDNA transcription (52, 117, 106, 18, 19); however compensatory mechanisms may exist, since the levels of Complex I subunits ND1 and ND3 increase in platelets from aged individuals, as observed in our laboratory (262). Other reports also showed decreased mitochondrial protein synthesis and decreased levels of mtDNA-encoded proteins (12, 253, 333).

Mitochondrial oxidative stress in mouse skeletal muscle is increased with age, leading to oxidative DNA damage and alterations in mitochondrial bioenergetic function (251).

Mitochondrial protein synthesis is unimpaired in mtDNA mutator mice (cf. previous section), consistent with the observed minor alterations of steady-state levels of mitochondrial transcripts. Despite that, the stability of several respiratory chain complexes is severely impaired, thus suggesting that the aging phenotype is caused by amino acid substitutions in the mtDNA-encoded protein subunits of the respiratory enzymes. In turn, the alteration of protein sequence leads to respiratory chain deficiency. It is worth mentioning that the mitochondrial respiratory proteins are specifically decreased in abundance in the brains of the mutator mice harbouring extensive mtDNA mutations (158). Those changes were not attributed to decreased transcription but to post-translational effects associated with the Poly mutation.

The notion that the respiratory chain is mainly controlled at the level of Complex I (393) suggests that the main alterations due to aging must be found at the level of this enzyme (414, 20, 227). In agreement with this observation, NADH-dependent State 3 respiration is decreased in aged rats (226, 227, 393).

The protein components of Complex I were found to be reduced or absent in aged motor neurons from elderly subjects (337), leading to neuron loss and sarcopenia. The activity of Complex I was investigated in human platelets from young and aged individuals (263). In this case, the most striking result was the decrease of rotenone sensitivity of the enzyme. This study demonstrates that bioenergetic alterations may exist not only in cells of post-mitotic tissues but also in cells directly deriving from mitotic divisions where it is usually assumed that selection washes away the damaged cells (44). Nevertheless, if cells can meet their energy needs by glycolysis, they will survive and maintain the mitochondrial alterations.

A recent study (360) demonstrated that fibroblasts from centenarians display significantly lower Complex I-driven ATP synthesis and higher production of H<sub>2</sub>O<sub>2</sub> in comparison with 75 years old subjects. Despite these changes, bioenergetics of the cells from centenarians appeared to operate normally, due to the significant increase of mitochondrial mass, allowing total ATP synthesis per cell to be unchanged. This lack of functional consequences appears to be due to a decreased mitophagy, induced by the presence of hyperfused, elongated mitochondria. The overall data indicate that longevity is characterized by a preserved bioenergetic function likely attained by a successful mitochondria remodelling that can compensate for functional defects through an increase in mass, i.e. a sort of mitochondrial "hypertrophy".

### 3.2. ROS signalling in aging

Recent investigations attempt to relate the mitochondrial changes with the cellular environment, and the cross-talk between mitochondrial and nuclear genome is receiving increased attention. The availability of techniques such as serial analysis of gene expression (391, 10, 168) has been applied to aging studies in *C. elegans* (187) and in rodent heart (9).

Also the yeast model has provided some important clues. Yeast is characterized by the retrograde response triggered by mitochondrial dysfunction (43) and activating specific signalling proteins. These proteins then migrate to the nucleus and induce numerous nuclear genes coding for metabolic enzymes and stress proteins (183). The result is an extension of yeast longevity.

Changes in the redox status of cellular components by oxidative stress during aging are considered the cause of the observed increased contents or DNA-binding activities of such transcription factors as NF- $\kappa$ B, AP-1 and HIF-1 (213, 50, 197), of heat-shock proteins (51) and of heme oxygenase (49). Their increased activity is considered a compensatory

mechanism for cellular protection and may depend either upon direct alteration of the factor or indirectly through activation of related transduction pathways (197).

A decrease of  $\text{NAD}^+$  and of the  $\text{NAD}^+/\text{NADH}$  ratio is a hallmark of aging (36); such decrease may be due to decreased mitochondrial respiration but also to the enhanced demand of  $\text{NAD}^+$ -requiring enzymes such as PARP and sirtuins. The decline in nuclear  $\text{NAD}^+$  during aging in mice leads to the accumulation of HIF-1 $\alpha$  under normoxic conditions, simulating the Warburg effect in cancer cells (133) (Figure 3).

Deleting SIRT1 accelerates this process, whereas raising  $\text{NAD}^+$  levels in old mice restores mitochondrial function to that of a young mouse in a SIRT1-dependent manner. Thus, a pseudohypoxic state that disrupts PGC-1 $\alpha/\beta$ -independent nuclear-mitochondrial communication contributes to the decline in mitochondrial function with age, a process that is apparently reversible. The activation of HIF1 $\alpha$  leads to selective loss of mtDNA-encoded, but not nuclear-encoded, OXPHOS subunits. It is important to note that this unbalance disrupts OXPHOS before the accumulation of evident DNA damage (133).

It must be considered in this connection, however, that also ROS appear to activate HIF1 $\alpha$  (see Section 2.2). In addition, HIF-1 $\alpha$  elevation can be elicited not only via  $\text{NAD}^+$ /sirtuins-mediated stabilization, but also via translation induced by TOR (Target Of Rapamycin), therefore it is suggested that the pseudohypoxic state is not necessarily caused by mitochondrial dysfunction, but rather driven by TOR hyperfunction (230) (see below).

### INSERT FIGURE 3

In accordance with the importance of oxidative stress in activation of redox-sensitive transcription factors, caloric restriction, the main known factor recognized to delay aging (13, 109), was found to prevent their activation (197). Similarly, hepatocytes from old

mice (233) and rats (175) showed reduced activation of ERK by H<sub>2</sub>O<sub>2</sub>, and the effect was suppressed by caloric restriction (175). Available data on redox-responsive transcription factors suggest that their uncontrolled activation in aging could lead to serious chronic pathogenic conditions characterized by what has been called “molecular inflammation” (65) or inflammaging (112).

We now know that the longevity response to dietary restriction is actively regulated by nutrient-sensing pathways involving the TOR, AMP kinase, sirtuins and insulin/insulin-like growth factor (IGF-1) signalling in a variety of model organisms (195). In nutrient replete conditions, organisms develop, grow, and age quickly. When nutrients become scarce as with dietary restriction, growth and development decline, stress response pathways become induced and organisms live longer.

According to Blagosklonny (34), aging is not a program, but it is a quasi-program, a useless and unintentional continuation of developmental programs. Similarly, cellular senescence is a continuation of cellular growth. The same TOR pathway, which drives developmental growth, later drives aging and its associated diseases. Actually, TOR has been proposed to be the central regulator of aging (34, 230, 403). Under nutrient-rich conditions TOR promotes growth by phosphorylating several factors, including the ribosomal protein S6K1, related with protein synthesis, whereas under starvation conditions its inhibition slows down cell proliferation and activates cell maintenance pathways involved in autophagy, apoptosis, and mitochondrial stress responses. Indeed TOR inhibition by starvation or by rapamycin has been shown to prolong the life span of several organisms (258). Reducing TOR activity increases autophagic flux, enhances mitochondrial membrane potential, reduces reactive oxygen species within the cell, and increases replicative life span. These effects appear to be mediated in part by an interaction between p62/SQSTM1 and Keap1 which allows nuclear accumulation of NRF2, increased expression of NRF1, and increased

expression of nuclear-encoded mitochondrial genes, such as the mitochondrial transcription factor A, and mitochondrial-encoded genes involved in oxidative phosphorylation (231, 31).

The TOR-centric theory of aging is not necessarily in contrast with the traditional view of the mitochondrial theory: antiaging drugs such as rapamycin, metformin, berberine, resveratrol, vitamin D3, 2-deoxyglucose, and acetylsalicylic acid, attenuate the level of constitutive TOR signalling (79). In parallel, they suppress the level of constitutive DNA damage induced by endogenous ROS. While the primary target of each of these agents may be different, the data obtained on several human cancer cell lines, WI-38 fibroblasts and normal lymphocytes suggest common downstream mechanism in which the decline in mTOR/S6K1 signalling and translation rate is coupled with a reduction of oxidative phosphorylation and ROS that leads to decreased oxidative DNA damage.

In this context, the adaptor protein  $p66^{Shc}$  (Section 2.1.4) could be a link between ROS and mTOR pathway (300). With its double identity as generator of mitochondrial oxidant species and as signalling adaptor in the insulin receptor cascade,  $p66^{Shc}$  has drawn major attention as a negative determinant of life span and healthy longevity in mammals.

In one scenario,  $p66^{Shc}$  action on S6K may lead to increased mitochondrial metabolism and, as a consequence, to a rise of mitochondrial ROS (385), as observed in cells where  $p66^{Shc}$  is overexpressed. In other words, TOR/S6K may mediate, at least in part, the pro-oxidant action of  $p66^{Shc}$ . Ablation of  $p66^{Shc}$ , by leading to reduced responsiveness of S6K to nutrients, creates a Rapamycin-like (although presumably milder) signalling block that conceivably promotes animal longevity, like caloric restriction.

More intriguingly, ROS may act upstream of the  $p66/S6K$  module, since  $p66^{Shc}$  not only generates ROS, but is also stimulated by oxidants (265). For instance, in fibroblasts exposed to oxidative stress, PI3K/AkT activation by ROS is mediated, at least to some extent, by  $p66^{Shc}$  (286). AkT can, in turn, activate mTOR. ROS are also generated in mitochondria in

response to energy substrates; these species may increase the phosphorylation/expression level of  $p66^{Shc}$ , thereby promoting its (redox-independent) stimulatory action on S6K. This would represent an intriguing alternative route for nutrients to signal, *via* mitochondria, ROS and  $p66^{Shc}$ , to the mTOR/S6K cascade.

Interestingly, oxidative stress and DNA damage can also induce the expression of miR-210, a micro-RNA that negatively regulates mitochondrial respiration under hypoxic conditions by limiting the functionality of the respiratory chain in many different cell types. In fact, at least two members of Complex I and II (i.e. NDUFA4 and SDHD, respectively) are targets of miR-210, with consequential transmembrane potential reduction, ROS production increase and induction of an apoptotic-like mitochondrial phenotype (321). Another senescence-induced miRNA that preferentially localizes to mitochondria, miR-494, was found to be even more effective than miR-210 in inducing a senescent phenotype in fibroblasts upon pre-miRNA transfection, thus indicating a role for these molecules as transducers of ROS or DNA damage associated signalling (102). Since these miRNAs can induce ROS production, a self-sustaining ROS vicious cycle that involves these two miRNAs could be hypothesized.

The analysis of young and old mouse brains further confirmed the role of miRNAs in reducing the mitochondrial activity during the senescence process and aging. The profiling of both miRNAs and proteins showed 70 differentially regulated miRNAs of which 27 were predicted to target subunits of Complex III, Complex IV and ATPase, as confirmed by protein profiling (234), supporting the fact that miRNA-driven mitochondrial dysfunction and ROS increase are common traits shared by aging tissues as well as senescent cells (212)

### *3.2.1. Beneficial effects of ROS in aging: a sort of preconditioning*

Some studies suggest that ROS are beneficial and not detrimental for life (119, 332, 211) when they serve as molecular signals to ultimately induce endogenous defence mechanisms culminating in increased stress resistance and longevity (76, 424, 332, 247), an adaptive response more specifically named mitochondrial hormesis or mitohormesis (376, 48, 417).

Knockdown of Complex I, III, IV, or V has been shown to increase lifespan in both *C. elegans* (90, 413) and *Drosophila* (68), whereas knockdown of Complex II does not extend lifespan (204). Accordingly, mutation of Complexes I or III subunits also increases lifespan, despite concomitantly increasing superoxide levels (105, 413), whereas the mutation of Complex II subunits that also elevates oxidative stress accelerates aging (176). It is possible that a specific ROS-mediated signal activates a prosurvival program that overcomes the deleterious effects of increased oxidative damage (73) caused by mutations in Complexes I or III, but that this prosurvival program cannot compensate for alterations in the Krebs cycle caused by mutations in Complex II.

Hydrogen peroxide enhances chronological lifespan in caloric-restricted budding yeast cells in parallel with a decrease in superoxide anions. This decrease is caused by induction by hydrogen peroxide of the activity of the cytosolic SOD1 and the mitochondrial SOD2, respectively (264). The induction of SOD activity by hydrogen peroxide is consistent with the earlier demonstration that the lifespan of budding yeast is extended by SOD overexpression (101, 157). These findings are also consistent with earlier reports that sublethal concentrations of hydrogen peroxide induce transcription of both the *SOD1* and *SOD2* genes as well as an increase in levels of the corresponding proteins (132).

A transient increase in intracellular hydrogen peroxide has also been implicated in lifespan extension associated with impaired insulin/IGF-1 signalling in *C. elegans*. Such

impairment disrupts glucose uptake and upregulates superoxide dismutases and other oxidative stress defences, leading to a subsequent decline in overall levels of ROS (419).

According to these results, the caloric restriction induces an initial stress response with an increase of ROS generation. The consequent induction of protective mechanisms, mainly antioxidant enzymes, results in an increased antioxidant defence and a lower level of ROS and ROS-induced damage (332). This interpretation agrees with the finding that the time necessary for caloric restriction to lower ROS production is of several months (140). Irrespective of the interpretation, it is clear that aging is linked to ROS-induced damage, and we can combat aging by eventually decreasing ROS levels in mitochondria.

It has been recently shown that increased lifespan in *C. elegans* is promoted by either dietary restriction or Complex I inhibition, but these two ways are not additive (351), indicating that impaired Complex I activity mimics dietary restriction. Consistent with the concept of mitohormesis, Complex I inhibition transiently increases mitochondrial formation of ROS that activate PMK-1/p38 MAP kinase and SKN-1/NRF-2. Sirtuins are also involved in ROS signalling (352): a sirtuin-dependent nicotinamide-N-methyltransferase generates 1-methylnicotinamide (MNA) from nicotinamide. MNA serves as a substrate for a newly identified aldehyde oxidase to generate hydrogen peroxide, which acts as a mitohormetic reactive oxygen species signal to promote *C. elegans* longevity.

Another aspect of defence against mitochondrial stress is the mitochondrial UPR (161, 309) that may be triggered by inhibition of the TOR pathway by rapamycin or by caloric restriction (403). The mtUPR is analogous to the better known endoplasmic reticulum UPR (see Section 2.3.1). The primary purpose of this initial stress response at low ROS concentration is to improve the folding environment of newly synthesized mitochondrial proteins and enhance mitochondrial functions.

### 3.3. Modifications not due to ROS: Are ROS really involved in aging?

Evidence has been presented that only a small fraction of the mtDNA mutations accumulating in *Drosophila* upon aging are G-C to T-A transversions, a signature of ROS oxidative damage to the bases (181). Moreover, loss-of-function mutations in the mitochondrial superoxide dismutase, Sod2 have no detectable influence on the somatic mtDNA mutation frequency, suggesting that mutations arise primarily from errors that occur during mtDNA replication.

These results are in line with the “Damage Theory” of Gladyshev (132) who proposes that infidelity, heterogeneity, and imperfectness of each and every biological process may be responsible for the inevitable accumulation of by-products and other damage forms. Thus, according to this view, aging is indeed due to damage to cellular structures, but the damage is not, or only in part, due to oxidative modifications by ROS.

The results, however, cannot be reconciled with the evidence shown in Section 3.1.1 that mtDNA mutations are proportional to the levels of 8-oxodG and to the extent of oxidative stress. It is however possible that both mechanisms of mtDNA mutagenesis exist. It has also to be borne in mind that mtDNA damage may not be the initial step leading to the aging phenotype, whilst lipid and protein damage by ROS may initially trigger the events leading to aging. We will more deeply discuss this aspect in Section 4.

ROS-dependent and ROS-independent mechanisms regulating lifespan through the mitochondrial respiratory chain have been reviewed (355), with particular emphasis on the metabolic consequences of changing the  $\text{NAD}^+/\text{NADH}$  ratio by changing the rate of respiration. Aging is characterized by a low  $\text{NAD}^+/\text{NADH}$  ratio, due to decrease of mitochondrial respiration (365, 36) (cf. Section 3.2).

If NADH is not reoxidized to  $\text{NAD}^+$  by Complex I, there is a shortage of  $\text{NAD}^+$  available for the conversion of glyceraldehyde-3-phosphate into 1,3-biphosphoglycerate

causing accumulation of dihydroxyacetone, which decomposes into methylglyoxal, the main initiator of nonenzymatic glycation (24, 323).

Furthermore, the ratio of  $\text{NAD}^+/\text{NADH}$ , together with AMP/ATP levels, is a major sensor of the energetic and redox state of the cell (171). Based on the information provided by these sensors, cells can choose between running under a proaging or prosurvival program. Dietary restriction that counteracts aging is characterized by a change shifting the  $\text{NAD}^+/\text{NADH}$  ratio to the oxidized form (237). Different experimental reports support the notion that it is possible to regulate longevity by manipulating the levels of NADH and  $\text{NAD}^+$ .

In addition, both sirtuins and poly(ADP-ribose) polymerase use  $\text{NAD}^+$  or some of its metabolites as cofactors. This implies that a change in the ratio of  $\text{NAD}^+/\text{NADH}$  elicited by a change in the electron transfer chain activity would alter the activity of these enzymes, thus activating or repressing a prosurvival genetic program (143).

Again, we wish to point out that even these mechanisms, that are apparently not dependent on ROS, are however the consequence of a deficiency of mitochondrial respiration that is largely a consequence of ROS attack (Section 3.1.2).

#### **4. A UNIFYING HYPOTHESIS INVOLVING SUPERCOMPLEX DESTABILIZATION IN AGING**

Contrary to the view of a random organization of the respiratory chain complexes prevailing in the last decades of the past century (146), evidence has now accumulated that a large proportion of the mitochondrial respiratory chain complexes in a variety of organisms is arranged in supramolecular assemblies called supercomplexes or respirasomes (348, 3, 224).

The natural assembly of the respiratory complexes I, III, and IV into supramolecular stoichiometric entities, such as  $\text{I}_1\text{III}_2\text{IV}_{0-4}$ , is not just a mere structural feature

but has deep functional implications on the properties of the respiratory chain (reviewed in 224). The most striking and obvious consequence of supercomplex dissociation is loss of enzymatic channelling (121, 122). Another functional consequence of disruption of the supercomplex (SC) core structure  $I_1III_2$  is loss of stability of Complex I. Flux control analysis in aerobic respiration in coupled liver mitochondria (393) showed that Complex I has little control in young rats but very high control in the old animals, meaning that defective operation induced by aging at the level of this enzyme is reflected on the entire OXPHOS process.

There is growing awareness that the free complexes can co-exist with supercomplexes. In this context, an integrated model, *the plasticity model*, has been proposed for the organization of the mitochondrial electron transport chain (3). The previous opposed models, solid vs. fluid, would be two possible extreme situations of a dynamic range of different molecular associations between respiratory complexes. The plasticity model and the dynamics of mitochondrial supercomplexes are widely discussed (1) in a recent review.

A fundamental prediction of the plasticity model is that, *in vivo*, the mitochondrial respiratory chain should be able to work both when supercomplexes are present and when the formation of supercomplexes is prevented. Indeed, several studies *in vitro* support the view that electron transfer in the respiratory chain can occur in absence of supercomplexes though with lower efficiency (122). Among the factors able to modulate supercomplex association are the mitochondrial membrane potential and posttranslational changes of the individual complexes, although these factors and the mechanisms through which they act are still unknown. In the reconstructed models of the  $I_1III_2IV_1$  supercomplex (96), only limited apparent interaction sites exist between neighbouring complexes. Some of the gap volumes may be lipid filled. It is worth noting that CL, the signature phospholipid of mitochondria, can also integrate into the structure of the respiratory complexes and act as a critical determinant

of the respiratory function. Consistent with this observation, impaired CL synthesis gives rise to more labile respiratory supercomplexes (cf. Section 4.1.2) in yeast and in human patients (267, 259). Another recent advance is the discovery of non-OXPHOS proteins that appear to adhere to and seal the individual respiratory complexes to form stable assemblages and to prevent electron leakage (408).

We first suggested an implication of supercomplex disorganization as the missing link between oxidative stress and energy failure (223). A dissociation of supercomplex association was proposed to occur under conditions of oxidative stress. The consequent loss of facilitated electron channelling leads to resumption of a less efficient random diffusional behaviour, with electron transfer depending upon the collisional encounters of the free ubiquinone molecules with the partner complexes. Dissociation of supercomplexes might have further deleterious consequences, such as disassembly of complex I and III subunits and loss of electron transfer and/or proton translocation (223); the consequent alteration of electron transfer may elicit further induction of ROS generation. Following this line of thought, the different susceptibility of different types of cells and tissues to ROS damage may be a consequence of the extent and tightness of supercomplex organisation in their respiratory chains. Supercomplex stability depends on phospholipids content and composition of their mitochondrial membranes. It would be interesting to show if a correlation exists between longevity of a species and tightness of supercomplex association, although no data presently exist on this issue. Supercomplex dissociation may have deep metabolic consequences, as depicted in the scheme in Figure 4. An initial enhanced ROS generation due to different possible reasons and originating in different regions of the cell besides mitochondria (229) would induce supercomplex disorganization eventually leading to possible decrease of Complex I assembly. Both the lack of efficient electron channelling and the loss of Complex I

would decrease NAD-linked respiration and ATP synthesis. In the following sections we briefly summarize the experimental evidence pertaining to this hypothesis.

## INSERT FIGURE 4

### 4.1 Supercomplex dissociation and aging

#### 4.1.1. Evidences for supercomplex dissociation in aging

Analysis of the occurrence of respiratory supercomplexes comprised of various stoichiometries of complexes I, III and IV reveals age-related variations, suggesting that destabilization of their supramolecular organization may be crucial for the development of the aging-phenotype (84, 135). In cardiac mitochondria from old rats (136), supercomplexes of the highest molecular weight decline to the greatest extent with age. It is noteworthy that this supercomplex disintegration is not caused by age-associated decrements in a particular enzyme component. In another study in mitochondria from rat skeletal muscle (243), proteomic analysis revealed an age-associated increase of the heavier supercomplexes, which was hypothesized to be a compensation for the significant loss of the smaller supercomplex I<sub>1</sub>III<sub>2</sub>.

Also in mitochondria of brain cortex from aged rats (30-months), profound adverse changes in the supramolecular organization of the respiratory chain complexes as well as of the FoF1 ATP synthase were evidenced (114); notably, the overall decline with age (40%) in the supercomplexes containing Complex I is caused to large extent by the pronounced decline (58%) of abundance of the supercomplex I<sub>1</sub>III<sub>2</sub>.

Studies of the effect of heart failure on cardiac mitochondria (334) showed that electron transfer in absence of supercomplex organization is decreased even if activity of the individual complexes is normal. In proteoliposomes enriched in supercomplex I<sub>1</sub>III<sub>2</sub> (at

protein to phospholipid ratio 1:1), electron transfer between Complex I and Complex III (NADH-cytochrome *c* oxidoreductase) is more efficient than predicted by random behaviour on the basis of the activities of the individual complexes (124). However, when the supercomplex is dissociated into its individual enzyme components (e.g. by lipid dilution of the proteoliposomes to a ratio of 1:30) the rate of NADH-cytochrome *c* oxidoreductase is much lower. Similar results were obtained by dissociation of the supercomplexes by detergents (252). This is a demonstration that supercomplex formation indeed enhances the rate of electron transfer above that occurring via a ubiquinone pool in the membrane (27, 124, 252), whereas loss of supercomplex organization induces less efficient pool activity of the quinone.

The progeroid profile of the mutator mice harbouring a mutation in the Poly proof-reading domain and hence exhibiting multiple mtDNA mutations (Section 3) is accompanied by decreased respiration and impeded assembly of respiratory complexes (98). Despite that only Complex IV subunits were significantly reduced, the steady state levels and activities of the other complexes, particularly Complex I, were secondarily affected and strongly decreased. This is in line with the idea that a decreased assembly of Complex IV may affect the stability of Complex I through disruption of the supercomplex organization (80). Nevertheless, although mtDNA deletions and concomitant loss of respiratory activity have been shown to occur in an age-dependent fashion in the rat kidney, no significant differences were found when comparing the protein abundance of individual respiratory complexes and the supercomplex profiles between young and old mitochondria (287). It must be noted that in this latter study the respiratory activities were only marginally decreased but it is possible that kidney mitochondria are not yet compromised at the age studied (24 months).

#### *4.1.2 Peroxidised phospholipids prevent supercomplex formation*

Lipids impose on membrane proteins the correct conformation for optimal activity (219, 173) and a correct template for membrane protein topogenesis (92).

The phospholipids in closest vicinity to the protein surface, as well as those in the free bilayer, are actually highly mobile and free to exchange, but crystallographic studies have shown the presence of a few tightly bound CL molecules in each of the crystal structures of Complex III (210) and Complex IV (291). These results suggest that CL is an integral component of these proteins, and that its presence is critical to protein folding and function. There are now extensive indications that CL stabilises respiratory supercomplexes as well as the individual complexes (405, 302).

The availability of a CL-lacking yeast mutant provided the opportunity to demonstrate that mitochondrial membranes still contained the III<sub>2</sub>-IV<sub>2</sub> supercomplex, but that it was significantly less stable than supercomplexes in the parental strain (421, 318).

Mutations of tafazzin, an acyltransferase involved in the synthesis of mature tetralinoleyl CL (169), result in Barth syndrome, a cardio-skeletal myopathy with neutropenia, characterised by respiratory chain dysfunction. The CL defect in Barth syndrome results in destabilisation of the supercomplexes by weakening the interactions between respiratory complexes (259, 137).

Flux control analysis (124) showed that the maintenance of a supercomplex I-III in proteoliposomes is abolished if lipid peroxidation is induced by 2,2'-azobis-(2-amidinopropane) dihydrochloride before reconstitution. Evidently, the distortion of the lipid bilayer induced by peroxidation and the alteration of the tightly bound phospholipids determine dissociation of the supercomplex originally present in the preparation.

Moreover, mitochondrial-mediated ROS production affects complex I and IV activity through CL peroxidation in beef heart submitochondrial particles (303, 304). CL liposomes added exogenously to mitochondria from aged rats almost completely restored the

activity of these enzyme complexes to the values of young control animals (316, 317). Neither other major phospholipid components of mitochondria, such as phosphatidylcholine and phosphatidylethanolamine, nor peroxidised CL could replace this effect of CL. Altogether, these results indicate that the defect in complex I and IV activities observed in mitochondria from aged rats could be ascribed, at least in part, to oxidative damage to mitochondrial CL.

#### *4.1.3. Loss of supercomplexes decreases the stability of Complex I and enhances generation of ROS*

Analysis of the state of supercomplexes in patients with an isolated deficiency of single complexes (349) and in cultured cell models harbouring cytochrome b mutations (2, 80, 87, 259) provided evidence that the formation of respirasomes affects the stability of Complex I. From these findings, supercomplex assembly emerged as a necessary step for respiration, its defect setting the threshold for respiratory impairment in mtDNA mutant cells.

Genetic alterations leading to a loss of Complex III prevented respirasome formation and led to secondary loss of Complex I, and therefore primary Complex III assembly deficiencies presented as Complex III/I defects. Conversely, Complex III stability was not influenced by the absence of Complex I.

The disassembly of Complex I does not appear to result from altered biogenesis assembly (1). The available evidence would favour the following course of events: misassembled Complex III prevents formation of supercomplex; the lack of the supercomplex association induces an enhanced ROS generation from Complex I (see below), with consequent damage to neighbour molecules and to Complex I itself (86, 88) which is vulnerable to oxidative stress both directly and through the lipid peroxidation, particularly of CL (303).

The possibility that loss of supercomplex organisation may enhance ROS generation by the respiratory chain has been advanced on theoretical grounds (223, 224, 84): a tighter organisation of the respiratory enzymes may hide auto-oxidisable prosthetic groups hindering their reaction with oxygen. Alternatively, slowing electron flow in the chain may keep the prosthetic groups in a more reduced form allowing them to interact with oxygen.

The molecular structure of the individual complexes would not seem to allow a close apposition of the matrix arm of Complex I, where the prosthetic groups are localised, with either Complex III or IV (347, 314). However, the actual shape of the I<sub>1</sub>III<sub>2</sub>IV<sub>1</sub> supercomplex from bovine heart (347) suggests a slightly different conformation of Complex I in the supercomplex, with the matrix arm showing a higher bending towards the membrane (and presumably Complex III). This observation agrees with the notion that Complex I may undergo important conformational changes (325). Moreover, the observed destabilisation of Complex I in absence of supercomplex may render the 51 kDa subunit containing the FMN more “loose” allowing it to interact with oxygen.

It was suggested (301) that supercomplex organization of Complex I within the chain prevents excessive superoxide production on oxidation of NAD-linked substrates because the efficient channelling helps maintaining the chain in the oxidized state. On the other hand, on succinate oxidation, the backward electron flow keeps the centres in Complex I more reduced favouring production of superoxide. In relation to this it is interesting to note that Complex II does not form a respirasome.

Additional circumstantial evidence on the role of supercomplex organisation comes from the observation that high mitochondrial membrane potential elicits ROS generation, while uncoupling strongly reduces ROS production (222, 184). Although other explanations may be given to these observations (cf. section 2.2.1), they are compatible with the suggestion

(322) that high membrane potential may dissociate the supercomplexes into the individual units.

A recent study (252) gave the first direct demonstration that loss of supercomplex organization causes an enhancement of ROS generation by Complex I (Fig. 5). This is clearly evident both in reconstituted proteoliposomes undergoing mild detergent treatment in the presence of dodecyl maltoside (DDM), where the dissociation of Complex I from the respirasome is accompanied by a three/four-fold increase of ROS generation, and in mitochondrial membranes. In the latter case, the existence of endogenous systems operating to reduce ROS levels in the mitochondrial sample (i.e. mitochondrial glutathione peroxidase, Mn-superoxide dismutase and non-enzymatic endogenous antioxidants) might have counteracted the dramatic effects of the complete dissociation of Complex I, thus leading to a two-fold only increase of the measured ROS production.

A further demonstration of the enhanced ROS generation due to supercomplex disruption was similarly obtained in a model system of reconstituted binary Complex I/Complex III at high lipid to protein ratio (30:1) (252) where formation of the supercomplex  $I_1III_2$  is prevented. Likewise, the generation of superoxide is several folds higher than in the same system reconstituted at a 1:1 ratio, which is rich in supercomplexes. It is worth noting that in the experiments reported in the above mentioned study, ROS production is investigated in the presence of inhibitors (mucidin and rotenone) that prevent electron transfer to any possible acceptor. For this reason the redox centres in Complex I are maximally reduced both in the situations where supercomplexes are maintained and in the situations where Complex I is free. We therefore exclude the possible reasoning that facilitation of electron flow by substrate channelling within the respirasome could maintain the redox components of the complexes in the oxidised state and limit ROS formation.

**INSERT FIGURE 5**

The *in vitro* study quoted above (252) is supported by several observations in cellular and animal models linking together supercomplex dissociation and enhanced ROS production. In particular, very recent observations (86) show that diminished stability of SC and Complex I is associated with increased levels of ROS in mouse lung fibroblasts lacking the Rieske iron-sulphur protein of Complex III and hence devoid of the supercomplexes containing Complex I.

Also in mouse fibroblasts expressing the activated form of the k-ras oncogene, a strong decrease of high molecular weight supercomplexes correlated with higher ROS generation in comparison with wild type fibroblasts (225). Moreover, enhanced ROS generation and oxidative stress were found in yeast mutants lacking the supercomplex assembly factor Rcf1 and thus devoid of supercomplexes III-IV (64, 371, 398). Since the yeast *S. cerevisiae* lacks Complex I, in this case we may consider the origin of the extra ROS being presumably Complex III.

Recently, the biochemical properties of cybrids carrying a human cytochrome b missense mutation m.15579A>G were analysed (128). This mutation causes a dramatic reduction in the Complex III activity and in Complex III-driven mitochondrial ATP synthesis, but does not induce disassembly of the supercomplex containing Complex I. However, the mutation enhances superoxide production, as indicated by direct measurements in mitochondria and by the imbalance of glutathione homeostasis in intact cybrids. The amounts of CIII dimer and III<sub>2</sub>IV<sub>1</sub> were reduced, whereas those of I<sub>1</sub>III<sub>2</sub>IV<sub>n</sub> slightly increased, suggesting that the deleterious effects of p.278Y>C mutation on cytochrome b are palliated when Complex III is assembled into the supercomplexes I<sub>1</sub>III<sub>2</sub>IV<sub>n</sub>, in contrast to when it is

found alone. In this case it is likely that the enhanced ROS formation is the result of derangement of the Q-cycle in Complex III.

It is tempting to suggest that physiological changes in the dynamic equilibrium of respiratory supercomplexes with isolated complexes may be aimed to regulation of ROS levels in the cell, in view of the well documented role of ROS in cellular redox signalling.

#### **4.2. Concluding remarks: is there an evidence for the vicious circle?**

The model we have just proposed poses supercomplex dissociation as having a double influence in relation to ROS. On the one hand, ROS contribute to dissociate supercomplexes, but on the other hand, supercomplex dissociation enhances ROS generation. This means that if these events are not tightly controlled they may initiate a vicious circle of ROS generation. Is this the series of events that conduce to aging? We have discussed in the previous sections the controversial existence of a vicious circle of ROS generation and mitochondrial failure during aging. Although the mitochondrial theory of aging does not require that a vicious circle be operative (14), there is wide evidence that ROS generation increases in aging (Section 3). However, exceptions exist to this rule, the most striking being the lack of increase of ROS in the mutator mouse, in which extensive mitochondrial DNA mutations lead to mitochondrial defect and premature aging (383). Nevertheless the reason for such a lack of ROS generation may be in the excessive disruption of the respiratory chain. It is obvious that completely non-functional complexes cannot catalyse reactions, nor generate ROS.

Taken together, the observations collected in this review locate supercomplex dissociation in a physiological signalling network that can be easily altered and lead to a catastrophic event when the generation of ROS loses control.

We may envisage supercomplex association/dissociation to occur under physiological conditions, according to the plasticity model (3), in response to such stimuli as mitochondrial membrane potential and protein phosphorylation/dephosphorylation of the respiratory complexes. The ensuing changes in ROS generation modulate the ROS-dependent signalling pathways. These changes are reversible and are kept under strict control by changes in the initial stimuli.

We propose that the primary event responsible for aging is the structural damage induced by ROS in mitochondria, as predicted by the original mitochondrial theory of aging. This structural damage is likely to initiate preferentially within the membrane proteins and lipids, and may be influenced by the signalling pathways that sense the nutrition state of the organism, via a modulation of mitochondrial activity and ROS generation. Retrograde signals starting from mitochondria may also induce compensatory mechanisms attempting to counteract the ROS generation and consequent damage. MtDNA mutations, although present, may not necessarily be an early phenomenon in the aging process.

A possible series of events might be the following (Fig. 4). Progressive damage is induced by ROS to the mitochondrial membrane lipids and proteins. The level of ROS is affected by such factors as the nutrition state and the activity of the mTOR and insulin/IGF pathways. Direct protein damage and increased CL peroxidation hamper supercomplex association (124); this leads to further increase of ROS generation. ROS at low concentration may induce a retrograde response inhibiting TOR pathway and thus leading to a protective life-extending response, but at higher concentration they may induce further damage with loss of coordination of the signalling pathways. Mutations in mtDNA at a later stage would make the overall process irreversible and lead to the final aging phenotype.

## LIST OF ABBREVIATIONS

AA, Antimycin A

AOX, alternative oxidase

BHM, bovine heart mitochondria

CL, cardiolipin

CoQ, Coenzyme Q (ubiquinone)

COX, cytochrome oxidase

DDM, dodecyl maltoside

DHO, dihydroorotate

ERK, extracellular signal-regulated kinase

ETF, electron transfer flavoprotein

GSH, glutathione

$\alpha$ GP, glycerol-3-phosphate

HIF-1 $\alpha$ , hypoxia-inducible factor

I, NADH-ubiquinone oxidoreductase

II, succinate-ubiquinone oxidoreductase

IGF-1, insulin-like growth factor

III, ubiquinol-cytochrome *c* oxidoreductase

IMM, inner mitochondrial membrane

IMS, intermembrane space

IV, cytochrome *c* oxidase

MNA, 1-methylnicotinamide

mtDNA, mitochondrial DNA

ND, alternative NAD(P)H dehydrogenases

NOX, NADPH NOX, NADPH oxidases

P66, cytochrome p66

Poly, mitochondrial DNA polymerase  $\gamma$

Prx, Peroxiredoxins

PTPs, phosphoprotein phosphatases

Q, ubiquinone (Coenzyme Q)

ROS, reactive oxygen species

SC, supercomplex

SMP, submitochondrial particles

SOD, Superoxide dismutase

TOR, Target Of Rapamycin

UPR, Unfolded Protein Response

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## AUTHOR DISCLOSURE STATEMENT

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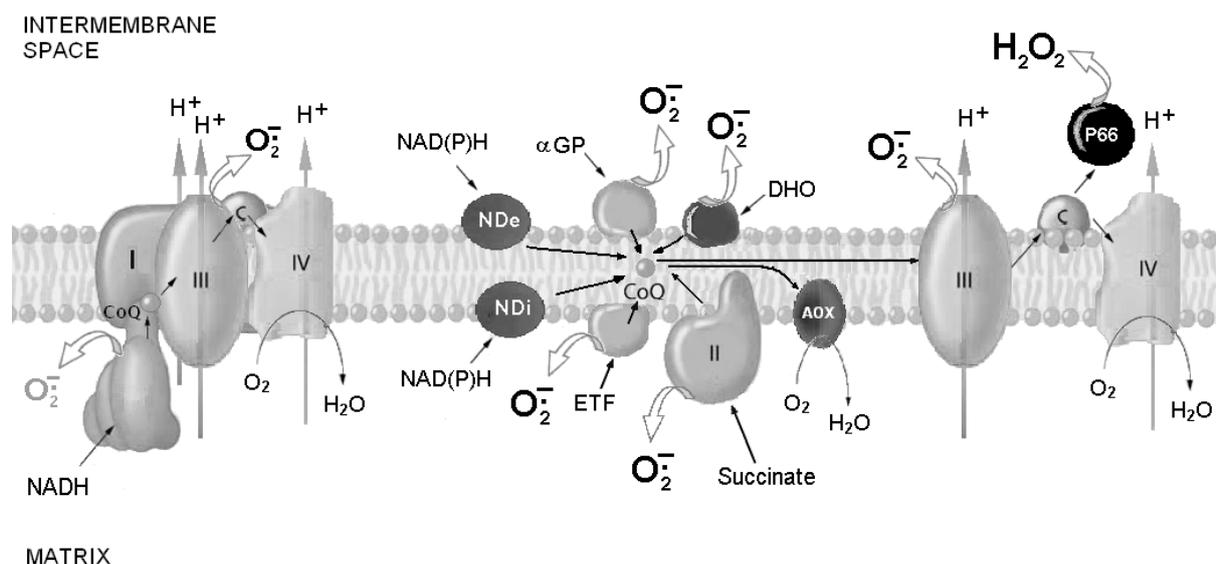
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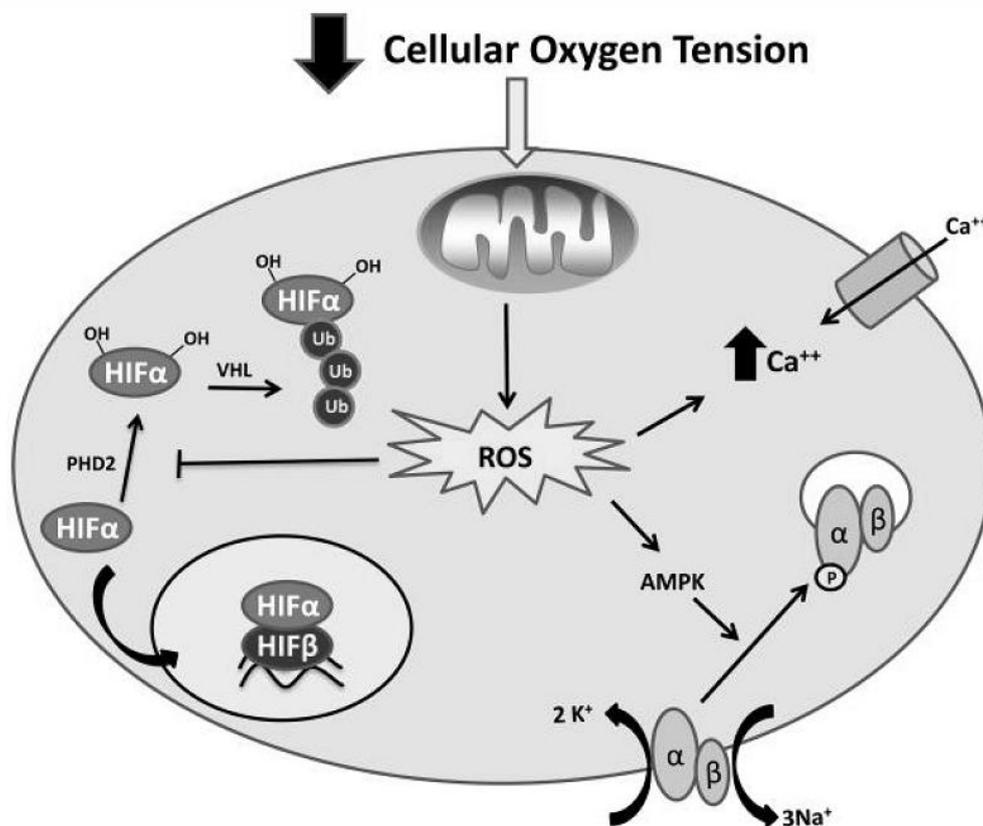
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## FIGURE LEGEND

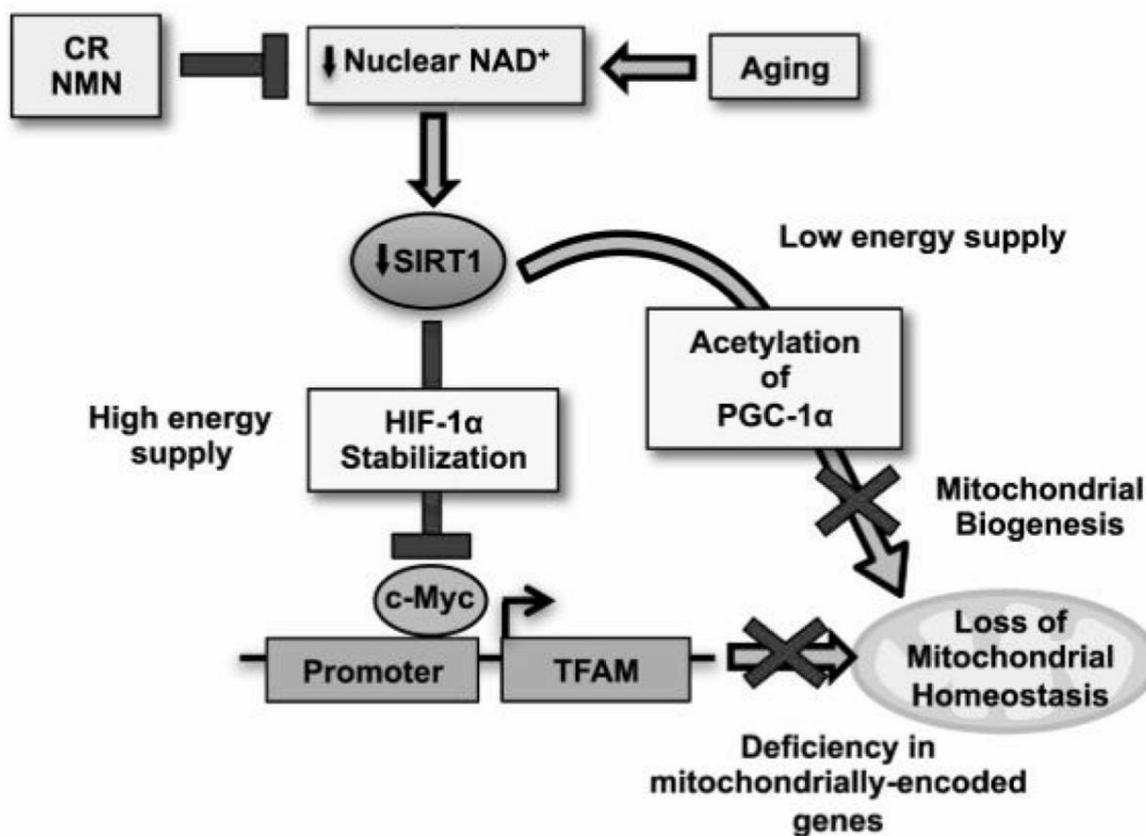
Antioxidants & Redox Signaling  
THE INTERPLAY BETWEEN RESPIRATORY SUPERCOMPLEXES AND ROS IN AGING (doi: 10.1089/ars.2014.6214)  
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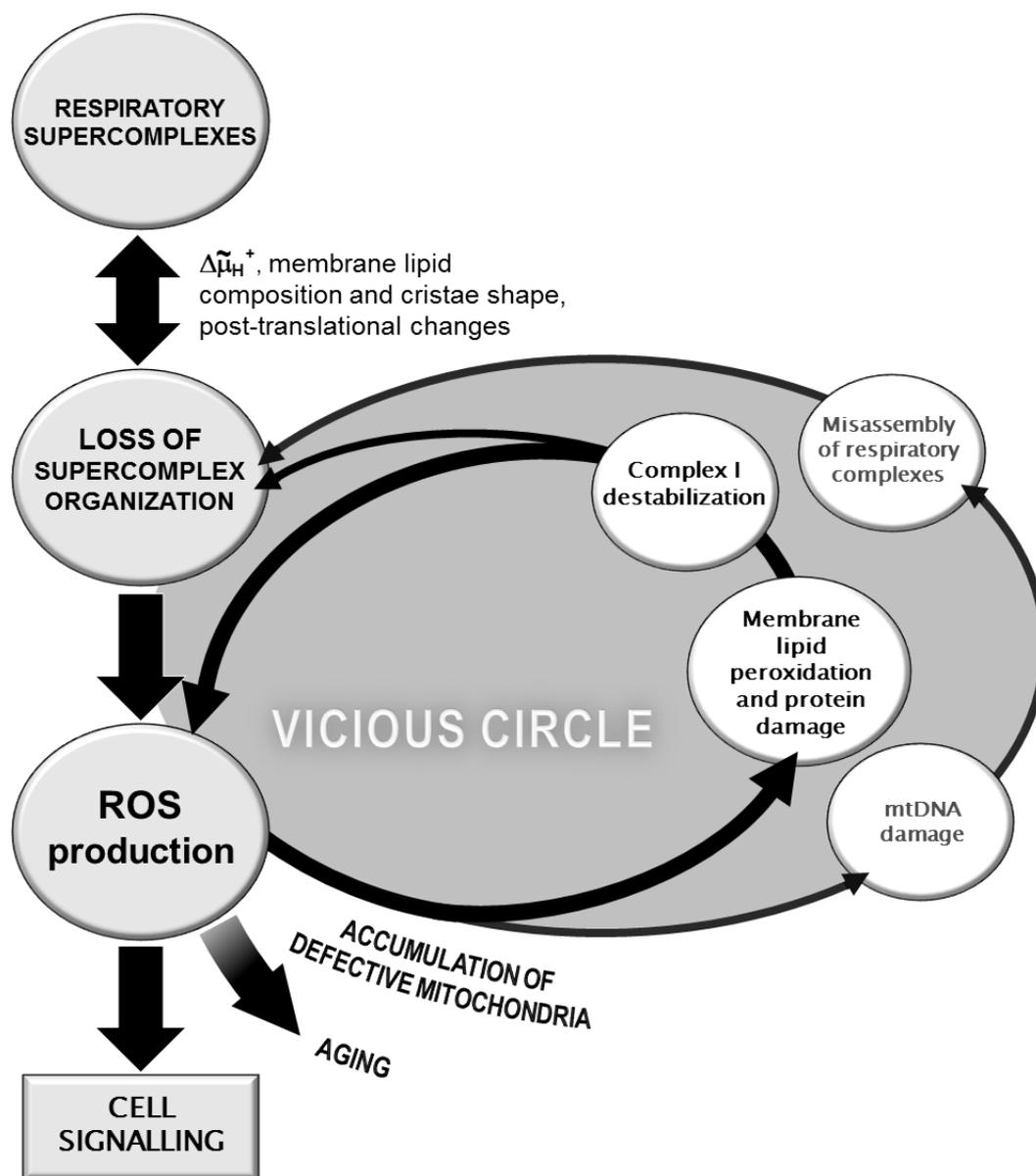
**Figure 1 - A schematic drawing of the respiratory chain depicting the protein complexes and their substrates.** Complex I is depicted as a component of the  $I_1III_2IV_1$  supercomplex; whereas Complex III and Complex IV are also shown in their free form. The white arrows represent the sources of superoxide at different sites in relation to the inner mitochondrial membrane. I, NADH-ubiquinone oxidoreductase; II, succinate-ubiquinone oxidoreductase; III, ubiquinol-cytochrome *c* oxidoreductase; IV, cytochrome oxidase; NDi and NDe, internal and external alternative NAD(P)H dehydrogenases; AOX, alternative oxidase;  $\alpha$ GP, glycerol-3-phosphate; ETF, electron transfer flavoprotein; DHO, dihydroorotate; CoQ, Coenzyme Q (ubiquinone); C, cytochrome *c*; P66, cytochrome p66 (see text for details).



**Figure 2 - Mitochondrial ROS regulate the cellular response to hypoxia.** Hypoxia induces the production of mitochondrial ROS that inhibit the activity of prolyl hydroxylases (PHD2) and stabilize HIF $\alpha$ . Under normoxic conditions, hydroxylation tags HIF $\alpha$  for recognition by the Von Hippel-Lindau tumor suppressor protein and subsequent proteosomal degradation. Mitochondrial ROS generated during hypoxia also regulate increases in cellular calcium uptake and lead to activation of AMPK, allowing increased cellular energy conservation. AMPK phosphorylates the  $\alpha$ -subunit of the Na/K ATPase leading to endocytosis. Figure taken from Hamanaka and Chandel (152), with permission from Elsevier.



**Figure 3 - Nuclear-mitochondrial communication and its decline during aging.** Nuclear  $\text{NAD}^+$  levels regulate mitochondria via a PGC-1 $\alpha$ -independent pathway that ensures the correct stoichiometry of OXPHOS subunits, but over time, a chronic pseudohypoxic response is activated, inhibiting OXPHOS. Mitochondrial encoded genes were *ND1*, *Cytb*, *COX1*, and *ATP6*. Reprinted from Gomes et al. (133), with permission from Elsevier.

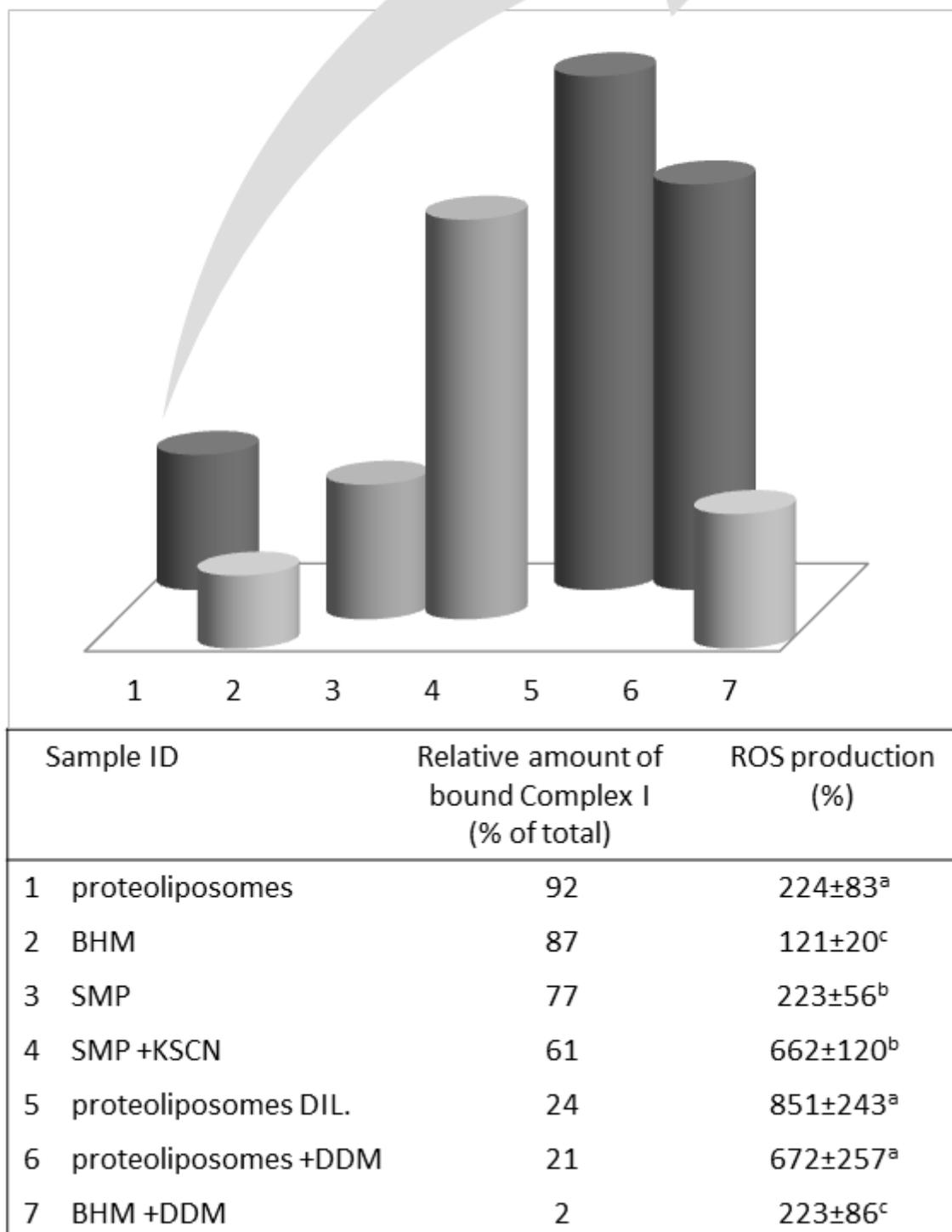


**Figure 4** – Scheme showing how the loss of supercomplex organisation may be involved in a vicious circle of oxidative stress and energy failure. ROS production by Complex I is enhanced as a consequence of supercomplex disassembling. Membrane phospholipid peroxidation, mtDNA damage and subsequent misassembly of the respiratory complexes with further loss of supercomplex organisation may occur due to enhanced mitochondrial oxidative

stress, thus perpetuating the vicious circle. Depending on the amount produced, ROS can operate as signalling molecules from mitochondria to the nucleus. See text for explanations.

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# ROS production



**Figure 5 - Production of ROS by mitochondrial Complex I in different situations where supercomplexes are maintained or disassembled.** The percent value of ROS production measured in all the samples listed in the table (*lower panel*) is plotted in the graph (*upper panel*) in descending order of the corresponding amount of Complex I-containing supercomplexes. The ratio of bound Complex I vs. total Complex I was determined by densitometric analysis of immunoblots obtained after 2D BN/SDS-PAGE as described in Maranzana et al. (252). The NADH-stimulated production of ROS was measured as the relative fluorescence intensity of dichlorofluorescein in the presence of 1.8  $\mu\text{M}$  mucidin and 4  $\mu\text{M}$  rotenone, and expressed as percentage value of the corresponding reference samples: <sup>a</sup>proteoliposomes (cf. text for details), <sup>b</sup>SMP (submitochondrial particles) and <sup>c</sup>BHM (bovine heart mitochondria) respectively assayed in the presence of 1.8  $\mu\text{M}$  mucidin only, as in Maranzana et al. (252). In the case of BHM, the existence of endogenous systems operating to reduce ROS levels in the mitochondrial sample might have counteracted the dramatic effects of the complete dissociation of Complex I, thus leading to a two-fold only increase of the measured ROS production. DIL., dilution at high lipid to protein ratio (30:1 w:w); DDM, dodecyl maltoside; KSCN, potassium thiocyanate.