

The mitochondrial energy transduction system and the aging process

Ana Navarro and Alberto Boveris

Am J Physiol Cell Physiol 292:670-686, 2007. First published Oct 4, 2006;
doi:10.1152/ajpcell.00213.2006

You might find this additional information useful...

This article cites 155 articles, 59 of which you can access free at:

<http://ajpcell.physiology.org/cgi/content/full/292/2/C670#BIBL>

Updated information and services including high-resolution figures, can be found at:

<http://ajpcell.physiology.org/cgi/content/full/292/2/C670>

Additional material and information about *AJP - Cell Physiology* can be found at:

<http://www.the-aps.org/publications/ajpcell>

This information is current as of March 28, 2007 .

The mitochondrial energy transduction system and the aging process

Ana Navarro¹ and Alberto Boveris²

¹Department of Biochemistry and Molecular Biology, School of Medicine, University of Cádiz, Cádiz, Spain; and ²School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina

Navarro A, Boveris A. The mitochondrial energy transduction system and the aging process. *Am J Physiol Cell Physiol* 292: C670–C686, 2007; doi:10.1152/ajpcell.00213.2006.—Aged mammalian tissues show a decreased capacity to produce ATP by oxidative phosphorylation due to dysfunctional mitochondria. The mitochondrial content of rat brain and liver is not reduced in aging and the impairment of mitochondrial function is due to decreased rates of electron transfer by the selectively diminished activities of complexes I and IV. Inner membrane H⁺ impermeability and F₁-ATP synthase activity are only slightly affected by aging. Dysfunctional mitochondria in aged rodents are characterized, besides decreased electron transfer and O₂ uptake, by an increased content of oxidation products of phospholipids, proteins and DNA, a decreased membrane potential, and increased size and fragility. Free radical-mediated oxidations are determining factors of mitochondrial dysfunction and turnover, cell apoptosis, tissue function, and lifespan. Inner membrane enzyme activities, such as those of complexes I and IV and mitochondrial nitric oxide synthase, decrease upon aging and afford aging markers. The activities of these three enzymes in mice brain are linearly correlated with neurological performance, as determined by the tightrope and the T-maze tests. The same enzymatic activities correlated positively with mice survival and negatively with the mitochondrial content of lipid and protein oxidation products. Conditions that increase survival, as vitamin E dietary supplementation, caloric restriction, high spontaneous neurological activity, and moderate physical exercise, ameliorate mitochondrial dysfunction in aged brain and liver. The pleiotropic signaling of mitochondrial H₂O₂ and nitric oxide diffusion to the cytosol seems modified in aged animals and to contribute to the decreased mitochondrial biogenesis in old animals.

oxidative damage; survival; complexes I and IV; nitric oxide synthase

EARLY OBSERVATIONS by Leloir and Muñoz (92) indicated that fatty acid oxidation depends on a labile particulate material of liver homogenates. Later, Kennedy and Lehninger (82) reported that the oxidation of fatty acids and of citric acid cycle intermediates was carried out by osmotically active structures with coupled ADP phosphorylation to ATP. Simultaneously, Sjostrand (146) and Palade (126) recognized by electron microscopy the characteristic double membrane of mitochondria. The concept of oxidative phosphorylation as the mitochondrial function evolved from the confluence of structural and biochemical knowledge and by 1952, mitochondria were described by Lehninger (91) as intracellular “power plants”.

BIOLOGICAL ENERGY TRANSDUCTION: INTRODUCTION AND HISTORICAL PERSPECTIVE

The isolation of mitochondrial proteins with specific electron transfer properties by Green and co-workers provided the concept of “complexes” of the respiratory chain (65). By 1955, Chance and co-workers (32), by using spectrophotometric techniques, described the reduction-oxidation levels of the components of the respiratory chain in the transition following

ADP addition and identified the sites that are thermodynamically and kinetically compatible with energy transduction.

The knowledge of ATP synthase followed contributions from morphology and biochemistry. Electron microscopy of heart mitochondria by Fernandez-Moran (52) revealed regularly spaced “elementary particles” of 8–10 nm diameter, later identified as the F₁ subunit of ATP synthase. The properties of ATP-synthase, called F₁-ATPase at that time, were described by the work of Racker and co-workers (130) and of Boyer (18). The modern knowledge of the vectorial biochemistry of mitochondrial energy transduction was developed after the formulation of Mitchell’s chemiosmotic theory (106, 107). The subsequent landmark contribution of Walker (156) was the understanding of the mechanism of ATP synthesis by the molecular rotor driven by proton (H⁺) flow through the ATP synthase complex.

We now understand oxidative phosphorylation as a process catalyzed by constitutive proteins of the inner mitochondrial membrane that encompasses electron transfer between the complexes of the respiratory chain, vectorial H⁺ release into the intermembrane space, and H⁺ reentry to the matrix through F₀ with ATP synthesis by the F₁-ATP synthase. In addition, mitochondria are recognized to contribute to intracellular signaling and regulation with a central role in keeping homeostatic cell ionic composition (44).

Address for reprint requests and other correspondence: A. Navarro, Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Plaza Fragela 9, 11003 Cádiz, Spain (e-mail: ana.navarro@uca.es).

The Mitochondrial Membranes

Mitochondria are characterized by two membranes, the inner and the outer membranes, with two compartments separated by the inner membrane. Cristae are invaginations of the inner membrane and the intermembrane and intracristal spaces are continuous and form a single compartment. The matrix, surrounded by the folding of the inner membrane, comprises the second compartment (126, 146).

The outer mitochondrial membrane is permeable to small molecules and ions, which move through transmembrane channels formed by a family of integral membrane proteins called porins. Voltage-dependent anion channels are the potential-sensitive porins of the outer membrane that allow metabolite exchange between mitochondria and the cytoplasm (67).

The inner membrane is impermeable to H^+ and this property provides the basis of mitochondrial energy transduction. Chemical species as ions and small molecules that cross the inner membrane have specific transporters. The inner membrane bears, as integral proteins, the key catalysts of oxidative phosphorylation: the electron transfer respiratory complexes and the ATP synthase complex (106).

Components of Mitochondrial Respiratory Chain and Respiratory Assemblies

The mitochondrial respiratory chain consists of a series of electron carriers that function as redox pairs and that are mainly prosthetic groups of integral proteins. There are four electron transfer or respiratory complexes (complexes I–IV), each capable of catalyzing electron transfer in a partial reaction of the respiratory chain.

Complex I (NADH-ubiquinone oxidoreductase; NADH dehydrogenase) is composed of 42–43 different polypeptides, including a FMN-containing flavoprotein and 6 iron-sulfur centers (73, 74). Complex I has an L-shaped form, with the long arm as a hydrophobic integral membrane protein and the short arm extending into the matrix with the hydrophilic part that contains the FMN and the NADH active center (56). The two arms of the L-shaped complex I have separated genetic origin and independent assembly (75).

Rotenone is the specific and stoichiometric inhibitor of complex I in mitochondrial fragments and in isolated mitochondria (33). Some complex I preparations, obtained by membrane solubilization or by further resolution of the classic rotenone-sensitive Hatefi's complex I (73), catalyze rotenone-insensitive NADH oxidation by artificial electron acceptors (93). It is understood that all rotenone-insensitive NADH dehydrogenase preparations are denatured forms of complex I, a point that illustrates the convenience of using native preparations in the assessment of complex I dysfunction in aging and neurological diseases.

Ubiquinone is a lipid soluble benzoquinone with a long isoprenoid side chain that is laterally diffusible in each of the two layers of the phospholipid bilayer of the inner membrane and adapted to shuttle electrons between membrane proteins. Complex I, prepared by the Hatefi's procedure, contains ~4 mol of ubiquinone per mol of FMN (74).

Complex II (succinate dehydrogenase; succinate-ubiquinone reductase) is the membrane-bound component of the citric acid cycle that also functions as a component of the mitochondrial respiratory chain. The integral protein has a covalently bound

FAD and iron-sulfur centers in the membrane extrinsic domain that catalyze electron transfer to ubiquinone and *b* heme in the hydrophobic membrane domain (28).

Complex III (cytochrome *bc*₁ complex; ubiquinol-cytochrome *c* oxidoreductase) is composed of 9–10 polypeptides, 3 of which are associated with redox centers. These centers are *b*₅₆₂, *b*₅₆₆, and *c*₁ hemes and a [2Fe-2S] cluster (73). In addition, two ubisemiquinone bind to two separate domains of complex III (42).

Cytochrome *c* is a peripheral protein facing the intermembrane space, easily solubilized by salt treatments, that transfers electrons from complex III to the Cu_A of complex IV.

Complex IV (cytochrome *c* oxidase, cytochrome oxidase; cytochrome *c*-O₂ oxidoreductase) is the final catalyst of the respiratory chain. Complex IV reduces O₂ to H₂O with four electrons from reduced cytochrome *c* in a process that consumes 4 H⁺ from the matrix. It is worth noting that complex IV dysfunction is common in aging and in neurological diseases.

The stoichiometry of the complexes of the respiratory chain is an interesting subject of current studies. The spectrophotometric determination of the respiratory carriers in isolated mitochondria (32) and the determination of the content of respiratory complexes in mitochondrial fragments prepared by sonic or chemical treatments (65) showed that they occur in simple molar ratios. This finding indicates that respiratory carriers and complexes are arranged in "assemblies" with specific protein-protein interactions, which are effective for rapid electron transfer by limiting the intermolecular distances that slow-diffusing proteins should otherwise travel between collisions (32). A single liver mitochondrion contain ~10,000 complete respiratory assemblies, the number is higher in kidney and about twice in heart mitochondria.

Complexes I, III, and IV interact to form supercomplexes with defined stoichiometric composition in mitochondria from yeast, higher plants, and mammals. Supercomplexes I₁III₂ and I₁III₂IV₁ were reported in bovine heart mitochondria (140).

Oxidation and electrochemical potentials in the mitochondrial respiratory chain

The electron flow from the electron donors, NADH or succinate, to the acceptor, O₂, occurs following the oxidation potential of the components of the electron transfer chain. Electrons move toward compounds with more positive oxidation potentials as given by the standard redox potential and the ratio of the oxidized and reduced forms, according to the Nernst equation. The differences in redox potential of the electron carriers define the reactions that are exergonic enough to provide the free energy required for the coupled endergonic pumping of H⁺ into the intermembrane space.

Complexes I, III, and IV function as H⁺ pumps, acting in series with respect to electron flux and in parallel with respect to the H⁺ circuit. The H⁺ pumps are driven by the free energy of the coupled oxidation reactions and have the characteristic that the catalyzed oxidation and the H⁺ release are both vectorial and coupled. The movements of H⁺ occur in a specific direction from the matrix to the intermembrane space, with the matrix becoming negatively charged with the departure of H⁺ (N side, negative), and the intermembrane space becoming positively charged (P side, positive). For instance, complex I catalyzes two simultaneous and obligatory pro-

cesses: the exergonic transfer of one hydrogen atom and one electron from NADH and of a H^+ from the matrix to ubiquinone, coupled to the endergonic transfer of 4 H^+ from the matrix to the intermembrane space (155).

The chemical free energy of the fall in redox potential of the electrons passing through the respiratory complexes is used to generate a H^+ electrochemical potential gradient, $\Delta\mu_H$, expressed in electric potential units as the proton-motive force (Δp) (106) as Δp (mV) = $\Delta\Psi_m - (2.3 RT/F) \Delta pH$, that at 37°C, results: $\Delta p = \Delta\Psi_m - 60 \Delta pH$. In the equation, $\Delta\Psi_m$ is the electric potential across the inner mitochondrial membrane, ΔpH is the pH gradient across the inner membrane, and R , T , and F refer to the gas constant, the absolute temperature, and the Faraday constant, respectively. Under most conditions, $\Delta\Psi_m$ is the dominant component of Δp , accounting for 150–180 mV of a Δp of 200–220 mV (107). The Δp drives ADP phosphorylation and stops electron flow in the controlled metabolic condition of absence of ADP. In the biophysical basis of respiratory control, Δp is able to stop exergonic substrate oxidation by a free energy equilibrium between electron flow between redox pairs and H^+ flow between compartments. Moreover, membrane potential provides the driving force for cation, such as K^+ and Ca^{2+} , uptake by mitochondria.

Electrochemical Potential and F_1 -ATPase Molecular Rotor are used to Phosphorylate ADP

The whole process of oxidative phosphorylation depends critically on the integrity and impermeability of the inner mitochondrial membrane. The first part of the process converts the chemical potential of NADH and succinate oxidation into a H^+ electrochemical gradient, and the second part of the process, catalyzed by the ATP synthase uses the H^+ electrochemical gradient to drive the endergonic ATP synthesis. The process is thermodynamically possible because electron transfer releases and the proton-motive force conserves enough free energy, about 34 kJ per mol of electron pair, to drive the formation of one mol of ATP, which requires ~32 kJ.

Mitochondrial ATP synthase (complex V) is a F-type ATPase that has two distinct components: F_1 , a peripheral membrane protein, and F_0 , which is integral to the membrane (18, 130, 156). The F_1 catalytic domain is a globular assembly of 5 proteins α , β , γ , δ , and ϵ with the stoichiometry 3:3:1:1:1. The γ -, δ -, and ϵ -subunits form a central stalk linking the $(\alpha\beta)_3$ subcomplex of the F_1 domain to F_0 . The $(\alpha\beta)_3$ subcomplex and the F_0 domain are linked by a peripheral stalk. The γ -subunit protrudes from $(\alpha\beta)_3$ subcomplex and the δ - and ϵ -subunits are associated with its foot. The movement of the subunits of ATP synthase is critical to its function, with the central stalk rotating at 50–100 times/s. The rotation is produced in F_0 , which is in contact with the foot of the central stalk and is fuelled by the H^+ flow (156).

The rate of mitochondrial respiration depends on ADP availability to F_1 -ATP synthase. The gradients of ADP and ATP across the inner membrane are equilibrated by the adenine nucleotide translocase activity, which is functional to provide ADP to the matrix and ATP for energy-dependent processes to the cytosol.

The experimental value for the H^+ required to drive the synthesis of one ATP molecule is ~3–4 (30, 106). The

phosphorylation efficiency is determined in isolated coupled mitochondria as the ADP/O ratio, with experimental values of 2.6–2.7 for NAD-dependent substrates and 1.6–1.7 for succinate oxidation. These values are close to the theoretical ADP/O ratios of 2.5 and 1.5, respectively, considering that the H^+ pumped out per pair of electron are 10 for NADH and 6 for succinate.

Mitochondrial Ca^{2+} Sequestration

When mitochondria are supplemented with Ca^{2+} , even in the presence of ADP, Ca^{2+} ions are internalized into mitochondria in an energy-dependent process and ATP synthesis is abolished. Mitochondria take up Ca^{2+} in exchange for H^+ by an ATP-powered or membrane potential driven Ca^{2+}/H^+ pump with a stoichiometry of 1:1. The stimulation of respiration by 2 Ca^{2+} ions yield the same amount of extra O_2 uptake as one molecule of ADP (26). Mitochondria accumulate large amounts of Ca^{2+} with simultaneous uptake of P_i and precipitation in the matrix of an insoluble salt similar to hydroxylapatite, in a way that the ionic Ca^{2+} concentration in the matrix is only moderately altered (26). Another route of Ca^{2+} uptake is functionally related with the opening of the Ca^{2+} -mediated permeability pore (136), a sudden increase in inner membrane permeability induced by a large series of compounds that collapses membrane potential and permits the efflux and influx of large molecules and ions. Intramitochondrial Ca^{2+} is recognized as an allosteric activator of matrix dehydrogenases, such as NAD⁺-isocitrate dehydrogenase, pyruvate dehydrogenase and α -ketoglutarate dehydrogenase (104) and as co-factor of mitochondrial nitric oxide (NO) synthase (mtNOS) (59).

REGULATION OF MITOCHONDRIAL RESPIRATION

It is now known that mitochondrial oxidative phosphorylation is regulated by three metabolites: ADP, O_2 , and NO. As early recognized, the rate of respiration and of ATP synthesis are determined by cellular energy needs, which are expressed as cytosolic ADP concentration or as phosphorylation potential ($[ATP]/[ADP][P_i]$), in which the operational part is the $[ATP]/[ADP]$ ratio. When the cellular demand for energy increases, ATP breakdown to ADP and P_i increases and lowers the phosphorylation potential. With more ADP available, the rate of respiration increases, causing regeneration of ATP. Usually, the phosphorylation potential is finely regulated and fluctuates only slightly in most tissues. Chance and Williams (32) defined the operational concepts of mitochondrial metabolic states and respiratory control. State 4, with availability of respiratory substrate but not of ADP, was described as “controlled or resting respiration”, whereas state 3, with ample respiratory substrate and ADP availability, was defined as the “active respiration”, i.e., the maximal physiological rate of ATP production and O_2 consumption. The rates of respiration of isolated mitochondria are 5 to 8 times faster in state 3 than in state 4, i.e., the respiratory controls are 5 to 8. Considering the rates of mitochondrial O_2 uptake in both states 4 and 3, and of O_2 uptake of perfused organs, it was estimated that mammalian mitochondria under physiological conditions are mostly (60–70%) in state 4, whereas the rest (30–40%) are in state 3 (15). This means that in a physiological setting there is only a 30–40% utilization of the ATP-producing capacity. The linear rates of O_2 uptake shown by tissue slices and perfused organs

are interpreted as a fast switching of mitochondria between metabolic states 3 and 4.

NO is a physiological regulator of mitochondrial respiration. In the arterioles, NO produces vasodilatation and increases blood flow and O₂ delivery to the tissues IV (37). However, in mitochondria NO inhibits respiration, by a rapid, selective, potent, and reversible inhibition of complex IV (21, 27, 35, 128). The inhibition occurs in competition with O₂, so that NO dramatically increases the O₂ concentration that yield half-maximal rate of O₂ uptake (2), from 1.4 μM O₂ in the absence of NO to 3.4 μM O₂ in the presence of 0.2 μM NO (16).

In mitochondria, synaptosomes and mitochondrial membranes half-inhibition of respiration occurs at O₂/NO ratios of 150 (2) to 600 (21). Macrophages, astrocytes, and endothelial cells under inflammatory conditions and with increased iNOS expression produce sufficient NO to inhibit their own respiration and also the O₂ uptake of surrounding cells (99).

GENERATION OF MITOCHONDRIAL FREE RADICALS

Mitochondria isolated from liver and heart were described by Boveris and Chance in 1972–1973 as an active source of H₂O₂ that diffuses to the cytosol (14, 17). Shortly after, O₂⁻ was recognized as the stoichiometric precursor of mitochondrial H₂O₂ and ubisemiquinone (UQH[•]) was indicated as the main source of O₂⁻ upon autooxidation (13, 25, 47). Mitochondria are considered the main quantitative source of O₂⁻ and H₂O₂ in mammalian organs (31). However, there is a standing criticism by Nohl and colleagues that mitochondria *in vivo* are not an effective source of O₂⁻ and H₂O₂ and that the determined rates are artifactual (121).

A few years ago, mitochondria were also recognized as a source of NO by two independent research groups, Ghafourifar and Richter (58) and Giulivi et al. (62). Mitochondrial NO is produced by an isoform of NOS, mtNOS, that carries out a classic NOS reaction.

Mitochondrial production of O₂⁻ and H₂O₂

The majority of mitochondrial O₂⁻, 70–80%, is vectorially released to the mitochondrial matrix and 20–30% is released into the intermembrane space. Two main O₂⁻ generating reactions have been described by auto-oxidation of intermediate semiquinones: UQH[•] for the ubiquinol/ubiquinone redox pair (UQH[•] + O₂ => UQ + H⁺ + O₂⁻) (13) and the FMN[•] of the FMNH₂/FMN coenzyme of NADH dehydrogenase (FMNH[•] + O₂ => FMN + H⁺ + O₂⁻) (150). The semiquinones are collisionally and nonenzymatically oxidized by molecular O₂ to yield O₂⁻. Ubisemiquinone is the quantitative main source of univalent O₂⁻ reduction to yield O₂⁻ (129, 150). A role of the Fe-S cluster N1a of complex I has been reported in preventing O₂⁻ generation (74).

The mitochondrial production of H₂O₂ is modulated by the mitochondrial metabolic state and by the intramitochondrial concentration of NO. The production of H₂O₂ in state 4 is ~4 to 5 times higher than in state 3: 0.3–0.8 nmol H₂O₂·min⁻¹·mg protein⁻¹ for state 4 and 0.05–0.15 nmol H₂O₂·min⁻¹·mg protein⁻¹ for state 3 (12). Ion movements through the inner membrane strongly affect the rates of H₂O₂ production, indicating a membrane potential regulation of the auto-oxidation of UQH[•] (24). In the rat heart and liver, mitochondrial H₂O₂ production accounts for ~0.5% of the physi-

ological organ O₂ uptake with succinate or malate-glutamate as substrate (12) and for ~0.15% with palmitoyl carnitine as substrate (148).

Mitochondrial Production of NO

NO is synthesized by the NOS enzymatic reaction that requires arginine, NADPH₂ and O₂ as substrates and that produces citrulline, H₂O, and NO (NADPH₂ + Arg + O₂ => NADP + H₂O + Cit + NO). Three different genomic NOS are known: neuronal NOS (nNOS or NOS-1); inducible or macrophage NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3). The mtNOS has been identified as the splice variant α of the nNOS with the posttranslational modifications of myristilation and phosphorylation (49, 55). The intramitochondrial concentrations of NADPH₂, arginine, O₂, and Ca²⁺ are in excess or in the range needed for enzymatic activity (152). Mitochondria and submitochondrial preparations yield rates of 0.25–0.90 nmol NO·min⁻¹·mg protein⁻¹. However, there is still some controversy concerning the existence of mtNOS (20).

The intramitochondrial steady-state concentrations of NO are calculated as 50–200 nM NO (129) and a release of 29 nM NO was electrochemically measured after supplementation of a single mitochondrion with Ca²⁺ (80). Under physiological conditions tissues are oxygenated at ~25 μM O₂ and mitochondria are exposed, by the NO generated by mtNOS, to [O₂]/[NO] ratios of ~150–300, which inhibit cytochrome oxidase by 50–25% (2, 16).

NO, produced by NO donors or by mtNOS, inhibits complex III electron transfer (half inhibition of electron transfer between cytochromes *b* and *c* occurs at 0.2–0.4 μM NO) and increases O₂⁻ and H₂O₂ production in submitochondrial particles and in mitochondria (128). The reaction is frequently referred as involved in the molecular mechanism of neurological diseases.

Intramitochondrial metabolism of O₂⁻, H₂O₂, and NO and diffusion of NO and H₂O₂ out of mitochondria

The intramitochondrial metabolites O₂⁻, H₂O₂, NO, and ONOO⁻ are pro-oxidants potentially leading to oxidative stress and damage and are nowadays considered as the chemical species that afford the molecular mechanism of tissue dysfunction in inflammation, neurological diseases, and aging. Two of them, O₂⁻ and NO, are free radicals; however, they are unreactive and sluggish and do not participate in propagation reactions and only show termination reactions yielding H₂O₂ and ONOO⁻. The latter two species are potentially harmful after homolytic scission due to the generation of the reactive hydroxyl radical. In the case of ONOO⁻, the homolytic scission also yields the free radical NO₂, which is involved in protein nitration (12, 132).

The main part of mitochondrial O₂⁻ is released into the matrix where it encounters specific intramitochondrial manganese superoxide dismutase (MnSOD) that catalyzes the reaction: 2 O₂⁻ + 2 H⁺ => O₂ + H₂O₂. Steady-state concentrations of 0.2–0.3 nM O₂⁻ were estimated for the mitochondrial matrix, with a content of 10–40 μM MnSOD reaction centers (12). The O₂⁻ released in the intermembrane space (69) reacts with cytochrome *c*, located on the P side of the inner

membrane, and with the Cu,Zn-SOD of the intermembrane space (123).

Glutathione peroxidase catalyzes H_2O_2 and ROOH reduction by GSH [H_2O_2 (ROOH) + 2 GSH \Rightarrow GSSG + 2 H_2O (ROH + H_2O)] and is the unique enzyme that uses H_2O_2 in the mitochondria of most mammalian organs (31), the exception being heart, where a mitochondrial catalase has been described (133). Mitochondrial glutathione peroxidase activity accounts for ~60% of the rate of H_2O_2 production and indicates both a role of H_2O_2 as messenger and a function of glutathione peroxidase in the reduction of mitochondrial hydroperoxides. Glutathione deficiency is associated with widespread mitochondrial dysfunction leading to cell damage (105). Glutathione peroxidase requires a continuous supply of GSH, since the co-factor becomes oxidized by H_2O_2 or hydroperoxide. A separate enzyme, NADPH₂-dependent glutathione reductase, is located in the mitochondrial matrix and shows a high activity that keeps the GSH/GSSG couple in a highly reduced state (31).

Boveris and co-workers (152) reported that mitochondria isolated from rat heart, liver, kidney, and brain release NO and H_2O_2 with rates that are higher in state 4 than in state 3. NO release from rat liver mitochondria shows an exponential dependence on membrane potential (152), as reported for mitochondrial H_2O_2 production by Korshunov et al. (84). The data support the speculation that NO and H_2O_2 diffusion to the cytosol reflect a high mitochondrial energy charge and signal such metabolic situation to the cytosol promoting mitochondrial biogenesis.

NO and O_2^- metabolism in the mitochondrial matrix are linked by the very fast, diffusion limited, reaction between NO and O_2^- to produce peroxynitrite ($\text{NO} + \text{O}_2^- \Rightarrow \text{ONOO}^-$) (4, 83). This oxidative utilization of NO is the main (80%) pathway of NO metabolism but only a minor part (15%) of O_2^- utilization, whereas the reductive utilization of NO by ubiquinol and cytochrome oxidase provides minor (20%) pathways of NO catabolism (129).

Peroxynitrite is a powerful oxidant that, as a charged species, is badly diffusible from the intramitochondrial space. Peroxynitrite inhibits both complex I and III (36, 135); the effects are irreversible and are usually observed in situations in which sustained high levels of ONOO^- lead to mitochondrial dysfunction and apoptosis. A level of 2–5 nM ONOO^- has been estimated for the mitochondrial matrix under physiological conditions (129, 151), and levels above 20–30 nM are considered cytotoxic. The existence of a stable low ONOO^- concentration is indicated by the detection of nitrotyrosine in normal mitochondria. At high levels, ONOO^- oxidizes and nitrates lipids and proteins and impairs mitochondrial function. The whole syndrome of mitochondrial dysfunction appears driven by excess NO and ONOO^- as postulated for ischemia-reperfusion, inflammation, and aging (5, 63).

There are effective reductants for matrix ONOO^- due to their reaction constants and concentrations. In rat liver, 3.8 mM NADH₂, 2.6 mM UQH₂ and 3.0 mM GSH in the matrix account for 33, 46, and 21% of the matrix capacity to reduce ONOO^- (151). Peroxynitrite readily reacts with CO_2 to yield the adduct ONOOCO_2^- ($k = 6 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) (11) that also participates in oxidation and nitration reactions. Formation of the adduct decreases the steady-state level of ONOO^- from 30 nM to 2 nM (151) and is considered a detoxification pathway

for mitochondrial ONOO^- that takes advantage of the high mitochondrial CO_2 concentration (1 mM) (132). Finally, NO has been found an effective chain-breaker antioxidant by termination reactions with the free radical intermediates of lipid peroxidation (137).

EFFECTS OF AGING ON MITOCHONDRIAL ENERGY TRANSDUCTION

Mitochondria were brought to attention in aging biology due to 1) the central role of mitochondria in producing chemical energy (ATP) to meet cellular requirements, and 2) the declines of basal metabolic rate and of physical performance in energy-requiring tasks, which are characteristic of the aging process (103).

An age-dependent impairment of mitochondrial function may comprise: 1) decreased electron transfer rates, 2) increased H^+ permeability of the inner membrane, and 3) impairment of the H^+ -driven ATP synthesis.

Decreased Electron Transfer in Aging

A widely recognized experimental fact concerning aging and energy transduction is that a decreased electron transfer activity is observed in mitochondrial membranes and mitochondria isolated from rat and mice tissues upon aging. Complexes I and IV show a selectively decreased enzymatic activity in mitochondria isolated from rat and mice liver, brain, heart, and kidney upon aging, whereas complexes II and III are largely unaffected (7, 87, 93, 101, 110, 112–115, 161). The decreased activity of complex I is determined in mitochondrial fragments as a decreased NADH-cytochrome *c* reductase activity with a simultaneously unchanged succinate-cytochrome *c* reductase activity (115) or as decreased NADH-ubiquinone reductase activity (93), and in coupled mitochondria as a decreased respiratory rate in state 3 with malate-glutamate, or other NAD-dependent substrates with a simultaneous unchanged respiratory rate with succinate as substrate (114) (Fig. 1). In our experience, direct assay of complex I using artificial quinone electron acceptors that exhibit relatively slow rates of electron transfer, may underestimate enzyme activity and miss the impairment in complex I function. Lenaz et al. (93) reported decreased complex I activity in aging and proposed a procedure to calculate complex I activity from aerobic NADH oxidation.

The decreased activity of complex IV is usually determined by the enzymatic assay in mitochondrial fragments (115). Figure 1 illustrates the determination of the enzymatic activity of mitochondrial electron transfer complexes.

The histochemical determinations of NADH-dehydrogenase in rat kidney (111), and of cytochrome oxidase in human *substantia nigra* (76), rat hippocampal dentate gyrus (8), and monkey tissues (109) show decreased activities upon aging.

The decreased enzymatic activities of complexes I and IV could be a consequence either of an enzyme inhibition by aging-produced inhibitors, or of aging-mediated enzyme modification, or of decreased protein expression. Western blot analysis showed that aging is associated with a selectively decreased content of complexes I, II, and IV in human skeletal muscle, whereas complex III was not altered (10). In the same line, aging did not alter the content of catalytic centers of complex III (cytochromes *b* and *c*₁ and [Fe-S] protein) in heart

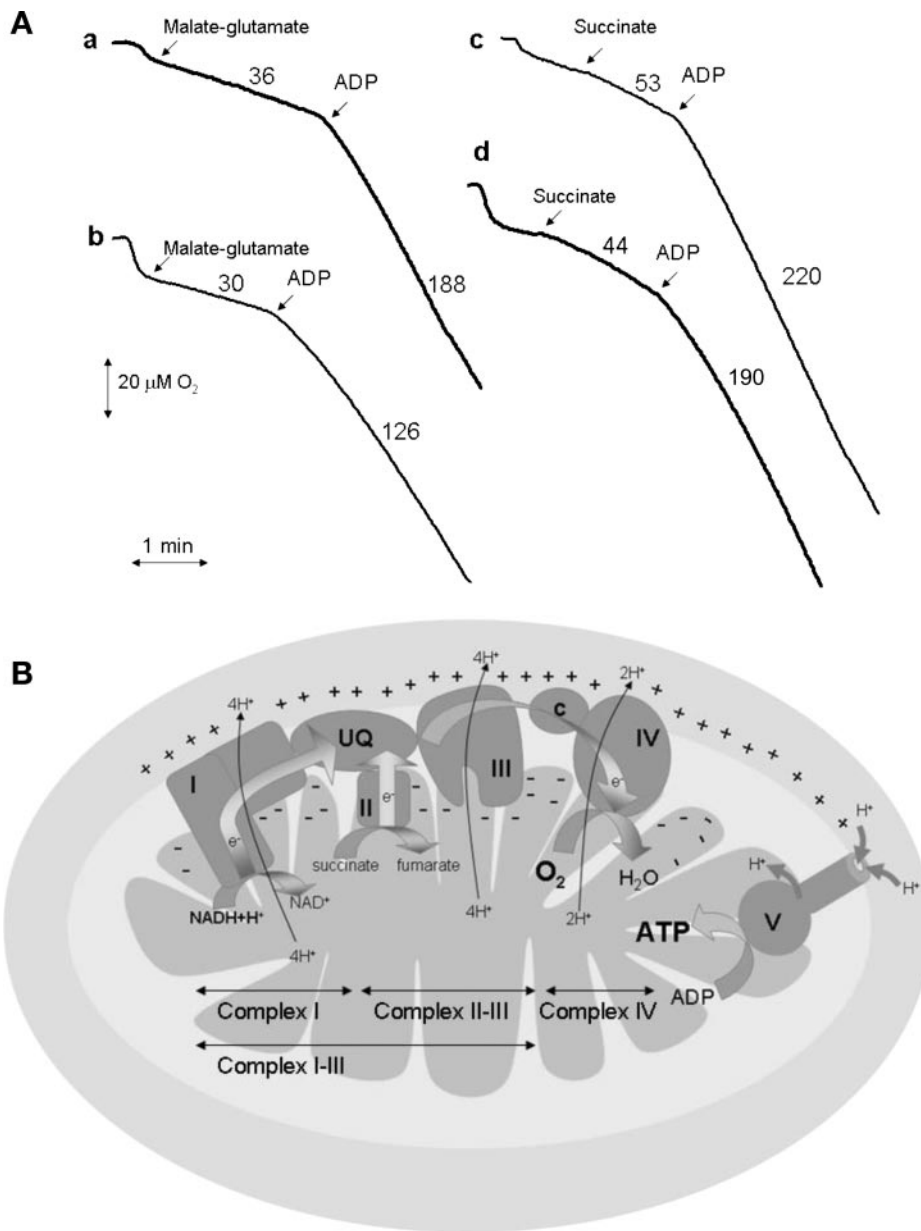


Fig. 1. Mitochondrial electron transfer activities and O_2 uptake. *A*: O_2 uptake of rat brain mitochondria. Respiratory rates were determined with mitochondria at 30°C and the additions were 5 mM malate-glutamate or 10 mM succinate and 0.5 mM ADP. The numbers near the traces indicates O_2 uptake in $\text{ng-at } O/\text{min} \times \text{mg protein}$. *a*, mitochondria (1.15 mg/ml) from a young (3 mo) rat; *b*, mitochondria (1.12 mg/ml) from an old (24 mo) rat; *c*, mitochondria (1.07 mg/ml) from a young (3 mo) rat; and *d*, mitochondria (1.2 mg/ml) from an old (24 mo) rat. *B*: scheme of the mitochondrial respiratory chain. In aging, the decreased activity of complex I is usually determined in mitochondrial fragments as a decreased NADH-cytochrome *c* reductase activity with a simultaneously unchanged succinate-cytochrome *c* reductase activity. The activity of complex IV is determined by the enzymatic assay in mitochondrial fragments.

interfibrillar mitochondria (94). The content of ubiquinone was reported decreased in the skeletal muscle of old mice (90).

Northern blot analysis of the respiratory complexes in mice brain mitochondria revealed an increased expression of mitochondrial-encoded genes in complexes I, III, IV, and V in 12- and 18-mo-old mice compared with 2-mo-old mice, suggesting a compensatory mechanism of overproduction of electron transfer proteins. However, the mRNA expression of all genes was decreased in 24-mo-old mice, suggesting that compensation by gene upregulation cannot be sustained for a long time and that downregulation of expression shows up in the late stage of aging (98).

Mitochondrial respiration can be limited by the activities of the NAD-dependent dehydrogenases. There is no clear evidence if the activities of the dehydrogenases of the citric acid cycle are modified during aging. In mitochondria isolated from kidneys of old mice, aconitase activity exhibited a significant

decrease with age (160), whereas α -ketoglutarate dehydrogenase exhibited a modest decrease and NADP^+ -isocitrate dehydrogenase activity increased moderately. The activities of citrate synthase, NAD^+ -isocitrate dehydrogenase, succinyl-CoA synthetase, succinate dehydrogenase, fumarase, and malate dehydrogenase were not affected. The ratio of the intramitochondrial redox indicator, $\text{NADPH}_2/\text{NADP}$, was higher in young compared with old animals, while the NADH_2/NAD ratio remained unchanged (160). In another study, the activities of citric acid cycle enzymes of heart and skeletal muscle mitochondria, such as isocitrate dehydrogenase, α -ketoglutarate dehydrogenase, succinate dehydrogenase, and malate dehydrogenase, were found decreased in aged rats (87).

Acyl carnitine transferase, that catalyzes fatty acid transport to the mitochondrial matrix, is essential for mitochondrial function and has been reported selectively decreased in aging (96). The proteomic analysis of aged male *Macaca fascicularis*

heart showed decreases in the expression of key glycolytic enzymes (e.g., pyruvate kinase, α -enolase, triosephosphate isomerase), glucose oxidation (e.g., pyruvate dehydrogenase E1- β -subunit), and tricarboxylic acid cycle (α -ketoglutarate dehydrogenase) in old male monkeys. Interestingly, these changes were not observed in old female monkeys (158).

Increased H⁺ Permeability of the Inner Membrane

There are speculations about an increased H⁺ permeability of the inner mitochondrial membrane and a consequent failure in maintaining the H⁺ electrochemical gradient in aging. However, there is no reported evidence of an increased H⁺ permeability in old animals. Indirect evidence from decreased membrane potential in astrocytes from old animals is consistent with an impairment of the H⁺ impermeability of the inner membrane (95). Brain mitochondria isolated from aged rats had a larger volume and increased water permeability when challenged by a hypotonic medium, but liver mitochondria did not show such difference between young and old animals (112). Liver mitochondria from old rats showed decreased membrane potential, an impairment that was improved by treatment with an antioxidant extract (139). Cortical and striatal mitochondria of aged rats showed lower membrane potentials with increased susceptibility to the permeability transition at advanced ages (88). Mitochondria isolated from thoraces of *Drosophila melanogaster*, mainly flight muscle mitochondria, showed a significant decrease in state 3 respiration, respiratory control ratios, and uncoupled respiration rates, as a function of age, using either NAD- or FAD-linked substrates, but there were no differences in state 4 respiration rates, which does not indicate increased H⁺ permeability in aging (51).

Conservation of the H⁺-Driven ATP Synthesis in Aging

The H⁺-driven ATP synthesis in aging has been estimated from the determination of the ADP/O ratios in coupled mitochondria. There is no report on the effect of aging on the biochemical activity of the ATP synthase complex or on F₁-ATPase activity. Two studies reported slightly depressed and not affected ADP/O ratios in aged brain (114) and liver (110, 114) mitochondria. In both cases, the almost no effect on the ADP/O ratios was simultaneous to a decreased state 3 respiration rate, which indicated an impairment of electron transfer.

The activity of adenine nucleotide translocase, that catalyzes the fast ADP/ATP exchange between cytosol and mitochondria, was reported decreased in aging (159).

FREE RADICALS, OXIDATIVE DAMAGE, MITOCHONDRIAL DYSFUNCTION AND APOPTOSIS

The free radical theory of aging is based in the works of Gerschman (57) and Harman (70), and when focused in mitochondria emerged as the mitochondrial hypothesis of aging (5, 71, 154). Mitochondria are considered the pacemakers of tissue aging due to the continuous production of free radicals, oxygen, and nitrogen free radicals and related reactive species, and to the selective oxidative damage that leads to mitochondrial dysfunction. Gerschman postulated in 1954 that oxygen free radicals were the common biochemical mechanism of O₂ and radiation toxicity after finding synergism between radiation

and hyperbaric O₂ in decreasing the survival of exposed mice (57).

Oxidative Damage and Mitochondrial Dysfunction

Dysfunctional mitochondria are characterized by decreased state 3 respiration, respiratory controls, and membrane potential, and increased rates of state 4 respiration and mitochondrial size and fragility associated to an increased content of oxidation products. Dysfunctional mitochondria are observed in aging, and also in pathological situations as ischemia-reperfusion and inflammation (5, 63, 112, 114).

The experimental determination of increases in oxidation products that correspond to the free-radical mediated oxidation of mitochondrial constituents, such as phospholipids, proteins and DNA, constitutes the usual finding in aging studies. Phospholipid oxidation products, as TBARS (112–115) and ROOH (139, 145), protein oxidation products, as protein carbonyls (54, 112–114, 149), DNA oxidation products, as 8-HO-dG (5, 138) were found increased in aged animals. Protein oxidation and nitration appear to have capital importance in the molecular mechanisms involved in cell turnover and cell cycle and in triggering apoptosis. Oxidized and nitrated proteins appear as the sand grains of the sand clock that determines cell life. The proteolytic enzymes that degrade modified proteins decline with aging which implies a less efficient removal and an accumulation of oxidized proteins (22, 66). Kozlov et al. (85) identified skeletal muscle, heart and lung as the main sources of oxygen radicals in vivo in old rats by injection of a spin trap. The spin trap reacts with O₂⁻ and with ONOO⁻ and provides conclusive evidence of the generation of these species under physiological conditions.

Proteomic techniques used with rat heart homogenate and mitochondria showed protein nitration in aging. A total of 48 nitrated proteins was identified; among them were α -enolase, α -aldolase, desmin, aconitate hydratase, methylmalonate semialdehyde dehydrogenase, 3-ketoacyl-CoA thiolase, acetyl-CoA acetyltransferase, malate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, creatine kinase, electron-transfer complex I flavoprotein, MnSOD, F₁-ATPase, and the voltage-dependent anion channel (81). The MnSOD activity in mice brain mitochondria decreased linearly upon aging from 28 to 76 wk of age, up to 67% (114).

Aging is characterized by a general decline of physiological performances with a more marked effect in the functions that depend on the central nervous system. In a mechanistic approach, the decreases in neuromuscular coordination and maze performance in mice upon aging were found directly related to the brain content of lipid and protein oxidation products (54, 113–115).

Mitochondrial inner membrane enzymes that are markers of aging

Accumulation of oxidative damage and decrease of mitochondrial energetic competence to produce ATP in the organs and tissues of aged mammals are two underlying concepts of the mitochondrial hypothesis of aging. Concerning the decreased capacity to produce ATP, a reduction of the mitochondrial mass in brain and liver as well as the phosphorylating capacity of the ATP synthase were ruled out (112, 114) and decreased rates of electron transfer were identified as the

mechanism of mitochondrial dysfunction in aging (112–115). Accordingly, the activities of mitochondrial complexes I and IV were found decreased upon aging. A 17–33% decrease of NADH-cytochrome c reductase activity (complexes I + III) at 52–76 wk of mice age, is interpreted as complex I decreased activity, since succinate cytochrome c reductase activity (complex I + II) was not affected by aging (113–115). Cytochrome oxidase activity was also decreased by 24–36% at the same time points (113–115). Thus the activities of both complex I and complex IV were decreased by about one-third in senescent mice and are considered effective markers of aging. Moreover, brain mitochondrial enzyme activities keep a quantitative relationship and correlate significantly with survival (Fig. 2).

Oxidative damage, as determined by the mitochondrial content of protein carbonyls and TBARS, correlated negatively with the activities of complex I and complex IV. The observation supports the view that oxidatively modified proteins and the increased level of lipid peroxidation products are part of the molecular mechanism of the decreased enzymatic activity (113–115). Moreover, it is likely that the inhibition of complex I activity upon aging occurs with increased reduction of complex I, i.e., higher levels of FMNH₂ and FMNH[•], which lead to an increased rate of O₂⁻ generation (Fig. 3).

Interestingly, mice neurological function, as determined by the tightrope test to evaluate neuromuscular coordination and by the T-maze test to evaluate memory and exploratory capacity, was linearly related to brain complex I and IV enzymatic activities (113, 114) and negatively correlated with the mitochondrial content of brain lipid and protein oxidation products (54, 113, 115) (Fig. 4). Applying the concept of rate limiting step in complex systems, it follows that decreased rates of electron transfer and a limiting energy supply by brain mitochondria are factors in the neurological dysfunction inherent to physiological aging.

The activity of mtNOS, an integral inner mitochondrial membrane enzyme, decreased significantly (40–65%) in the brain and liver of senescent mice and old rats (112, 114). The magnitude of this change makes mtNOS the most sensitive marker of aging, more sensitive than the already considered complexes I and IV. The decreased activity of mtNOS suggests a loss in critical regulatory roles of mitochondrial NO in aging (114). The association between mtNOS activity and cellular homeostasis has been called the pleiotropic effect of mtNOS and was originally used to describe preserved kidney and heart functions (161).

Recently, NO has been implicated in mitochondrial biogenesis by the stimulation of guanylate cyclase, generation of cGMP and activation of PGC1- α (120). It was claimed that the pleiotropic effect occurs through NO and H₂O₂ diffusion from mitochondria to the cytosol, a signal that indicates a high mitochondrial energy charge (117). MtNO production seems to be the main signal for the cell changes in the ovarian cycle (117) and to provide the 70% of cellular NO in the heart (161). The general role of mitochondria as ATP provider, as source of NO to signal for mitochondrial proliferation and for mitochondria-dependent apoptosis, and as the main intracellular source of O₂⁻ and H₂O₂ (31) appears well adapted to serve the proliferation-apoptosis sequence of the ovarian cycle (117). Thus, the marked decrease in mtNOS activity in aged brain mitochondria is interpreted as a decrease in mitochondrial signaling that hinders a sustained neuronal homeostasis in aging (114).

In summary, the inner membrane mitochondrial enzymes mtNOS, complex I, and complex IV are markers of tissue aging (Fig. 2).

Mitochondria-dependent cell death program

The pivotal role of mitochondria in the execution of cell death is well established (86). The sequence of biochemical and cellular events in mitochondria-dependent apoptosis has been reported by Bustamante et al. (23) in thymocytes, describing the time course and the quantitative consideration of the signals and the involved molecular markers. At the initial phase (2.5–30 min), cytosolic [Ca²⁺] and [NO] exerted their signaling by marked (7–8 times) changes in their levels (23). The first intracellular signal was the increased cytosolic [Ca²⁺] which occurred in a 2–3 min and is due to inhibition of endoplasmic reticulum Ca²⁺-ATPase and to depletion of intracellular Ca²⁺ stores (78). The next rapid observed effects, with a estimated half-life ($t_{1/2}$) of ~15 min, were the parallel increases in mitochondrial NO production and in cellular H₂O₂ steady-state concentration (23). As said, the rate of mitochondrial H₂O₂ production is directly regulated by mtNOS activity,

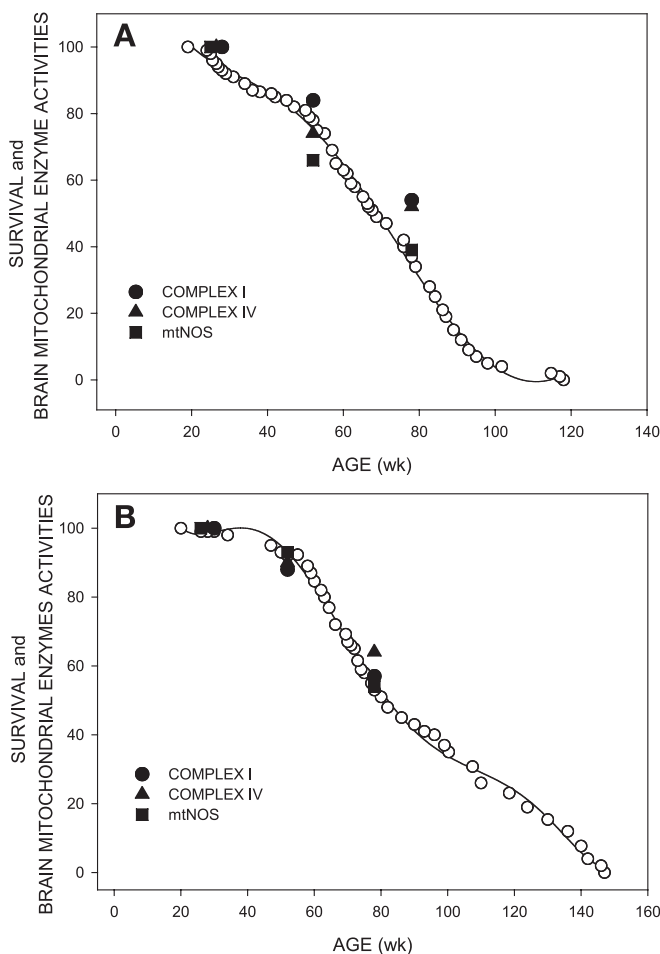


Fig. 2. Activity of brain mitochondrial enzymes that are markers of aging in relation to mice survival. Complex I, complex IV, and mitochondrial nitric oxide (NO) synthase (mtNOS) activities in male (A) and female (B) mice.

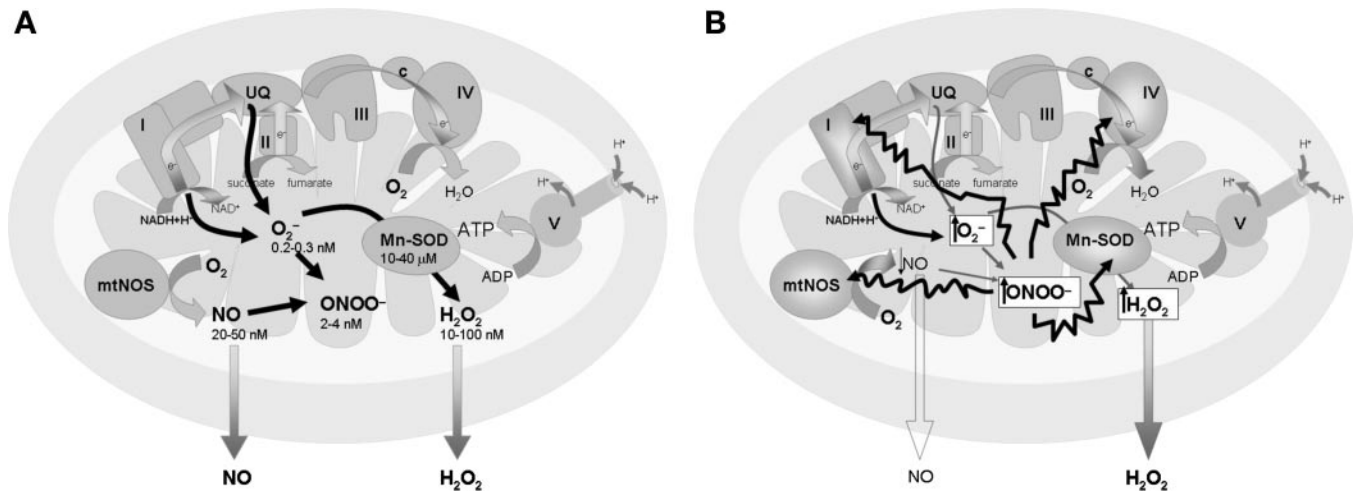


Fig. 3. Mitochondrial metabolism of O_2^- and NO in physiological conditions and upon aging. *A*: numbers below the symbols indicate steady-state concentrations for mammalian organs under physiological conditions. *B*: selective damage of mitochondrial enzymes in aging by increased complex I O_2^- production and intramitochondrial $ONOO^-$ generation with the positive feedback between complex I inhibition, O_2^- generation, and oxidative damage and nitration.

since the enzyme product NO augments the rate of O_2^- production by inhibition of electron transfer at complex III (128). Increased intracellular Ca^{2+} is associated with the activation of the whole NOS family (34) and with cell death.

The increased levels of NO , O_2^- , and H_2O_2 constitute the molecular mechanism for the increased free radical-dependent lipid peroxidation process, with increases in cellular ROOH and TBARS production and with $t_{1/2}$ of 27–30 min in thymocytes. Thymocyte mitochondria were able to stand up cellular oxidative stress and remained functional for ~1 h, as an indication of the resistance of these organelles to oxidants. At the intermediate phase (30–130 min) in which apoptosis is still reversible, the molecular contribution or signaling by H_2O_2 and ROOH occurs with their intracellular levels increased by a factor of ~2 (23).

Mitochondrial dysfunction followed, with a $t_{1/2}$ of 101–133 min in thymocytes, as described by the abolition of respiratory

control, inner membrane depolarization, and cytochrome *c* release (23). Increased intramitochondrial steady-state levels of NO and O_2^- lead to increased rates of formation and steady-state levels of $ONOO^-$ with further inhibition of complex III (27, 128) and inactivation of complex I (36, 135). The molecular markers of the final and irreversible phase of apoptosis, such as caspases-3 activity and DNA fragmentation, were again markedly increased, by a factor of 4, in agreement with the irreversibility of the process at that point (23).

Tissues, such as brain and specific brain areas, as the hippocampus, that have a slow turnover of mitochondria and mitochondrial components, show a cellular accumulation of dysfunctional mitochondria with increased apoptosis upon aging, a condition that drives the tissue to a physiological deficit (64).

PHYSIOLOGY OF THE DECREASED CAPACITY OF ENERGY SUPPLY

Reduction of the capacity to produce ATP in the organs and tissues of old mammals is one of the basic concepts of the mitochondrial hypothesis of aging. The experimental evidence, as mentioned in section 5.2, points out to a selectively decreased rate of electron transfer as the molecular mechanism of the mitochondrial impairment associated with aging.

The ~35% decreased activity of complex I in brain and liver of old rats is close to the limit of a tolerable functional damage in terms of basal energy production. In rat liver, ATP is provided by the respiration of about 36% of the mitochondrial mass in metabolic state 3 (15). Under conditions of increased ATP demand, liver mitochondria will be able to increase ATP synthesis up to 2.8 times by switching more mitochondria from the resting state 4 to the active state 3. Aged hepatocytes, with a 30% reduction in complex I activity, will be able to increase ATP production only up to 1.9 times by switching mitochondria from state 4 to state 3. The situation is clearly worst in the brain due to the lower mitochondrial mass and where aged neurons are close to be unable to respond to any increased ATP demands (112).

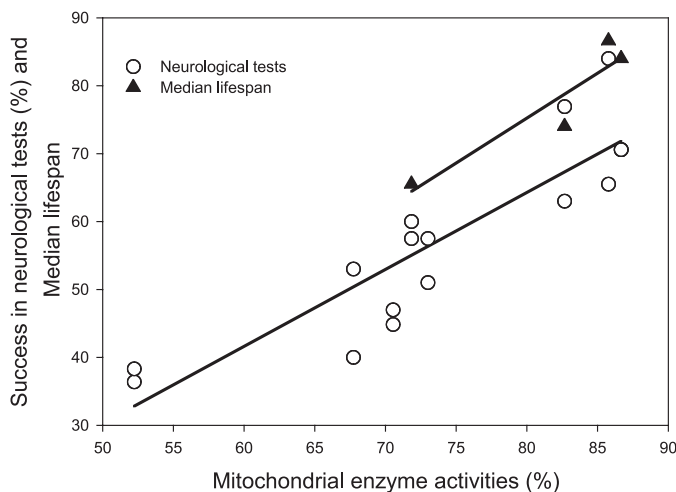


Fig. 4. Correlation between mitochondrial inner membrane enzymatic activities [mean value of complex I (estimated as NADH-cytochrome *c* reductase), complex IV and mtNOS activities] of brain mice at 28, 52, and 76 wk of age with success in the T-maze and in the tightrope tests at the same age points. $r^2 = 0.78$, $P < 0.05$ and with median lifespan, $r^2 = 0.87$, $P < 0.05$.

The observed age-dependent decrease in marker activities in brain mitochondria that in senescent animals reaches 35–65% is simultaneous with the existence of a mitochondrial subpopulation with increased size (139) and fragility (112). Most neurons are long living cells, with a slow turnover of mitochondria and of mitochondrial components, that age at the same time as the animal ages with neurogenesis spatially restricted and quantitatively negligible (64).

Liver mitochondria isolated from young and old animals did not exhibit a significant difference in fragility (112). In hepatocytes, mitochondria are continuously subjected to protein and phospholipid turnover and to elimination of dysfunctional organelles. The cellular population of damaged mitochondria is likely to become determinant in the signaling for apoptosis as hepatocytes reach their life span limit. A $t_{1/2}$ of mitochondrial proteins and mitochondrial fractions gives 7–10 days for liver mitochondria and 4–6 wk for brain mitochondria (64). The slower turnover of brain mitochondria makes possible the detection of a fraction of dysfunctional mitochondria with impaired electron transfer and increased fragility, which may correspond to subpopulations of enlarged mitochondria in morphological or flow cytometry studies.

Considering the evolution of the knowledge on brain aging and the complexity and variety of metabolic conditions in brain nuclei and areas, it is nowadays necessary to attempt the determination of mitochondrial function in mitochondria isolated from brain nuclei, as hippocampus, that are specially sensitive to aging. Preliminary results in our laboratory show a selective mitochondrial damage, similar to the one described here for whole brain, in the hippocampus of aging rats.

CONDITIONS THAT IMPROVE SURVIVAL AND MITOCHONDRIAL FUNCTION

Some conditions that are able to increase survival and to improve the neurological deficits of mice senescence have been found to also decrease the age-related oxidative damage and dysfunction of brain mitochondria. The age-related oxidative damage is, in a first approximation, due to an increased rate of generation of oxidants. The hypothesis of a decrease in the activity of the antioxidant enzymes, in spite of numerous studies that measured age-related changes in antioxidants defenses, failed to produce a clear evidence of decreased antioxidant enzymes in mice and rats in aging (5). However, the interspecies comparisons of oxidative damage, antioxidant content and oxidant generation provide some of the most compelling evidence that oxidants are significant determinants of life span (43).

The selected life conditions that were able to improve survival in mice are similar to recommendations that are commonly followed by many aging humans, i.e., antioxidant supplementation, caloric restriction, high spontaneous neurological activity, and moderate physical exercise. There is evidence that these conditions increase mice survival associated with improved brain mitochondrial function (113–115). However, most of the studies show a clear increase in median lifespan without a significant modification in maximal lifespan. The kinetics of aging are described by the survival curves (Fig. 5) which shows mice survival curves with increased either median or maximal life span in modified life conditions.

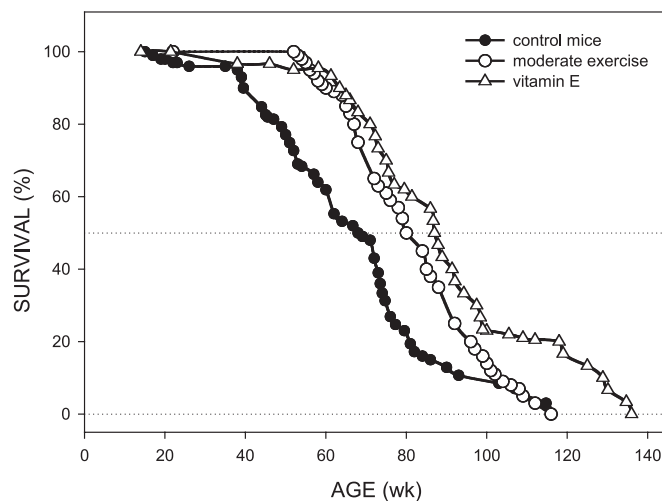


Fig. 5. Survival curves of Swiss CD1-UCadiz male mice subjected to selected life conditions. Control mice ($n = 50$): median lifespan, 61 ± 4 wk; maximal lifespan, 116 ± 4 wk. Moderate physical exercise ($n = 40$): median lifespan, 80 ± 4 wk; maximal lifespan, 109 ± 4 wk. Vitamin E-supplemented mice ($n = 40$): median lifespan, 85 ± 4 wk; maximal lifespan, 136 ± 4 wk.

Antioxidant Supplementation

Mice of the strain CD-1/UCadiz chronically supplemented with high doses of vitamin E (5.0 g *dl*-RRR- α -tocopherol acetate/kg of food) showed increased survival, with a 40% increased median life span and a 17% increased maximal life span (Fig. 5) (114). The strain CD-1/UCadiz has similar survival to AKR, SAM, NZB/Lac and SJL/J that are senescence accelerated strains and that are used as models of aging, considering that the whole aging process is similar to normal mice and that can be extrapolated to primates (53). Vitamin E also retarded the neurological deficits associated with aging, since mice that received vitamin E exhibited better performances in behavioral tests. Moreover, both the increase in mitochondrial oxidative damage, and the diminished rates of electron transfer in brain mitochondria of aged and senescent mice were ameliorated by vitamin E supplementation (Table 1) (114). The effects of aging and vitamin E on mitochondrial O_2 uptake and oxidative phosphorylation were determined in mice brain and liver after 24 wk of vitamin E supplementation. The rate of respiration of brain mitochondria in active state 3 was 24% decreased in 52 wk mice, an effect that was 50% prevented by vitamin E supplementation. Respiratory control and ADP/O ratios were neither affected by aging nor by vitamin E supplementation (114). The functional activity of mtNOS in the regulation of state 3 O_2 uptake was decreased $\sim 50\%$ by aging and the loss was about 64% prevented by vitamin E, in clear agreement with the effects observed in mtNOS biochemical activity (Table 1) (114). The effects of aging and vitamin E supplementation on liver mitochondria, considering respiration, oxidative phosphorylation and mtNOS functional activity, were qualitatively similar to the ones observed in brain mitochondria, but less marked (114).

Dietary supplementation with acetyl-carnitine and lipoic acid (68, 96) and with a flavonoid-rich vegetable extract (9) produce a prevention of the age-associated decline in mouse physiological functions and interpreted as due to protection or remediation of an oxidative damage in mitochondria.

Table 1. Activities of enzyme markers of aging in brain mitochondria of young, aged, and senescent male mice subjected to selected life conditions

Age and Enzyme Activity/Group	Young (28 wk)	Aged (52 wk)	Senescent (76 wk)
Complexes I-III			
Control mice	330 ± 10	273 ± 10*	212 ± 10*
High neurological activity		285 ± 10	250 ± 10†
Moderate exercise		291 ± 10	275 ± 10†
Vitamin E supplementation		290 ± 10	283 ± 11†
Complexes II-III			
Control mice	127 ± 9	125 ± 9	131 ± 9
High neurological activity		131 ± 9	127 ± 9
Moderate exercise		127 ± 9	132 ± 9
Vitamin E supplementation		129 ± 9	128 ± 9
Complex IV			
Control mice	124 ± 8	96 ± 8*	79 ± 8*
High neurological activity		105 ± 8†	95 ± 8†
Moderate exercise		112 ± 9†	100 ± 8†
Vitamin E supplementation		107 ± 8	102 ± 8†
mtNOS			
Control mice	0.65 ± 0.05	0.36 ± 0.04*	0.20 ± 0.03*
High neurological activity		0.50 ± 0.05†	0.33 ± 0.03†
Moderate exercise		0.53 ± 0.04†	0.31 ± 0.04†
Vitamin E supplementation		0.54 ± 0.05†	0.41 ± 0.05†

Values are means ± SE. mtNOS, mitochondrial nitric oxide (NO) synthase. Swiss CD1-UCadiz mice were used in the study. Complexes I-III, II-III, and IV activities are expressed in nmol cytochrome *c* (reduced or oxidized)/min mg protein; and mtNOS in nmol NO/min mg protein for 12 mice in each group. * $P < 0.05$ for aging, compared with 28-wk-old mice. † $P < 0.05$ for high neurological activity, moderate exercise, or vitamin E supplementation compared with control mice.

The addition to the diet of low molecular weight antioxidants, such as 2-mercaptoethylamine (71), ethoxiquin (40) and 2-ethyl-6-methyl-3-hydroxy-pyridine (50), was able to produce a 13–29% increase in median life span.

Other substances, as *N*-acetyl cysteine (119), ubiquinone (122), and melatonin (124), were reported to improve mitochondrial function in aging and the effect was interpreted as an antioxidant action. *N*-acetyl cysteine prevented, in rat brain, the aged-related changes in mitochondrial gene expression of subunit 39 kDa and ND-1 of complex I, complex IV, subunit α of F_1 -ATP synthase (complex V) and of adenine nucleotide translocator isoform 1 (119). Ubiquinone supplementation improved mitochondrial function in heart mitochondria of aged rats (122) and melatonin protected hepatic mitochondrial respiratory activity in senescence-accelerated mice (124).

Mitochondrial oxidative dysfunction is accelerated by many common micronutrient deficiencies. One major mechanism is inhibition of the pathway of heme biosynthesis in mitochondria, which causes a deficit of heme *a*. Minerals such as iron or zinc, and several vitamin deficiencies, such as biotin or pantothenic acid, increase mitochondrial oxidative dysfunction and turnover through this mechanism. Ames et al. (1) suggested that an optimum intake of micronutrients could tune up metabolism and give a marked increase in health, particularly for the poor, elderly, and obese, at little cost.

Caloric restriction

Limitation in the dietary intake, namely caloric restriction, is a well-established way to extend the life span in mammals (157). Early expectations that caloric restriction would lower metabolic rate have not been confirmed, then if caloric restriction attenuates oxidative damage, it is not by a simple reduction in O_2 consumption (147). In liver, heart, brain, and kidney mitochondria, there were no significant effects of caloric restriction in state 4 mitochondrial respiration rate (89) and in

mitochondrial ATP content (48). There is now evidence that indicates that caloric restriction acts by decreasing oxidative stress and damage and by increasing antioxidant defenses and repair systems (134). However, the issue is not free of conflicting reports and caloric restriction was reported without enhancing effect in the antioxidant defenses.

Mitochondrial function and caloric restriction are apparently related and that understanding is the main concept of a series of experimental approaches. Caloric restriction was reported to induce a hypometabolic state characterized by decreased reactive oxygen species production and decreased mitochondrial H^+ leak, with decreased O_2^- production in brain mitochondria at complex I without changes in O_2 consumption (138). Caloric restricted rats showed structural and functional liver mitochondrial properties (fatty acid pattern, respiratory chain activities, antioxidant levels, and hydroperoxide contents) similar to those of younger rats (3). It was suggested that caloric restriction provides neuroprotection through apoptosis repression by suppressing cytochrome *c* release and caspase-2 activation, but neither age nor caloric restriction had any effect on caspase-3 and caspase-9 activities (142). Recently, it was reported that caloric restriction induces mitochondrial biogenesis and increases bioenergetic efficiency (97).

In yeast, caloric restriction delays aging by activating Sir2 deacetylase. The expression of mammalian Sir2 (SIRT1) is induced in caloric-restricted rats as well as in human cells that are treated with serum from these animals. SIRT1 deacetylates the DNA repair factor Ku70, causing it to sequester the proapoptotic factor Bax away from mitochondria, thereby inhibiting stress-induced apoptotic cell death. Thus, caloric restriction could extend life-span by inducing SIRT1 expression and promoting the long-term survival of irreplaceable cells (38). SIRT3 is one of the seven mammalian sirtuin homologs of the yeast *Sir2* gene, which mediates the effect of caloric restriction in lifespan extension in yeast and *Caeno-*

rhabditis elegans. Murine SIRT3 is expressed in brown adipose tissue and is localized in the mitochondrial inner membrane. Caloric restriction activates SIRT3 expression in white and brown adipose tissue. Additionally, cold exposure upregulates SIRT3 expression in brown fat, whereas elevated temperature reduces expression. Enforced expression of SIRT3 in HIB1B brown adipocytes enhances the expression of the uncoupling protein UCP1, and a series of mitochondria-related genes. Functionally, sustained expression of SIRT3 would decrease membrane potential and reactive oxygen species production while increasing cellular respiration. In summary, it seems that SIRT3 activates mitochondrial functions and has a role in the adaptive thermogenesis of brown adipose tissue (144).

Mice dietary supplemented with thioproline (*l*-thiazolidine-4-carboxylic acid), a physiological metabolite of 5-hydroxytryptamine, show a 29% increased median life span and a 23% increased maximal life span, associated with improved mitochondrial function and neurological functions. The thioproline effect seems secondary to caloric restriction, since thioproline induces an anorexic effect with lower food intake and lower body weight (116).

High Spontaneous Neurological Activity

Aging is characterized by a decline of the physiological functions of the central nervous system (61). Associations of the quality of the response to stressful stimuli with longevity and decreased neurodegeneration were reported (61, 115). Mice with high spontaneous neurological activity have 11–14% increased median lifespan and a marked improvement in the performance of behavioral tests (115). Concerning sex difference, female mice with high spontaneous neurological activity showed the longest life span. The longer life span of females is in agreement with the lower mitochondrial production of oxidants in females and with the downregulation of oxidant production by estrogenic hormones (153). Figure 2 shows the relationship between mice survival and the mitochondrial enzyme activities of complex I, complex IV, and mtNOS. Mice were considered with high spontaneous neurological activity by selection according to their performance in two neurological tests. Individual mice were subjected every 2 wk to the tightrope test and the T-shaped maze test (115). In the tightrope test, to evaluate neuromuscular coordination, mice were placed hanging from their anterior legs in the middle of a 60-cm tightrope and the test was considered successful when mice reached the end of the rope in <30 s. In the test to evaluate the spontaneous exploratory and cognitive activities, mice were challenged in a T-shaped maze of 50 cm arms and the test was considered successful when mice moved toward the T-intersection in <30 s. The study showed an inverse statistical relationship between brain oxidative damage and mice performance in the tests and mitochondrial electron transfer. The higher mitochondrial enzyme activity and the better neurological activity were associated to lower oxidative damage (115).

Moderate Physical Exercise

Exercise has been long considered associated with oxidative stress based on the experimental data showing an increase of

oxidative stress markers after high-intensity exercise and on the unsupported assumption that the production of oxygen free radicals is linearly related to the rate of respiration (41, 77). Chronic moderate exercise started at young age in mice increased median lifespan (Fig. 5), decreased oxidative damage and prevented the decline of cytochrome oxidase activity and behavioral performance at middle age but not at old age (Table 1) (113).

Regular physical exercise seems to retard the accumulation of cell damage and the physiological dysfunction that are characteristic of the aging process (131). There is ample evidence of the reduction of skeletal muscle mass associated to aging and of the beneficial effects of regular exercise in increasing muscle mass and strength in elderly individuals. The available evidence extends from experimental animals to humans and from biochemical markers to physiological parameters and behavioral performances (102). A series of reports documented that the beneficial effects of exercise are extended to other organs, such as the heart (19, 113), kidney (113), liver (113), and brain (39). However, aerobic exercise of high intensity and duration is not beneficial for aged rats (118). The positive effects of moderate exercise, observed in heart, kidney, liver, and brain support the concept that the effects involve genomic modulation. Radak et al. (131) suggested that moderate exercise activates DNA repair systems and the resistance against oxidative stress in rat skeletal muscle. Exercise by lifelong voluntary wheel running reduces subsarcolemmal and interfibrillar mitochondrial H₂O₂ production in the heart (79). Oxidant production in skeletal muscle is increased in old age and during prolonged exercise, with both the mitochondrial respiratory chain and NADPH oxidase as potential sources, and decreased GSH/GSSG ratios (6).

Overexpression of mitochondrial antioxidant enzymes

The development of transgenic animals that overexpress antioxidant enzymes and other regulatory enzymes in mitochondria and that exhibit increased life span will certainly increase our understanding of the mechanisms of aging. Schriener et al. (141) reported that transgenic mice that overexpressed human catalase in heart mitochondria have increased median and maximal life span. This transgenic mice strain have reduced age-dependent atherosclerosis and increased genomic stability, as indicated by a decrease in oxidative stress markers and in mitochondrial deletions in heart and muscle. Oxidative damage was reduced, H₂O₂ production and H₂O₂-induced aconitase inactivation were attenuated, and mitochondrial deletions were reduced. It was disappointing that the Gompertz plot of this transgenic mice strain ran parallel to the wild-type control, indicating a delay in the onset of aging rather than a decrease in aging rate. Nevertheless, the results support the notion of mitochondrial oxidative stress and damage as determinants of both health and life span (141). The relatively large increase in life span resulting from the upregulation of a single gene suggests the possibility that the upregulation of relatively few longevity genes may result in dramatic increases in life span (43).

Previous experiments with transgenic *Drosophila melanogaster* that overexpressed Cu,Zn-SOD failed to show an increased maximal life span. However, the overexpression of

Cu,Zn-SOD and catalase in these flies extended median and maximal life span (125). Therefore, decreased mitochondrial O_2^- and H_2O_2 steady-state concentrations appear as able to induce an extended survival.

HUMAN AGING AND NEURODEGENERATIVE DISEASES

The increase in human lifespan in the industrialized countries is accompanied by a marked prevalence of neurodegenerative diseases. These diseases are preferential lines of study in gerontology and geriatrics because healthy aging is the first aim in those fields. Human neurodegenerative diseases are characterized by progressive cellular damage which is encompassed by particular neurological deficits once neuronal loss reaches, in most instances, more than half of the neuron population in a given region. For example, Parkinson's disease is suspected to evolve for years before typical motor signs appear, a moment when there is a loss of dopaminergic neurons of ~60% in *substantia nigra pars compacta*.

There is evidence that mitochondrial dysfunction and impairment of the respiratory complexes have a role in the neuronal loss of neurodegenerative diseases. Impaired complex IV activity has been reported in Alzheimer's disease (29) and decreased complex I activity is usually reported in the *substantia nigra* of *postmortem* samples obtained from patients with Parkinson's disease (108) and in platelets from Parkinson's patients (100). Also, a decreased activity of complexes I and II has been reported in Huntington's disease (127).

Inhibition of complex I creates an environment of mitochondrial oxidative damage and of nitration that may lead to opening of the permeability transition pore and to apoptotic cell death (72), or to aggregation of α -synuclein with the subsequent death of dopaminergic neurons (46). These results show that impairment in complex I activity is central to the pathogenesis of the dopaminergic neuronal demise in Parkinson's disease (143). Reduced complex I activity predisposes to excitotoxicity by altering ATP levels and by impairing Ca^{2+} homeostasis (45). Reduced ATP levels decrease the activity of plasma membrane $Na^+-K^+-ATPase$, resulting in partial neuronal depolarization that decreases the voltage-dependent Mg^{2+} blockade of the *N*-methyl-D-aspartate glutamate receptor. Under these conditions, even normal levels of extracellular glutamate may cause excitotoxic activation of *N*-methyl-D-aspartate receptors and elevation of intracellular Ca^{2+} . Complex I defects have been reported to disrupt normal Ca^{2+} signaling in neurons (60).

It is to be expected that prevention or slowing down the processes of mitochondrial dysfunction upon aging will decrease the neurological deficits in aged people.

CONCLUSIONS AND PERSPECTIVES

It is now clear that mammalian life span is negatively related to the mitochondrial production of oxidizing free radicals and that dysfunctional mitochondria determine mitochondrial and cellular turnover. Mitochondrial impairment and cellular dysfunction upon aging determine tissue physiological function. Tissues, such as brain and brain areas that have a slow turnover for their mitochondria and mitochondrial components, are

driven to a physiological deficit by mitochondrion-dependent apoptosis.

Aged mitochondria show a selective diminished activity of the enzymes that are integral and constitutive proteins of the inner mitochondrial membrane. Complexes I and IV and mtNOS are selectively affected in aging and constitute markers of tissue aging. These three enzymes interact by physical contact, according to the model of the supercomplexes of the respiratory chain. The enzyme mtNOS has been reported associated to complexes I and IV by evidence of complementary sequences and immuno-coprecipitation (55).

The slower rate of mitochondrial electron transfer in aging favors the mitochondrial generation of O_2^- by complex I, and produces a positive feedback between complex I inhibition and the mitochondrial production of O_2^- and peroxyxynitrite.

Mitochondrial biogenesis seems regulated by the signaling given by NO and H_2O_2 diffusion from mitochondria to the cytosol. The study of mitochondrial NO and H_2O_2 production and diffusion as a function of aging will contribute to the understanding of mitochondrial biogenesis, cell proliferation and apoptosis in determining the physiological failure of aging tissues. The diffusion of mitochondrial NO and H_2O_2 depends on the mitochondrial metabolic state, with rates that are different in state 4 and in state 3. In the case of NO diffusion there is an exponential dependence on the membrane potential and in the case of H_2O_2 diffusion the process is regulated by the redox state of the respiratory chain. Moreover, the regulation of mtNOS activity by membrane potential makes mtNOS a regulable enzyme that in turn regulates mitochondrial O_2 uptake and H_2O_2 production. The altered signaling by mitochondrial NO and H_2O_2 appears as a likely explanation for the decreased mitochondrial biogenesis in aging, a process that certainly contributes to cellular energy deficits, apoptosis, and tissue physiological failure in aging.

GRANTS

This study was supported by Grants FIS PI021354 and FIS PI050636 from Ministerio de Sanidad y Consumo de España, Fondo de Investigación Sanitaria, Instituto de Salud Carlos III; by Plan Andaluz de Investigación 2003–2005 (CTS-194) of Spain, and by Grant B-027 from the University of Buenos Aires, Agencia Nacional de Promoción Científica y Tecnológica Grant PIP 2271-00, and Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina Grant PIP 6320-05.

REFERENCES

1. Ames BN, Atamna H, Killilea DW. Mineral and vitamin deficiencies can accelerate the mitochondrial decay of aging. *Mol Aspects Med* 26: 363–378, 2005.
2. Antunes F, Boveris A, Cadenas E. On the mechanism and biology of cytochrome oxidase inhibition by nitric oxide. *Proc Natl Acad Sci USA* 101: 16774–16779, 2004.
3. Armeni T, Principato G, Quiles JL, Pieri C, Bompadre S, Battino M. Mitochondrial dysfunctions during aging: vitamin E deficiency or caloric restriction—two different ways of modulating stress. *J Bioenerg Biomembr* 35: 181–191, 2003.
4. Beckman JS. Ischaemic injury mediator. *Nature* 345: 27–28, 1990.
5. Beckman KB, Ames BN. The free radical theory of aging matures. *Physiol Rev* 78: 547–581, 1998.
6. Bejma J, Ji LL. Aging and acute exercise enhance free radical generation in rat skeletal muscle. *J Appl Physiol* 87: 465–470, 1999.
7. Benzi G, Pastoris O, Marzatico F, Villa RF, Dagani F, Curti D. The mitochondrial electron transfer alteration as a factor involved in the brain aging. *Neurobiol Aging* 13: 361–368, 1992.
8. Bertoni-Freddari C, Fattoretti P, Giorgetti B, Solazzi M, Balietti M, Casoli T, Di SG. Cytochrome oxidase activity in hippocampal synaptic

- mitochondria during aging: a quantitative cytochemical investigation. *Ann NY Acad Sci* 1019: 33–36, 2004.
9. **Bickford PC, Shukitt-Hale B, Joseph J.** Effects of aging on cerebellar noradrenergic function and motor learning: nutritional interventions. *Mech Ageing Dev* 111: 141–154, 1999.
 10. **Boffoli D, Scacco SC, Vergari R, Solarino G, Santacroce G, Papa S.** Decline with age of the respiratory chain activity in human skeletal muscle. *Biochim Biophys Acta* 1226: 73–82, 1994.
 11. **Bonini MG, Radi R, Ferrer-Sueta G, Ferreira AM, Augusto O.** Direct EPR detection of the carbonate radical anion produced from peroxynitrite and carbon dioxide. *J Biol Chem* 274: 10802–10806, 1999.
 12. **Boveris A, Cadenas E.** Mitochondrial production of hydrogen peroxide regulation by nitric oxide and the role of ubiquinone. *IUBMB Life* 50: 245–250, 2000.
 13. **Boveris A, Cadenas E, Stoppani AO.** Role of ubiquinone in the mitochondrial generation of hydrogen peroxide. *Biochem J* 156: 435–444, 1976.
 14. **Boveris A, Chance B.** The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem J* 134: 707–716, 1973.
 15. **Boveris A, Costa LE, Cadenas E.** The mitochondrial production of oxygen radicals and cellular aging. In: *Understanding the Process of Aging: the Roles of Mitochondria, Free Radicals, and Antioxidants (Antioxidants in Health and Disease* (vol. 8), edited by Cadenas E and Packer L. New York: Dekker, 1999, p. 1–20.
 16. **Boveris A, Costa LE, Poderoso JJ, Carreras MC, Cadenas E.** Regulation of mitochondrial respiration by oxygen and nitric oxide. *Ann NY Acad Sci* 899: 121–135, 2000.
 17. **Boveris A, Oshino N, Chance B.** The cellular production of hydrogen peroxide. *Biochem J* 128: 617–630, 1972.
 18. **Boyer PD.** ATP synthase—past and future. *Biochim Biophys Acta* 1365: 3–9, 1998.
 19. **Brechue WF, Pollock ML.** Exercise training for coronary artery disease in the elderly. *Clin Geriatr Med* 12: 207–229, 1996.
 20. **Brookes PS.** Mitochondrial nitric oxide synthase. *Mitochondrion* 3: 187–204, 2004.
 21. **Brown GC, Cooper CE.** Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. *FEBS Lett* 356: 295–298, 1994.
 22. **Buchczyk DP, Grune T, Sies H, Klotz LO.** Modifications of glyceraldehyde-3-phosphate dehydrogenase induced by increasing concentrations of peroxynitrite: early recognition by 20S proteasome. *Biol Chem* 384: 237–241, 2003.
 23. **Bustamante J, Di LE, Fernandez-Cobo M, Monti N, Cadenas E, Boveris A.** Kinetic analysis of thapsigargin-induced thymocyte apoptosis. *Free Radic Biol Med* 37: 1490–1498, 2004.
 24. **Cadenas E, Boveris A.** Enhancement of hydrogen peroxide formation by protophores and ionophores in antimycin-supplemented mitochondria. *Biochem J* 188: 31–37, 1980.
 25. **Cadenas E, Boveris A, Ragan CI, Stoppani AO.** Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome c reductase from beef-heart mitochondria. *Arch Biochem Biophys* 180: 248–257, 1977.
 26. **Carafoli E.** Historical review: mitochondria and calcium: ups and downs of an unusual relationship. *Trends Biochem Sci* 28: 175–181, 2003.
 27. **Cassina A, Radi R.** Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport. *Arch Biochem Biophys* 328: 309–316, 1996.
 28. **Cecchini G.** Function and structure of complex II of the respiratory chain. *Annu Rev Biochem* 72: 77–109, 2003.
 29. **Chagnon P, Betard C, Robitaille Y, Cholette A, Gauvreau D.** Distribution of brain cytochrome oxidase activity in various neurodegenerative diseases. *Neuroreport* 6: 711–715, 1995.
 30. **Chance B.** Electron transfer: pathways mechanisms and controls. *Annu Rev Biochem* 46: 967–980, 1977.
 31. **Chance B, Sies H, Boveris A.** Hydroperoxide metabolism in mammalian organs. *Physiol Rev* 59: 527–605, 1979.
 32. **Chance B, Williams GR.** The respiratory chain and oxidative phosphorylation. *Adv Enzymol Relat Subj Biochem* 17: 65–134, 1956.
 33. **Chance B, Williams GR, Hollunger G.** Inhibition of electron and energy transfer in mitochondria. I. Effects of Amytal, thiopental, rotenone, progesterone, and methylene glycol. *J Biol Chem* 238: 418–431, 1963.
 34. **Clapham DE.** Calcium signaling. *Cell* 80: 259–268, 1995.
 35. **Cleeter MW, Cooper JM, Darley-Usmar VM, Moncada S, Schapira AH.** Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases. *FEBS Lett* 345: 50–54, 1994.
 36. **Clementi E, Brown GC, Feelisch M, Moncada S.** Persistent inhibition of cell respiration by nitric oxide: crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. *Proc Natl Acad Sci USA* 95: 7631–7636, 1998.
 37. **Clementi E, Brown GC, Foxwell N, Moncada S.** On the mechanism by which vascular endothelial cells regulate their oxygen consumption. *Proc Natl Acad Sci USA* 96: 1559–1562, 1999.
 38. **Cohen HY, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de CR, Sinclair DA.** Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science* 305: 390–392, 2004.
 39. **Colcombe SJ, Erickson KI, Raz N, Webb AG, Cohen NJ, McAuley E, Kramer AF.** Aerobic fitness reduces brain tissue loss in aging humans. *J Gerontol A Biol Sci Med Sci* 58: M176–M180, 2003.
 40. **Comfort A.** Likelihood of human pheromones. *Nature* 230: 432–433, 1971.
 41. **Cooper CE, Vollaard NB, Choueiri T, Wilson MT.** Exercise, free radicals and oxidative stress. *Biochem Soc Trans* 30: 280–285, 2002.
 42. **Crofts AR.** The cytochrome bc1 complex: function in the context of structure. *Annu Rev Physiol* 66: 689–733, 2004.
 43. **Cutler RG.** Oxidative stress and aging: catalase is a longevity determinant enzyme. *Rejuvenation Res* 8: 138–140, 2005.
 44. **Darley-Usmar V.** The powerhouse takes control of the cell; the role of mitochondria in signal transduction. *Free Radic Biol Med* 37: 753–754, 2004.
 45. **Dauer W, Przedborski S.** Parkinson's disease: mechanisms and models. *Neuron* 39: 889–909, 2003.
 46. **Dawson TM, Dawson VL.** Molecular pathways of neurodegeneration in Parkinson's disease. *Science* 302: 819–822, 2003.
 47. **Dionisi O, Galeotti T, Terranova T, Azzi A.** Superoxide radicals and hydrogen peroxide formation in mitochondria from normal and neoplastic tissues. *Biochim Biophys Acta* 403: 292–300, 1975.
 48. **Drew B, Leeuwenburgh C.** Method for measuring ATP production in isolated mitochondria: ATP production in brain and liver mitochondria of Fischer-344 rats with age and caloric restriction. *Am J Physiol Regul Integr Comp Physiol* 285: R1259–R1267, 2003.
 49. **Elfering SL, Sarkela TM, Giulivi C.** Biochemistry of mitochondrial nitric-oxide synthase. *J Biol Chem* 277: 38079–38086, 2002.
 50. **Emanuel NM.** Free radicals and the action of inhibitors of radical processes under pathological states and ageing in living organisms and in man. *Q Rev Biophys* 9: 283–308, 1976.
 51. **Ferguson M, Mockett RJ, Shen Y, Orr WC, Sohal RS.** Age-associated decline in mitochondrial respiration and electron transport in *Drosophila melanogaster*. *Biochem J* 390: 501–511, 2005.
 52. **Fernandez-Moran H, Oda T, Blair PV, Green DE.** A macromolecular repeating unit of mitochondrial structure and function correlated electron microscopic and biochemical studies of isolated mitochondria and sub-mitochondrial particles of beef heart muscle. *J Cell Biol* 22: 63–100, 1964.
 53. **Finch CE.** *Longevity, Senescence, and the Genome*. Chicago: University Chicago Press, 1990.
 54. **Forster MJ, Dubey A, Dawson KM, Stutts WA, Lal H, Sohal RS.** Age-related losses of cognitive function and motor skills in mice are associated with oxidative protein damage in the brain. *Proc Natl Acad Sci USA* 93: 4765–4769, 1996.
 55. **Franco MC, Arciuch VG, Peralta JG, Galli S, Levisman D, Lopez LM, Romorini L, Poderoso JJ, Carreras MC.** Hypothyroid phenotype is contributed by mitochondrial complex I inactivation due to translocated neuronal nitric-oxide synthase. *J Biol Chem* 281: 4779–4786, 2006.
 56. **Friedrich T, Bottcher B.** The gross structure of the respiratory complex I: a Lego System. *Biochim Biophys Acta* 1608: 1–9, 2004.
 57. **Gerschman R, Gilbert DL, Nye SW, Dwyer P, Fenn WO.** Oxygen poisoning and x-irradiation: a mechanism in common. *Science* 119: 623–626, 1954.
 58. **Ghafourifar P, Richter C.** Nitric oxide synthase activity in mitochondria. *FEBS Lett* 418: 291–296, 1997.
 59. **Ghafourifar P, Schenk U, Klein SD, Richter C.** Mitochondrial nitric-oxide synthase stimulation causes cytochrome c release from isolated

- mitochondria. Evidence for intramitochondrial peroxynitrite formation. *J Biol Chem* 274: 31185–31188, 1999.
60. **Giasson BI, Lee VM.** Are ubiquitination pathways central to Parkinson's disease? *Cell* 114: 1–8, 2003.
 61. **Gilad GM, Gilad VH.** Strain, stress, neurodegeneration and longevity. *Mech Ageing Dev* 78: 75–83, 1995.
 62. **Giulivi C, Poderoso JJ, Boveris A.** Production of nitric oxide by mitochondria. *J Biol Chem* 273: 11038–11043, 1998.
 63. **Gonzalez-Flecha B, Cutrin JC, Boveris A.** Time course and mechanism of oxidative stress and tissue damage in rat liver subjected to in vivo ischemia-reperfusion. *J Clin Invest* 91: 456–464, 1993.
 64. **Gould E, McEwen BS.** Neuronal birth and death. *Curr Opin Neurobiol* 3: 676–682, 1993.
 65. **Green DE, Vande ZH.** On the enzymic mechanism of oxidative phosphorylation. *Proc Natl Acad Sci USA* 79: 1064–1068, 1982.
 66. **Grune T, Merker K, Sandig G, Davies KJ.** Selective degradation of oxidatively modified protein substrates by the proteasome. *Biochem Biophys Res Commun* 305: 709–718, 2003.
 67. **Ha H, Hajek P, Bedwell DM, Burrows PD.** A mitochondrial porin cDNA predicts the existence of multiple human porins. *J Biol Chem* 268: 12143–12149, 1993.
 68. **Hagen TM, Ingersoll RT, Wehr CM, Lykkesfeldt J, Vinarsky V, Bartholomew JC, Song MH, Ames BN.** Acetyl-L-carnitine fed to old rats partially restores mitochondrial function and ambulatory activity. *Proc Natl Acad Sci USA* 95: 9562–9566, 1998.
 69. **Han D, Antunes F, Canali R, Rettori D, Cadenas E.** Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol. *J Biol Chem* 278: 5557–5563, 2003.
 70. **Harman D.** Aging: a theory based on free radical and radiation chemistry. *J Gerontol A Biol Sci Med Sci* 11: 298–300, 1956.
 71. **Harman D.** Free radical theory of aging: an update: increasing the functional life span. *Ann NY Acad Sci* 1067: 10–21, 2006.
 72. **Hartley A, Stone JM, Heron C, Cooper JM, Schapira AH.** Complex I inhibitors induce dose-dependent apoptosis in PC12 cells: relevance to Parkinson's disease. *J Neurochem* 63: 1987–1990, 1994.
 73. **Hatefi Y.** The mitochondrial electron transport and oxidative phosphorylation system. *Annu Rev Biochem* 54: 1015–1069, 1985.
 74. **Hinchliffe P, Sazanov LA.** Organization of iron-sulfur clusters in respiratory complex I. *Science* 309: 771–774, 2005.
 75. **Hofhaus G, Weiss H, Leonard K.** Electron microscopic analysis of the peripheral and membrane parts of mitochondrial NADH dehydrogenase (complex I). *J Mol Biol* 221: 1027–1043, 1991.
 76. **Itoh K, Weis S, Mehraein P, and Muller-Hocker J.** Cytochrome c oxidase defects of the human substantia nigra in normal aging. *Neurobiol Aging* 17: 843–848, 1996.
 77. **Ji LL.** Exercise at old age: does it increase or alleviate oxidative stress? *Ann NY Acad Sci* 928: 236–247, 2001.
 78. **Jiang S, Chow SC, Nicotera P, Orrenius S.** Intracellular Ca²⁺ signals activate apoptosis in thymocytes: studies using the Ca²⁺-ATPase inhibitor thapsigargin. *Exp Cell Res* 212: 84–92, 1994.
 79. **Judge S, Jang YM, Smith A, Selman C, Phillips T, Speakman JR, Hagen T, Leeuwenburgh C.** Exercise by lifelong voluntary wheel running reduces subsarcolemmal and interfibrillar mitochondrial hydrogen peroxide production in the heart. *Am J Physiol Regul Integr Comp Physiol* 289: R1564–R1572, 2005.
 80. **Kanai AJ, Pearce LL, Clemens PR, Birder LA, VanBibber MM, Choi SY, de Groat WC, Peterson J.** Identification of a neuronal nitric oxide synthase in isolated cardiac mitochondria using electrochemical detection. *Proc Natl Acad Sci USA* 98: 14126–14131, 2001.
 81. **Kanski J, Behring A, Pelling J, Schoneich C.** Proteomic identification of 3-nitrotyrosine-containing rat cardiac proteins: effects of biological aging. *Am J Physiol Heart Circ Physiol* 288: H371–H381, 2005.
 82. **Kennedy EP, Lehninger AL.** Oxidation of fatty acids and tricarboxylic acid cycle intermediates by isolated rat liver mitochondria. *J Biol Chem* 179: 957–969, 1949.
 83. **Kissner R, Nauser T, Bugnon P, Lye PG, Koppenol WH.** Formation and properties of peroxynitrite as studied by laser flash photolysis, high-pressure stopped-flow technique, and pulse radiolysis. *Chem Res Toxicol* 10: 1285–1292, 1997.
 84. **Korshunov SS, Skulachev VP, Starkov AA.** High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS Lett* 416: 15–18, 1997.
 85. **Kozlov AV, Szalay L, Umar F, Kropik K, Staniek K, Niedermuller H, Bahrami S, Nohl H.** Skeletal muscles, heart, and lung are the main sources of oxygen radicals in old rats. *Biochim Biophys Acta* 1740: 382–389, 2005.
 86. **Kroemer G, Dallaporta B, and Resche-Rigon M.** The mitochondrial death/life regulator in apoptosis and necrosis. *Annu Rev Physiol* 60: 619–642, 1998.
 87. **Kumaran S, Subathra M, Balu M, Panneerselvam C.** Supplementation of L-carnitine improves mitochondrial enzymes in heart and skeletal muscle of aged rats. *Exp Aging Res* 31: 55–67, 2005.
 88. **LaFrance R, Brustovetsky N, Sherburne C, Delong D, Dubinsky JM.** Age-related changes in regional brain mitochondria from Fischer 344 rats. *Aging Cell* 4: 139–145, 2005.
 89. **Lambert AJ, Wang B, Yardley J, Edwards J, Merry BJ.** The effect of aging and caloric restriction on mitochondrial protein density and oxygen consumption. *Exp Gerontol* 39: 289–295, 2004.
 90. **Lass A, Kwong L, Sohal RS.** Mitochondrial coenzyme Q content and aging. *Biofactors* 9: 199–205, 1999.
 91. **Lehninger AL.** Water uptake and extrusion by mitochondria in relation to oxidative phosphorylation. *Physiol Rev* 42: 467–517, 1962.
 92. **Leloir LF, Munoz JM.** Fatty acid oxidation in the liver. *Biochem J* 33: 734–746, 1939.
 93. **Lenaz G, Bovina C, Castelluccio C, Fato R, Formiggini G, Genova ML, Marchetti M, Pich MM, Pallotti F, Parenti CG, Biagini G.** Mitochondrial complex I defects in aging. *Mol Cell Biochem* 174: 329–333, 1997.
 94. **Lesnefsky EJ, Guduz TI, Moghaddas S, Migita CT, Ikeda-Saito M, Turkaly PJ, Hoppel CL.** Aging decreases electron transport complex III activity in heart interfibrillar mitochondria by alteration of the cytochrome c binding site. *J Mol Cell Cardiol* 33: 37–47, 2001.
 95. **Lin DT, Wu J, Holstein D, Upadhyay G, Rourk W, Muller E, Lechleiter JD.** Ca²⁺ signaling, mitochondria and sensitivity to oxidative stress in aging astrocytes. *Neurobiol Aging* 28: 99–111, 2007.
 96. **Liu J, Killilea DW, Ames BN.** Age-associated mitochondrial oxidative decay: improvement of carnitine acetyltransferase substrate-binding affinity and activity in brain by feeding old rats acetyl-L-carnitine and/or R- α -lipoic acid. *Proc Natl Acad Sci USA* 99: 1876–1881, 2002.
 97. **Lopez-Lluch G, Hunt N, Jones B, Zhu M, Jamieson H, Hilmer S, Cascajo MV, Allard J, Ingram DK, Navas P, de CR.** Calorie restriction induces mitochondrial biogenesis and bioenergetic efficiency. *Proc Natl Acad Sci USA* 103: 1768–1773, 2006.
 98. **Manczak M, Jung Y, Park BS, Partovi D, Reddy PH.** Time-course of mitochondrial gene expressions in mice brains: implications for mitochondrial dysfunction, oxidative damage, and cytochrome c in aging. *J Neurochem* 92: 494–504, 2005.
 99. **Mander P, Borutaite V, Moncada S, Brown GC.** Nitric oxide from inflammatory-activated glia synergizes with hypoxia to induce neuronal death. *J Neurosci Res* 79: 208–215, 2005.
 100. **Mann VM, Cooper JM, Krige D, Daniel SE, Schapira AH, Marsden CD.** Brain, skeletal muscle and platelet homogenate mitochondrial function in Parkinson's disease. *Brain* 115: 333–342, 1992.
 101. **Martinez M, Ferrandiz ML, De Juan E, Miquel J.** Age-related changes in glutathione and lipid peroxide content in mouse synaptic mitochondria: relationship to cytochrome c oxidase decline. *Neurosci Lett* 170: 121–124, 1994.
 102. **McArdle A, Vasilaki A, Jackson M.** Exercise and skeletal muscle ageing: cellular and molecular mechanisms. *Aging Res Rev* 1: 79–93, 2002.
 103. **McCarter RJM.** Aging. In: *Handbook of Physiology*. Oxford, UK: Oxford University Press, 1995, chapt. 11.
 104. **McCormack JG, Halestrap AP, Denton RM.** Role of calcium ions in regulation of mammalian intramitochondrial metabolism. *Physiol Rev* 70: 391–425, 1990.
 105. **Meister A.** Mitochondrial changes associated with glutathione deficiency. *Biochim Biophys Acta* 1271: 35–42, 1995.
 106. **Mitchell P, Moyle J.** Stoichiometry of proton translocation through the respiratory chain and adenosine triphosphatase systems of rat liver mitochondria. *Nature* 208: 147–151, 1965.
 107. **Mitchell P, Moyle J.** Estimation of membrane potential and pH difference across the cristae membrane of rat liver mitochondria. *Eur J Biochem* 7: 471–484, 1969.
 108. **Mizuno Y, Ohta S, Tanaka M, Takamiya S, Suzuki K, Sato T, Oya H, Ozawa T, Kagawa Y.** Deficiencies in complex I subunits of the respiratory chain in Parkinson's disease. *Biochem Biophys Res Commun* 163: 1450–1455, 1989.

109. Muller-Hocker J, Schafer S, Link TA, Possekel S, Hammer C. Defects of the respiratory chain in various tissues of old monkeys: a cytochemical-immunocytochemical study. *Mech Ageing Dev* 86: 197–213, 1996.
110. Nakahara H, Kanno T, Inai Y, Utsumi K, Hiramatsu M, Mori A, Packer L. Mitochondrial dysfunction in the senescence accelerated mouse (SAM). *Free Radic Biol Med* 24: 85–92, 1998.
111. Napoleone P, Bronzetti E, Amenta F. Enzyme histochemistry of aging rat kidney. *Mech Ageing Dev* 61: 187–195, 1991.
112. Navarro A, Boveris A. Rat brain and liver mitochondria develop oxidative stress and lose enzymatic activities on aging. *Am J Physiol Regul Integr Comp Physiol* 287: R1244–R1249, 2004.
113. Navarro A, Gomez C, Lopez-Cepero JM, Boveris A. Beneficial effects of moderate exercise on mice aging: survival, behavior, oxidative stress, and mitochondrial electron transfer. *Am J Physiol Regul Integr Comp Physiol* 286: R505–R511, 2004.
114. Navarro A, Gomez C, Sanchez-Pino MJ, Gonzalez H, Bandez MJ, Boveris AD, Boveris A. Vitamin E at high doses improves survival, neurological performance, and brain mitochondrial function in aging male mice. *Am J Physiol Regul Integr Comp Physiol* 289: R1392–R1399, 2005.
115. Navarro A, Sanchez-Pino MJ, Gomez C, Peralta JL, Boveris A. Behavioral dysfunction, brain oxidative stress, and impaired mitochondrial electron transfer in aging mice. *Am J Physiol Regul Integr Comp Physiol* 282: R985–R992, 2002.
116. Navarro A, Sanchez-Pino MJ, Gomez C, Bandez MJ, Cadenas E, Boveris A. Dietary thioproline decreases spontaneous food intake and increases survival and neurological function in mice. *Antioxid Redox Signal* 9: 131–191, 2007.
117. Navarro A, Torrejon R, Bandez MJ, Lopez-Cepero JM, Boveris A. Mitochondrial function and mitochondria-induced apoptosis in an over-stimulated rat ovarian cycle. *Am J Physiol Endocrinol Metab* 289: E1101–E1109, 2005.
118. Navarro-Arevalo A, Canavate C, Sanchez-Pino MJ. Myocardial and skeletal muscle aging and changes in oxidative stress in relationship to rigorous exercise training. *Mech Ageing Dev* 108: 207–217, 1999.
119. Nicoletti VG, Marino VM, Cuppari C, Licciardello D, Patti D, Purrello VS, Stella AM. Effect of antioxidant diets on mitochondrial gene expression in rat brain during aging. *Neurochem Res* 30: 737–752, 2005.
120. Nisoli E, Falcone S, Tonello C, Cozzi V, Palomba L, Fiorani M, Pisconti A, Brunelli S, Cardile A, Francolini M, Cantoni O, Carruba MO, Moncada S, Clementi E. Mitochondrial biogenesis by NO yields functionally active mitochondria in mammals. *Proc Natl Acad Sci USA* 101: 16507–16512, 2004.
121. Nohl H, Gille L, Staniek K. Intracellular generation of reactive oxygen species by mitochondria. *Biochem Pharmacol* 69: 719–723, 2005.
122. Ochoa JJ, Quiles JL, Huertas JR, Mataix J. Coenzyme Q10 protects from aging-related oxidative stress and improves mitochondrial function in heart of rats fed a polyunsaturated fatty acid (PUFA)-rich diet. *J Gerontol A Biol Sci Med Sci* 60: 970–975, 2005.
123. Okado-Matsumoto A, Fridovich I. Subcellular distribution of superoxide dismutases (SOD) in rat liver: Cu,Zn-SOD in mitochondria. *J Biol Chem* 276: 38388–38393, 2001.
124. Okatani Y, Wakatsuki A, Reiter RJ. Melatonin protects hepatic mitochondrial respiratory chain activity in senescence-accelerated mice. *J Pineal Res* 32: 143–148, 2002.
125. Orr WC, Sohal RS. Extension of life-span by overexpression of superoxide dismutase and catalase in *Drosophila melanogaster*. *Science* 263: 1128–1130, 1994.
126. Palade GE. Electron microscopy of mitochondria and other cytoplasmic structures. In: *Enzymes: Units of Biological Structure and Function*, edited by Gaebler OH. New York: Academic, 1956, p. 185.
127. Parker WD Jr, Boyson SJ, Luder AS and Parks JK. Evidence for a defect in NADH: ubiquinone oxidoreductase (complex I) in Huntington's disease. *Neurology* 40: 1231–1234, 1990.
128. Poderoso JJ, Carreras MC, Lisdero C, Riobo N, Schopfer F, Boveris A. Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particles. *Arch Biochem Biophys* 328: 85–92, 1996.
129. Poderoso JJ, Lisdero C, Schopfer F, Riobo N, Carreras MC, Cadenas E, Boveris A. The regulation of mitochondrial oxygen uptake by redox reactions involving nitric oxide and ubiquinol. *J Biol Chem* 274: 37709–37716, 1999.
130. Pullman ME, Penefsky H, Racker E. A soluble protein fraction required for coupling phosphorylation to oxidation in submitochondrial fragments of beef heart mitochondria. *Arch Biochem Biophys* 76: 227–230, 1958.
131. Radak Z, Naito H, Kaneko T, Tahara S, Nakamoto H, Takahashi R, Cardozo-Pelaez F, Goto S. Exercise training decreases DNA damage and increases DNA repair and resistance against oxidative stress of proteins in aged rat skeletal muscle. *Pflügers Arch* 445: 273–278, 2002.
132. Radi R, Cassina A, Hodara R, Quijano C, Castro L. Peroxynitrite reactions and formation in mitochondria. *Free Radic Biol Med* 33: 1451–1464, 2002.
133. Radi R, Turrens JF, Chang LY, Bush KM, Crapo JD, Freeman BA. Detection of catalase in rat heart mitochondria. *J Biol Chem* 266: 22028–22034, 1991.
134. Rao G, Xia E, Nadakavukaren MJ, Richardson A. Effect of dietary restriction on the age-dependent changes in the expression of antioxidant enzymes in rat liver. *J Nutr* 120: 602–609, 1990.
135. Riobo NA, Clementi E, Melani M, Boveris A, Cadenas E, Moncada S, Poderoso JJ. Nitric oxide inhibits mitochondrial NADH:ubiquinone reductase activity through peroxynitrite formation. *Biochem J* 359: 139–145, 2001.
136. Robertson JD, Zhivotovsky B, Gogvadze V, Orrenius S. Outer mitochondrial membrane permeabilization: an open-and-shut case? *Cell Death Differ* 10: 485–487, 2003.
137. Rubbo H, Radi R, Anselmi D, Kirk M, Barnes S, Butler J, Eiserich JP, Freeman BA. Nitric oxide reaction with lipid peroxyl radicals spares alpha-tocopherol during lipid peroxidation. Greater oxidant protection from the pair nitric oxide/alpha-tocopherol than alpha-tocopherol/ascorbate. *J Biol Chem* 275: 10812–10818, 2000.
138. Sanz A, Caro P, Ibanez J, Gomez J, Gredilla R, Barja G. Dietary restriction at old age lowers mitochondrial oxygen radical production and leak at complex I and oxidative DNA damage in rat brain. *J Bioenerg Biomembr* 37: 83–90, 2005.
139. Sastre J, Millan A, Garcia dIA, Pla R, Juan G, Pallardo O'Connor E, Martin JA, Droy-Lefaix MT, Vina J. A Ginkgo biloba extract (EGb 761) prevents mitochondrial aging by protecting against oxidative stress. *Free Radic Biol Med* 24: 298–304, 1998.
140. Schafer E, Seelert H, Reifschneider NH, Krause F, Dencher NA, Vonck J. Architecture of active mammalian respiratory chain supercomplexes. *J Biol Chem* 281: 15370–15375, 2006.
141. Schriener SE, Linford NJ, Martin GM, Treuting P, Ogburn CE, Emond M, Coskun PE, Ladiges W, Wolf N, Van RH, Wallace DC, Rabinovitch PS. Extension of murine life span by overexpression of catalase targeted to mitochondria. *Science* 308: 1909–1911, 2005.
142. Shelke RR, Leeuwenburgh C. Lifelong caloric restriction increases expression of apoptosis repressor with a caspase recruitment domain (ARC) in the brain. *FASEB J* 17: 494–496, 2003.
143. Sherer TB, Trimmer PA, Borland K, Parks JK, Bennett JP Jr., Tuttle JB. Chronic reduction in complex I function alters calcium signaling in SH-SY5Y neuroblastoma cells. *Brain Res* 891: 94–105, 2001.
144. Shi T, Wang F, Stieren E, Tong Q. SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes. *J Biol Chem* 280: 13560–13567, 2005.
145. Shigenaga MK, Hagen TM, Ames BN. Oxidative damage and mitochondrial decay in aging. *Proc Natl Acad Sci USA* 91: 10771–10778, 1994.
146. Sjostrand FS. The ultrastructure of mitochondria; the ultrastructure of the ground substance of the cytoplasm. In: *Fine Structure of Cells*. New York: Interscience, 1955, p. 16–222.
147. Sohal RS, Weindruch R. Oxidative stress caloric restriction, aging. *Science* 273: 59–63, 1996.
148. St-Pierre J, Buckingham JA, Roebuck SJ, Brand MD. Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J Biol Chem* 277: 44784–44790, 2002.
149. Stadtman ER. Importance of individuality in oxidative stress and aging. *Free Radic Biol Med* 33: 597–604, 2002.
150. Turrens JF, Boveris A. Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem J* 191: 421–427, 1980.
151. Valdez LB, Alvarez S, Arnaiz SL, Schopfer F, Carreras MC, Poderoso JJ, Boveris A. Reactions of peroxynitrite in the mitochondrial matrix. *Free Radic Biol Med* 29: 349–356, 2000.

152. Valdez LB, Zaobornyj T, Boveris A. Mitochondrial metabolic states and membrane potential modulate mtNOS activity. *Biochim Biophys Acta* 1757: 166–172, 2006.
153. Vina J, Borras C, Gambini J, Sastre J, Pallardo FV. Why females live longer than males? Importance of the upregulation of longevity-associated genes by oestrogenic compounds. *FEBS Lett* 579: 2541–2545, 2005.
154. Vina J, Sastre J, Pallardo F, Borras C. Mitochondrial theory of aging: importance to explain why females live longer than males. *Antioxid Redox Signal* 5: 549–556, 2003.
155. Walker JE. The NADH:ubiquinone oxidoreductase (complex I) of respiratory chains. *Q Rev Biophys* 25: 253–324, 1992.
156. Walker JE, Collinson IR, van Raaij MJ, Runswick MJ. Structural analysis of ATP synthase from bovine heart mitochondria. *Methods Enzymol* 260: 163–190, 1995.
157. Weindruch R. The retardation of aging by caloric restriction: studies in rodents and primates. *Toxicol Pathol* 24: 742–745, 1996.
158. Yan L, Ge H, Li H, Lieber SC, Natividad F, Resuello RR, Kim SJ, Akeju S, Sun A, Loo K, Peppas AP, Rossi F, Lewandowski ED, Thomas AP, Vatner SF, Vatner DE. Gender-specific proteomic alterations in glycolytic and mitochondrial pathways in aging monkey hearts. *J Mol Cell Cardiol* 37: 921–929, 2004.
159. Yan LJ, Sohal RS. Mitochondrial adenine nucleotide translocase is modified oxidatively during aging. *Proc Natl Acad Sci USA* 95: 12896–12901, 1998.
160. Yarian CS, Toroser D, Sohal RS. Aconitase is the main functional target of aging in the citric acid cycle of kidney mitochondria from mice. *Mech Ageing Dev* 127: 79–84, 2006.
161. Zaobornyj T, Valdez LB, La PP, Costa LE, Boveris A. Effect of sustained hypobaric hypoxia during maturation and aging on rat myocardium. II. mtNOS activity. *J Appl Physiol* 98: 2370–2375, 2005.

