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Forum Review

New Insights Into Structure and Function of Mitochondria and Their Role in Aging and Disease

GIORGIO LENAZ, ALESSANDRA BARACCA, ROMANA FATO, MARIA LUISA GENOVA, and GIANCARLO SOLAINI

ABSTRACT

This review covers some novel findings on mitochondrial biochemistry and discusses diseases due to mitochondrial DNA mutations as a model of the changes occurring during physiological aging. The random collision model of organization of the mitochondrial respiratory chain has been recently challenged on the basis of findings of supramolecular organization of respiratory chain complexes. The source of superoxide in Complex I is discussed on the basis of laboratory experiments using a series of specific inhibitors and is presumably iron sulfur center N2. Maternally inherited diseases due to mutations of structural genes in mitochondrial DNA are surveyed as a model of alterations mimicking those occurring during normal aging. The molecular defects in senescence are surveyed on the basis of the "Mitochondrial Theory of Aging", establishing mitochondrial DNA somatic mutations, caused by accumulation of oxygen radical damage, to be at the basis of cellular senescence. Mitochondrial production of reactive oxygen species increases with aging and mitochondrial DNA mutations and deletions accumulate and may be responsible for oxidative phosphorylation defects. Evidence is presented favoring the mitochondrial theory, with primary mitochondrial alterations, although the problem is made more complex by changes in the cross-talk between nuclear and mitochondrial DNA. *Antioxid. Redox Signal.* 8, 417–437.

INTRODUCTION

The involvement of mitochondria in a variety of pathological aspects and in aging (14, 67, 91, 138) has provided renewed interest in these cellular organelles. According to the mitochondrial theory of aging (138, 153, 177), somatic mutations of mitochondrial DNA (mtDNA) cause a progressive energy decline leading cells to senescence and death.

Mitochondria are known to be at the same time strong producers of Reactive Oxygen Species (ROS) and particularly susceptible to damage by their action on lipids, proteins and DNA (78, 79, 138). In particular, damage to mtDNA would induce damage to the polypeptides encoded by mtDNA in the respiratory complexes, with consequent decrease of electron transfer, leading to further production of ROS, and thus establishing a vicious circle of oxidative stress and energetic decline (144, 193). This fall of mitochondrial energetic capacity is considered to be the cause of aging and age-related degenerative diseases (67, 138). This vicious circle might be broken by agents capable to prevent a chain reaction of ROS formation and damage.

Genetic diseases due to hereditary mutations of mtDNA (mitochondrial cytopathies) may represent a useful model for the lesions that occur in aging.

Mitochondrial cytopathies (or encephalomyopathies) caused by mutations of mitochondrial DNA are well-known genetic and clinical entities, but their biochemical pathogenic mechanisms are often obscure (124, 142, 181). The reason may be in the presence of still unknown concomitant genetic and environmental factors, but also in insufficient knowledge of some basic structural and functional aspects of the mitochondrial respiratory chain. In particular the supramolecular

Dipartimento di Biochimica, Università di Bologna, Bologna, Italy.

organization of the chain (random or supercomplex) and the factors affecting formation of ROS may have deep implications in the pathogenesis of mitochondrial diseases.

Diagnosis of mitochondrial diseases is based on biochemical, morphological, and molecular genetic analyses of blood, muscle, or skin biopsies. Because it is often difficult to obtain enough material to study pathogenic mechanisms, patient-derived cell culture models have been used to establish bioenergetic deficits and to analyze the biochemical phenotypes. A cell culture model most extensively used for the study of mitochondrial disorders is the cybrid (cytoplasmic hybrids) system, first described by King and Attardi (123). Cybrids are generated by fusing enucleated cytoplasts from patients' cells harboring mtDNA mutations with mtDNA-less (ρ^o) cells, grown under selection, and subcloned. This technique permits the analysis of cybrids that are isogenic for mitochondrial DNA and have a neutral nuclear background. Cybrids may contain not only homoplasmic wild type and homoplasmic mutant mtDNA, but also different percentages of mutant molecules from the same patient. Several mtDNA mutations have been studied and characterized using this system (cf. 194).

STRUCTURAL ORGANIZATION OF THE MITOCHONDRIAL RESPIRATORY CHAIN

Two models exist of the organization of the mitochondrial respiratory chain (140): the model of a random distribution of mitochondrial complexes, with electron transfer ensured by collisional interactions of small connecting molecules (coenzyme Q and cytochrome *c*); and a model of stable supramolecular specific aggregates of respiratory complexes. The former model was favored until recently, when Schägger (214) found structural evidence by Blue-Native electrophoresis of specific aggregations, and introduced the model of the "respirasome." Concomitantly, biochemical evidence for homooligomeric ATP synthase competent for ATP hydrolytic activity has been provided (126) and recently specific associations of ATP synthase with other OXPHOS components building up "ATP synthasomes" have been proposed (47).

Although the structural evidence for the existence of specific aggregations increases (72, 81, 201), there is very little functional evidence of the existence of supercomplexes, if we except a study in yeast (22) showing that the pool function of ubiquinone (127, 139, 140) investigated by inhibitor titration was lost upon addition of chaotropic agents.

We have tested the model kinetically by exploiting flux control analysis that studies the extent of control that an enzyme exerts on a metabolic pathway (119). The flux control coefficient represents the fractional change of total activity of a pathway induced by a fractional change of the individual enzyme in the pathway. Such a change is usually obtained by stepwise addition of a specific inhibitor. A flux control coefficient approaching 1 means that the change of the individual step induces the same change on the whole pathway, so the enzyme is totally rate limiting; a flux control coefficient approaching zero means that the enzyme has no control on the whole pathway and its activity is in large excess.

A more evident way of showing flux control is by means of threshold plots (210), where the residual activity of the total pathway is plotted as a function of the extent of inhibition of the individual step.

We have applied flux control analysis on each segment of the respiratory chain using specific inhibitors of each Complex. In open bovine heart submitochondrial particles (SMP) (82, 97) where the system is simplified by the absence of membrane potential and ATP synthesis and by the lack of requirement of the carrier systems for substrates, we surprisingly found that both Complex I and Complex III are almost completely controlling aerobic NADH oxidation (18, 19). Threshold plots of NADH oxidase in bovine heart submitochondrial particles are shown in Figure 1. The NADH oxidase activity is plotted as a function of stepwise inhibition of Complex I by rotenone, of Complex III by mucidin and of Complex IV by KCN.

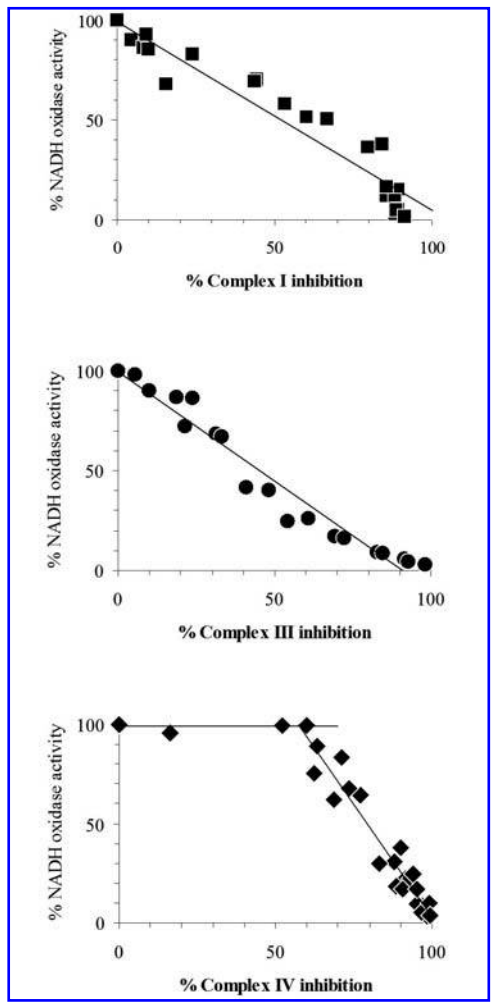

FIG. 1. Threshold plots of residual NADH oxidase activity versus percent inhibition. Complex I *(top)*, Complex III *(middle)*, and Complex IV activity *(bottom)* in bovine heart submitochondrial particles; the individual complexes were inhibited by stepwise addition of rotenone, mucidin, and KCN, respectively.

It clearly appears that a linear correlation exists between the residual NADH oxidation and both Complex I and Complex III inhibition, whereas Complex IV does not affect NADH oxidation until 60% inhibition of cytochrome oxidase has occurred. The most reasonable explanation is that Complex I and Complex III behave as a single enzyme (140), forming a supercomplex with metabolic channeling (i.e., direct interaction without diffusion-coupled steps) of the connecting intermediate, coenzyme Q. This is contrary to the common view of electron transfer (93, 137), but is in line with the recent structural findings by Schägger (214). Differing with that author, however, only Complex I and III appear to form a supercomplex, while Complex IV seems to behave independently. On the other hand, Complex II is rate limiting over succinate oxidation, but there appears to be no channeling between Complexes II and III.

Preliminary results indicate that treating the particles with a chaotropic agent (50 m*M* trichloroacetate adjusted to neutral pH) can affect the rate-limiting step at Complex III, thereby disrupting its interaction with Complex I. The arrangement of the respiratory chain in the inner membrane according to the above results is shown in Figure 2.

Can we reconcile the extensive evidence formerly gained in favor of the pool function of CoQ between Complexes I and III with the more recent evidence favoring the existence of supercomplexes? The subject has been discussed in a previous review (140) where it was reasoned that the pool equation of Kröger and Klingenberg (127), establishing the total observed rate of electron transfer (V_{obs}) as a function of rate of reduction (V_{red}) and of reoxidation (V_{ox}) of the CoQ pool,

$$V_{obs} = V_{red} \cdot V_{ox} / (V_{red} + V_{ox})$$

can be verified only for comparable rates of CoQ reduction and oxidation: in such case the resulting total rate is much lower than the individual rates, otherwise the observed total rate approaches that of the slower process and the result could not be distinguished from the kinetics when channeling occurs in a supercomplex.

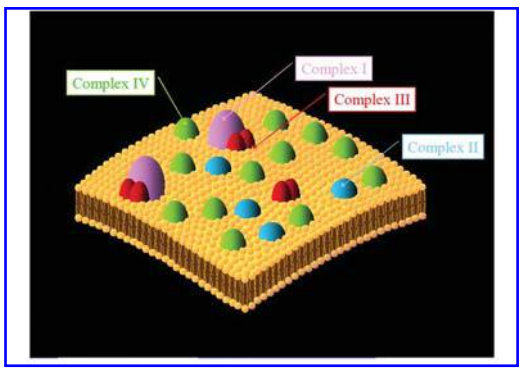

FIG. 2. A model of the respiratory chain, as viewed from the matrix surface of the inner mitochondrial membrane. The presence of supramolecular specific aggregates between Complex I and III is depicted according to the studies reported by Bianchi *et al.* (19).

A stable supercomplex consisting of Complex I and dimeric Complex III was recently purified from plant mitochondria (72): structural characterization by single-particle electron microscopy revealed that the ubiquinone-binding domain in Complex I does not appear to be in close proximity with the Complex I-Complex III interphase; although the authors exclude direct CoQ channeling, they postulate that a rate advantage must derive from supercomplex formation.

In a previous report (18) we demonstrated that dilution with phospholipids of a mitochondrial fraction enriched in Complexes I and III determines adherence to pool behavior for CoQ but only at dilution protein:phospholipids higher than 1:5, whereas at lower phospholipids content the turnover of NADH cytochrome c reductase is higher than expected by the pool equation.

This means that membrane lipids may modulate intercomplex aggregation; it is not unlikely that besides the quantity of lipids also their composition may affect supercomplex formation. It has been shown that cardiolipin is required for specific aggregation between complexes (260); a decrease in cardiolipin content, such as due to peroxidation by mitochondrial ROS production following decreased electron transfer of Complex I (196) and Complex III (200), might deeply affect intercomplex assembly. Indeed, preliminary results from our laboratory show that peroxidation of the lipids used to reconstitute the mitochondrial fraction enriched in Complexes I and III (see above) induces disaggregation of the Complexes even at low lipid to protein ratios.

The issue of the organization of the respiratory chain is not trivial in concern of mitochondrial diseases: Schägger (213) has recently published evidence that mutations of Complex III affect the assembly and stability of Complex I subunits.

Moreover the different tissue distribution of pathological aspects of mitochondrial cytopathies has been attributed, besides to different degrees of heteroplasmy, also to different extents of flux control by individual complexes of oxidative phosphorylation (129); in view of the results discussed above, it is tempting to speculate that different supercomplex organizations may be present in individual tissues. We have data available only in a plant mitochondrial system. Unpublished results of M.L. Genova and C. Bianchi from our laboratory, in collaboration with H.P. Braun (Hannover, Germany) showed that in potato tuber mitochondria both Complex III and Complex IV are completely rate-limiting for respiratory electron transfer, suggesting that they are linked in a supercomplex. Unfortunately in these mitochondria flux control analysis of Complex I could not be achieved because of the low rotenone sensitivity of total NADH-CoQ reductase activity, but structural studies reported that Complex I is assembled with both Complex III and IV (81).

MITOCHONDRIAL ROS GENERATION

Within a cell, mitochondria largely contribute to the production of ROS via the respiratory chain (138, 141). The relevance of mitochondrial production of ROS within a cell is indirectly revealed by the results of deficiency of mitochondrial antioxidant enzymes. Mitochondria contain an isozyme of superoxide dismutase (SOD-2, Mn-SOD) and glutathione per-

oxidase (GPx). In the matrix Mn-SOD forms hydrogen peroxide that may be converted to water by glutathione peroxidase; reduced GSH is regenerated by glutathione reductase (GR) in presence of NADPH formed by transhydrogenase reaction. This reaction is essential, since H_2O_2 in excess would be converted into the damaging hydroxyl radical by the Fenton reaction (141). The lack of SOD-2 (171) and of mitochondrial GPx (80) is deleterious to cells.

It is now recognized that the major sites of ROS production are within Complexes I and III (141). If we except few reports (48, 154), most authors consider Complex I rather than Complex III as the main source of ROS (128, 130, 248). In addition, also glycerol-3-phosphate dehydrogenase has been shown to be a major source of ROS (70).

In Complex I, superoxide can be generated during direct electron transfer and during reverse electron transfer in presence of high membrane potential, as shown by the stimulation by rotenone in the former case and by inhibition in the latter (45, 88, 106, 130, 169). Superoxide produced by Complex I is presumably released in the matrix.

In Complex III, antimycin induces strong stimulation of superoxide generation, and the activation is inhibited by myxothiazol and other center o inhibitors: in this case the site responsible for superoxide generation must be in center o or P side, and it is probably released in the intermembrane space (45, 183, 244).

Superoxide generated by mitochondria is disposed by mitochondrial and extramitochondrial enzymes. Under physiological conditions mitochondria may efficiently catalyze net H_2O_2 removal rather than its production (261). Alternatively, the excess H_2O_2 may be exported out of the mitochondrion and converted to water by external GPx and GR, with NADPH largely formed by the pentose pathway (211).

Superoxide formed in the intermembrane space may exit via the voltage-dependent anion channel (VDAC) and be converted into H_2O_2 by extramitochondrial SOD (96), then processed as above (Fig. 3). Any unbalance in the delicate pattern of antioxidant enzymes may lead to excess radical production and oxidative stress (67).

The first site of damage of the ROS produced by mitochondria is the mitochondrion itself: superoxide dismutates to H_2O_2 and the hydroxyl radical produced by H_2O_2 in presence of reduced metal ions may damage several biomolecules in the inner membrane and in the matrix (138). Another important effect of ROS is induction of permeability transition by opening the cyclosporin-sensitive pore in the inner membrane (16, 85, 120, 199).

An important question is whether free radicals produced by mitochondria are also physiologically released to the cytosol. Staniek and Nohl (234) applied a noninvasive detecting system for hydrogen peroxide and found that isolated intact rat heart mitochondria do not produce detectable H_2O_2, unless when using succinate in presence of antimycin. Korshunov *et al.* (125) also found no hydrogen peroxide formation by intact rat heart mitochondria, unless pretreated in such a way to deplete them of endogenous antioxidants. It may be inferred that under normal conditions ROS are not exported out of mitochondria, due to the high efficiency of the enzymatic protecting systems. Nevertheless, it has been shown that glutathione peroxidase and glutathione reductase are inhibited by low calcium concentrations in the physiological range (261): it is therefore likely that even physiological oscillations in intramitochondrial calcium concentration may condition the export of ROS from mitochondria. There is overwhelming evidence that ROS production detected in different cells under pathological conditions has a mitochondrial origin.

It is not easy to demonstrate that ROS detected in cells are produced by mitochondria; the effect of respiratory inhibitors appears to be a useful way to discriminate between mitochon-

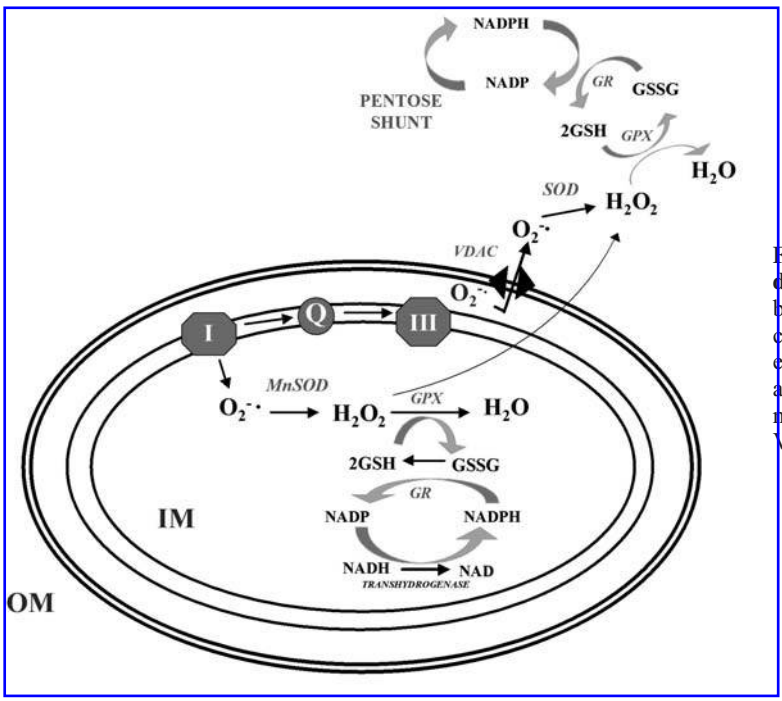

FIG. 3. Fate of ROS produced in the mitochondrion. OM, outer membrane; IM, inner membrane; Q, ubiquinone; I and III are the respective complexes of the respiratory chain (see text for explanations and abbreviations). H_2O_2 is depicted as freely permeable across the mitochondrial membranes, while $O_2^{-\cdot}$ is transported by the VDAC channel.

drial and nonmitochondrial ROS. However, the effect of inhibitors is ambiguous. Although antimycin is usually found to stimulate ROS production (23) in intact cells, as it does in mitochondria, the effect of rotenone is contradictory. Some studies showed that rotenone enhances ROS production in intact cells (13, 149), whereas others showed inhibition of cellular ROS production by the same inhibitor (150, 169, 249). We found that lymphocytes or HL60 cells produce less peroxide in the presence of rotenone than in noninhibited conditions, whereas papillary thyroid carcinoma cells produce more peroxide under the same conditions (A. Biondi and G. Lenaz, unpublished results). Rotenone decreases ROS production by Complex III while enhancing ROS production by Complex I and the relative contribution of the two Complexes to ROS production may vary in different cells. Therefore, if ROS production by Complex III is relatively high, rotenone inhibition would decrease total ROS production, whereas if ROS production by Complex III is low, then enhancement of ROS release by Complex I would prevail and total ROS would be increased (Fig. 4). Since ROS production by reverse flux of electrons is decreased by rotenone (130), another critical point may be represented by membrane potential and the contribution of reverse electron transfer in Complex I. Succinate-driven reverse electron transfer through Complex I is actually considered the main source of ROS in intact mitochondria (128, 261).

Mitochondrial ROS production is enhanced in State 4 and when the rate of electron transfer is lowered (222). The rationale is in a more reduced state of the respiratory carriers capable of donating electrons to oxygen. To this purpose uncoupling and release of excessive membrane proton potential may protect mitochondria from damage due to excessive free radical production. In rat hepatocytes the futile cycle of proton pumping and proton leak may be responsible for 20%–25% of respiration (24); in perfused rat muscle the value is even greater. Uncoupling may be obtained by activating proton leak through uncoupling proteins (251). In such way a tissue may dissipate a conspicuous part of the energy conserved by its mitochondria, however in such a way it keeps the mitochondrial respiratory chain under more oxidized conditions preventing the formation of damaging free radicals. Murine endothelial cells pretreated with antisense oligonucleotides directed against UCP-2 mRNA exhibited a significant and specific increase in membrane potential and intracellular ROS level (74). Conversely, superoxide was found to activate proton transport through uncoupling proteins (75). Both exogenously generated superoxide (75) and endogenous superoxide (236) can activate proton conductance via UCP3. It has been proposed that the main ancestral function of UCPs is to generate mild uncoupling thus preserving from oxidative damage (25).

STRUCTURE AND FUNCTION OF COMPLEX I AND ITS ROLE IN ROS GENERATION

Complex I (NADH coenzyme Q oxidoreductase) is a very large enzyme catalyzing the first step of the mitochondrial electron transport chain (220).The total number of subunits in the bovine enzyme is 46 (44) for a molecular mass of about 1000 KDa. Seven subunits are the products of the mitochondrial genome (49) and correspond to hydrophobic components named ND1-ND6 and ND4L. The molecular mechanism of catalysis of this enzyme is not completely understood due to lack of detailed structural information (167). Electron microscopy of Complex I from different organisms has shown an L-shaped structure with a hydrophobic arm residing in the membrane and a peripheral arm protruding into the mitochondrial matrix space (69). The minimal active form of the enzyme found in bacteria is composed of 14 subunits, all of which are homologous to their mitochondrial counterparts.

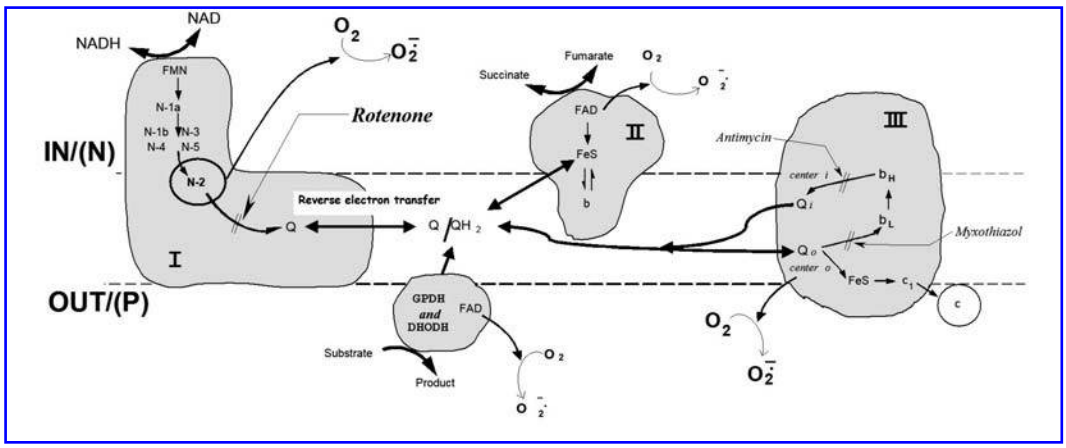

FIG. 4. Summarizing scheme of the sites of superoxide production by the mitochondrial respiratory chain. The sidedness of superoxide release is discussed in the text. I, II, III are the respective complexes, DHODH is dehydroorotate dehydrogenase, GPDH is glycerophosphate dehydrogenase. The production of ROS by the respiratory Complexes I and III may vary in different types of cells. Rotenone inhibition of Complex I enhances ROS formation by Complex I by direct electron transfer but inhibits ROS generation by Complex III and by reverse electron transfer through Complex I. The resulting effect of rotenone may be an increase or a decrease of ROS formation, depending on their prevailing origin.

Analysis of the midpoint potential of the redox active groups suggests that FMN is the entry point for electrons from NADH that are transferred to the iron–sulfur clusters. Enzymes from different sources have different numbers of iron–sulfur clusters, most of which share the same midpoint potential and are called "isopotential" clusters. Two clusters present different characteristics: N1a has the lowest midpoint potential ($E_m = -370$ mV) and N2 has the highest midpoint potential ($E_m = -150$ mV) and presents EPR magnetic interactions with the semiquinone radicals; for these reasons it is considered to be the direct electron donor to ubiquinone (159). N2 iron–sulfur cluster is most likely located in the connection between the peripheral and the membrane arm. Brandt *et al.* (27) recently hypothesized an amphipathic "ramp" guiding ubiquinone into the catalytic site.

Complex I is inhibited by more than 60 different families of compounds (63). These inhibitors have been grouped into three classes based on their effects on the kinetic behaviour of the enzyme, but it is commonly accepted that they share the same hydrophobic large pocket in the enzyme (188).

Complex I is also involved in the formation of the transmembrane proton gradient with a stoichiometry of 4 $H^+/2e^-$. In recent years many mechanistic schemes were proposed that combined features of a direct pump with a reversed Q-cycle type mechanism (26). Another possible mechanism is related to a conformational energy transfer and is supported by the recent evidences that all catalytic centers are located in the peripheral arm of the enzyme clearly separated from the membrane domain (235). An additional activity of Complex I is its univalent reaction with oxygen to produce superoxide anion, as anticipated in Section 3; an understanding of the site(s) of superoxide release from the enzyme is believed to provide hints for clarification of the electron paths in the Complex.

Previous studies from our laboratory suggested that the site responsible for one-electron transfer to oxygen is probably the iron–sulphur cluster N2 (88), from which electrons are addressed to bound ubiquinone molecules. It was found that rotenone and several other hydrophobic inhibitors stimulate superoxide production in submitochondrial particles (87, 88, 106); such stimulation occurs also in coenzyme Q-depleted mitochondria, indicating that bound CoQ semiquinone is not the site for one electron transfer to oxygen.

The short chain CoQ homolog, CoQ_1, has been used as a tool to investigate superoxide production (C. Bergamini, R. Fato, and G. Lenaz, unpublished observations). There is a common agreement on the capability of CoQ_1 to interact with the physiological acceptor site because its rotenone sensitivity and the high turnover number of the NADH-CoQ_1 activity (139). Nevertheless in most types of mitochondria a rotenone-insensitive component is present in the reduction of CoQ_1 that varies depending on the cell type (146). This component indicates the presence of a CoQ_1 reduction site situated upstream of the rotenone block. Since this rotenone-insensitive component is absent with more hydrophobic quinones, such as decyl ubiquinone (139), it is likely that it reflects a hydrophilic site. Due to the position of N2 at the interface between the hydrophobic and hydrophilic sector of the enzyme, it is tempting to speculate that also the hydrophilic CoQ_1 reduction site is represented by N2 itself.

Addition of CoQ_1 to NADH-supplemented particles inhibited with rotenone further enhances ROS production. Pieridicin A and rolliniastatin-1 and -2 had a behavior similar to rotenone, whereas other hydrophobic Complex I inhibitors such as stigmatellin, capsaicin, and reduced quinones strongly inhibit ROS formation both in the presence and absence of CoQ_1.

The possible prooxidant action exerted by a short-chain ubiquinone under some conditions, shared by other physiological quinone-like molecules such as adrenochrome (20), suggest caution in using these molecules as antioxidants; on the other hand, the physiological CoQ_{10} has never been found to exert prooxidant effects (140).

The results obtained following the ROS production by Complex I suggest the presence of two distinct sites for Complex I inhibitors: the rotenone-like inhibitors interact with a more hydrophobic site that is not able to interact with O_2, whereas the second class of inhibitors (Stigmatellin, etc.) interact directly with the escape site of electrons, responsible of the mono-electronic reduction of O_2. According to previous observations that refer to the center N2 as the site for electron leak, on the basis of this study Complex I inhibitors should be divided into two classes: (a) Class A inhibitors that favor the electron escape from the enzyme, and (b) Class B inhibitors that act blocking the mono-electronic reduction of O_2. On the other hand, if we consider full quinone reduction in the physiological site, both classes of inhibitors block the electron flux from NADH to the quinone. One possible explanation is that only class A inhibitors induce a conformational change of the enzyme making the site of escape of electrons more accessible to oxygen.

This model allows a rational picture of the superoxide production by Complex I (Fig. 5). Since rotenone prevents formation of the CoQ_{10} semiquinone form (189), the enhancement of superoxide production induced by rotenone cannot be due to reaction of the semiquinone with molecular oxygen. From our previous study (88), we consider it likely that the one-electron donor to oxygen in the presence of rotenone is cluster N2 that could release one electron to oxygen in a hydrophilic phase either directly or indirectly via the CoQ_1 semiquinone. The different behavior of the CoQ_1 and CoQ_{10} semiquinones toward oxygen would depend on the unavailability of the latter to protons that are required for reaction (187).

Other hydrophilic quinones, such as adrenochrome (20) and dopaminochrome (262), may accept electrons from the same site, thus establishing a vicious circle of superoxide production. The relevance of this mechanism to Parkinson's disease is evident (232, 239, 262) and it has long been known that Complex I is involved in its pathogenesis (197, 215).

It is unlikely that the major site of superoxide production in SMP under our experimental conditions is FMN, as suggested by Kudin *et al.* (128) since in that case, for an electron carrier situated upstream of the iron–sulphur clusters, all hydrophobic inhibitors should have the same effect.

MOLECULAR PATHOGENESIS OF MITOCHONDRIAL CYTOPATHIES

A genetic classification of mitochondrial cytopathies (66) distinguishes disorders due to defects of the mitochondrial genome and those due to nuclear DNA mutations. Only one

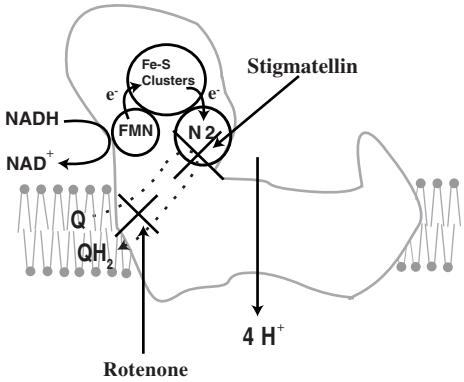

FIG. 5. Site of ROS generation by Complex I. The figure is freely redrawn from Brandt *et al.* (27). Rotenone and stigmatellin are drawn as interacting with different ends of the pocket or ramp for the access of ubiquinone. One can postulate (see text) that the inhibitors induce different conformations of the site responsible for O_2 reduction (presumably N2) in such way to favor superoxide formation (rotenone) or to inhibit it (stigmatellin).

third of the more than 150 pathogenic mtDNA mutations concerns structural genes, the others are either deletions or rearrangements or they affect mitochondrial tRNA or rRNA genes. The heterogeneity of the clinical patterns of mtDNA defects is related to the complexity of mitochondrial genetics (65): the degree of heteroplasmy usually differs in different tissues due to mitotic segregation and other less known phenomena; in addition, threshold effects (209) exist that allow normal biochemical phenotype until a well-defined threshold (usually high, up to 90% mutated mtDNA with respect to wild type) is reached. The phenotypic threshold may be explained by complementation of the altered products of mutated mtDNA by the normal products of wild-type mtDNA at different levels: transcription, translation, enzyme complex assembly, biochemical level, and cellular level. The possibility of mitochondrial transcomplementation is controversial (3, 77, 190). Recently D'Aurelio *et al.* (56) have fused two human cybrid cell lines, each containing a distinct pathogenic mtDNA mutation, and found direct evidence of recombination between these two mtDNA haplotypes. Three mechanisms may underlie the biochemical threshold (209), viz., an excess of active oxidative phosphorylation complexes, the presence of inactive complexes that are activated when the oxidative phosphorylation level becomes insufficient, and an increased turnover of the active complexes due to regulation mechanisms. Flux control analysis (cf. Section 2) has been critical for the understanding of the biochemical threshold.

Several diseases are due to point mutations of structural genes in mtDNA (142) (Table 1); here we survey some biochemical aspects of three selected examples (the LHON syndrome due to three mutations in Complex I ND subunits, the NARP syndrome due to a mutation in ATPase-6, and a form of MELAS due to a mutation in cytochrome oxidase subunit III).

Leber's hereditary optic neuropathy

Leber's Hereditary Optic Neuropathy (LHON) (38) is due to three main mutations in genes for Complex I subunits affecting subunits ND1, ND4, ND6. The clinical syndrome is characterized by retinal ganglion cells and optic nerve degeneration with sudden blindness. The disease mainly affects individuals with homoplasmic mutations, but not all subjects harboring the pathogenic mutations are affected, suggesting that other genetic and environmental factors are required for the development of the disease.

TABLE 1. PATHOGENIC POINT MUTATIONS OF MTDNA STRUCTURAL GENES

mtDNA mutation	*Disease*	*Subunit involved*	*Amino acid substitution*
G3460A	LHON	ND1	Ala 52 Thr
A4917G		ND2	Asp 150 Asn
G11778A		ND4	Arg 340 His
G13708A		ND5	Ala 458 Thr
T14484C		ND6	Met 64 Val
T9101C		ATP6	Ile 192 Thr
G9438A		COX III	Gly 78 Ser
G15257A		Cyt b	Asp 171 Asn
G15812A		Cyt b	Val 356 Met
G14459A	LHON/dystonia	ND6	Ala 72 Val
G15762A	Myopathy	Cyt b	Gly 339 Glu
T9957C	MELAS	COX III	Phe 251 Leu
G13513A		ND5	Asp 393 Asn
G6930A	Mitochondrial	COX I	Stop codon
G9952A	encephalomyopathy	COX III	Stop codon
T7587C	Multisystem disorders	COX II	Met 1 Thr
T7671A		COX II	Met 29 Lys
T88993G	NARP/Leigh syndrome	ATP6	Leu 156 Arg
T8993C		ATP6	Leu 156 Pro
T9176G		ATP6	Leu 217 Arg
T9176C		ATP6	Leu 217 Pro
T9176C	FBSN	ATP6	Leu 217 Pro

Three frequent mutations affecting ND subunits of Complex I (38, 253) occurring at positions G11778A/ND4, G3460A/ND1, and T14484C/ND6, are established as pathogenic.

Biochemical investigations of the three most frequent mutations revealed some subtle biochemical changes in Complex I function (28). Only the 3460/ND1 mutation showed a consistent reduction in Complex I electron transfer activity (41, 114, 161), while both 11778/ND4 and 14484/ND6 mutations had normal activities (40, 41, 51).

Further studies on the sensitivity of Complex I to different inhibitors showed a decreased sensitivity to rotenone and an enhanced sensitivity to quinol product inhibitors (160), while sensitivity to other Complex I inhibitors not interfering with the CoQ binding site, such as rolliniastatin-2 or amytal, was unchanged. These results suggest that the mutations interfere with the interaction of complex I with CoQ, indicating that the CoQ binding site may be affected by the mutations perhaps decreasing the stability of ubisemiquinone radical and favoring its dismutation (64, 142).

The complex I dysfunction in LHON may have three major consequences: (a) the release of quinol product may be affected, thus leading to decreased total respiratory activity; (b) due to alteration of the hydrophobic quinone binding site(s), proton pumping through complex I may be defective thereby affecting energy conservation; (c) an increase of reactive oxygen species (ROS) generation may occur as a consequence of altered electron flow, as reported in the case of nuclear complex I mutations (202), or because unstable ubisemiquinone radicals may rapidly react with oxygen (64). Studies in our laboratory using osteosarcoma-derived cybrids carrying each of the LHON mutations indicate that Complex I-dependent ATP synthesis is affected by all three mutations, as also reflected by the slight reduction of total ATP cellular content observed (7, 142). Complex II-dependent ATP synthesis does not appear to be significantly affected.

The present results are not in contrast with the currently favored hypothesis that besides an energy defect, overproduction of ROS may represent a major element in LHON pathophysiology (42). This hypothesis is supported by the increased ROS generation after partial Complex I inhibition (13). The apoptotic cell death occurring in LHON cybrids carrying the mutations, when incubated in galactose medium (89), may be the result, besides of decreased bioenergetics, of increased ROS generation (43).

NARP

The Neurogenic muscle weakness, Ataxia, and Retinitis Pigmentosa (NARP) syndrome and the maternally inherited Leigh syndrome (MILS), as well as familial bilateral striatal necrosis (FBSN) (217), are all caused by mutations affecting the ATPase-6 subunit gene (subunit a) of the mitochondrial ATP synthase complex. Of the five known pathogenic mutations causing these disorders, four are located at two base-pairs (8993 and 9176), each of which can undergo mutations converting a conserved Leu (-156 and -217) to either Arg or Pro. The T→G transversion at the 8993 position in mtDNA is the more frequent mutation and changes Leu-156 with Arg in the ATPase-6 subunit gene. The variable degree of heteroplasmy is responsible for the different clinical expressions (110, 212, 238). More recently a T→G transition at the 8851 position, changing Trp-109 with Arg may lead to a FBSN pathology closely resembling NARP (62, 113).

The biochemical effects of the T→G transversion at the 8993 position are not fully understood. A decrease of ATP synthesis has been observed in studies of fibroblasts (112), cybrids (164), and coupled submitochondrial particles prepared from platelets of NARP patients (5), all harboring a high percentage of mutation; however, the decrease of ATP synthesis was widely different in these studies, and maximal in the latter study.

Coupled submitochondrial particles prepared from platelets of NARP patients showed a dramatic decrease of ATP synthesis driven by succinate, but both ATP hydrolysis and ATP-driven proton translocation were minimally affected (5). This implies either a different effect of the mutation on the F_0F_1 function or a unidirectional impairment of proton flow, only when the enzyme works physiologically (i.e., protons flow from cytosol to matrix). There was a close relationship between tissue heteroplasmy, expression of the biochemical defect of ATP synthesis in platelets, and clinical involvement (39). The biochemical defect is strictly correlated with the degree of heteroplasmy without any evidence of a biochemical threshold. However, 60%–75% mutant mtDNA is required for clinical expression of typical central nervous systems symptoms.

In order to elucidate the pathogenic mechanism of the disease we are measuring the proton transport from cytosol to matrix during ATP synthesis in digitonin-permeabilized lymphocytes of NARP patients (cf. 142) exploiting the kinetics of Rhodamine-123 fluorescence quenching in state 3 and state 4 respiratory conditions: the overall quenching rate is proportional to the steady-state membrane potential (6).

Preliminary results show that lymphocytes from NARP patients exhibit a mitochondrial membrane potential, slightly enhanced with respect to controls, suggesting that the mutation hardly affects the F_0 permeability to protons and that ATP synthesis is impaired for a different reason, such as an impaired assembly of the mutated ATP synthase complex (112, 186). However, a study in NARP cybrids showed that ROS formation is strongly involved in the biochemical lesions due to the mutation (168). ROS overproduction might contribute to the loss of ATP synthesis in cells harboring the T8993G mutation but might also play a crucial role in the transition from the NARP to the severe Leigh clinical phenotype.

COX III mtDNA mutation associated with MELAS

A missense mutation in the gene encoding COX III at position 9957 of mtDNA has been reported in a patient affected by mitochondrial encephalomyopathy clinically resembling MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes); the mutation causes a Phe 251 to Leu amino acid substitution, a highly conserved residue among different species (165). The COX III residue Phe-251 in humans is located in the hydrophobic core of the last transmembrane helix of the subunit (151), and is homologous to Phe-263 in *Paracoccus denitrificans* (165). When this residue was converted to Leu there was a reduced growth of the mutant strain, reduction of proton pumping stoichiom-

etry of COX, and reduction of membrane potential (166). Therefore, an intrinsic uncoupling of COX, that is a reduced efficiency of $\Delta\mu_{H+}$ generation, has been suggested to be the cause of the mitochondrial dysfunction in the patient harboring the 9957 mtDNA mutation. A role of Subunit III in proton translocation through the D proton pathway has been suggested (90, 176). Alternatively, a defect of COX III might result in altered subunit assembly (95): there is increasing evidence for a role of subunit III in the assembly of the Complex (99, 107, 174, 240).

Cybrids produced from the patient's fibroblasts and an osteosarcoma-derived cell line have been kindly provided by G. Manfredi. We found no major decrease of either cytochrome oxidase electron transfer activity or total respiration nor any change of mitochondrial membrane potential in the cybrids in comparison with the parent osteosarcoma cell line; on the other hand we did find a significant increase of ROS production (142).

The mechanism of enhanced ROS production in the mutant cells is not clear: in general ROS are produced by Complexes I and III when the electron flow is lowered and the electron carriers are mostly in the reduced state (222). Since no change in oxidation rate of the chain is apparent, this mechanism has little support. On the other hand, no case has been described so far of ROS generation directly at the level of cytochrome oxidase, where the electron transfer to oxygen does not lead to release of partially reduced oxygen intermediates.

In accordance with the enhanced ROS production, the redox activities of the plasma membrane, both the NADH oxidase and membrane-bound DT-diaphorase, were also strongly activated in the mutant cybrids (60). It is believed that the plasma membrane NADH oxidase represents a defence mechanism when mitochondrial function is impaired or against oxidative stress (156, 185).

OUTLOOKS ON AGING AND AGE-RELATED DISEASES

What can we learn from the molecular pathogenesis of mitochondrial cytopathies in the understanding of aging and age-related diseases? The points to be clarified also in the light of knowledge of mitochondrial diseases are the following:

a) What is the precise role of ROS in aging?
b) What can we learn from mtDNA genetics in relation to aging and longevity?
c) What is the relevance of mtDNA deletions and mutations?
d) Can mtDNA damage be causally correlated with mitochondrial functional defects?
e) Are mitochondria self-sufficient for the increase and propagation of the damage or is the expression of nuclear genes also necessary for the aging phenotype?

The increasing availability of suitable animal models for aging (73, 170) and more generally for mitochondrial dysfunction (251, 252) adds novel perspectives to the solution of the problems.

Most research on the role of mitochondria in aging has been formerly performed using different tissues from aged animals and humans (cf. 138); the necessity of employing simpler systems at the same time allowing to investigate a number of spontaneous or induced genetic mutations and/or exerting metabolic manipulations during a reduced life span have focused attention more and more on invertebrates, such as *Drosophila* (105, 224, 231), the housefly *Musca domestica* (52), and the nematode *Caenorhabditis elegans* (2, 104, 243); besides these, even yeast *S. cerevisiae* (36, 117) and the filamentous fungus *Podospora anserina* (191, 192) have been usefully employed in aging research. Also naturally occurring mouse mutants, such as the senescence-accelerated mice (SAM) (111) and transgenic mice (179) have provided important clues. Information may be gained by the comparative approach exploiting different species (55) or different strains within the same species (229). Finally, although the use of cell cultures aging in vitro as a model for senescence is controversial (61, 76, 102, 103), primary cell cultures from senescent animals (e.g. 94, 103) represent a useful tool for studies of mitochondria in relation to aging, also considering an integrative approach where mitochondrial damage is compared and related to other lesions, such as to nuclear DNA and telomeres (247).

Aging and ROS production

The mitochondrial theory of aging states that the original damage to mtDNA is induced by the continuous generation of ROS and other toxic species; thus it is not necessary that an increase of ROS generation occurs in aging, since it is the damage that would accumulate. Nevertheless, a vicious circle (193) can be established only if the accumulated damage (to the respiratory chain) would enhance ROS generation (Fig. 6). In spite of a few studies reporting no increase of ROS production with aging (37, 98), many reports demonstrated that the rate of production of ROS from mitochondria increases with age in mammalian tissues (157, 205, 225); accordingly, an increase of ROS was found in hepatocytes from aged rats (11, 46); by flow cytometric analysis of peroxide production detected by dichlorofluorescin diacetate labeling, hepatocytes from old rats were found to have a higher peroxide level than hepatocytes from young animals; in addition, the peroxide production after an oxidative stress induced by adriamycin was much higher in the old animals.

In fibroblasts during replicative cell senescence, considered to represent a plausible model of *in vivo* aging (15, 102, 103), ROS production increases (115, 136). ROS production was also found to increase with aging in resting skeletal muscle and to be potentiated by strenuous exercise to exhaustion (17). An additional factor eliciting an increased ROS production in aging is the postulated spreading of damage from one cell to the surroundings by means of the plasma membrane oxidoreductase (58, 108, 182), thus triggering a chain reaction of oxidative damage.

The evidence existing on the role of oxidative damage as the main pathogenic factor for aging is overwhelming. Compelling evidence for the free radical and mitochondrial theory of aging derives from several observations: the strong negative correlation existing between expected lifespan and meta-

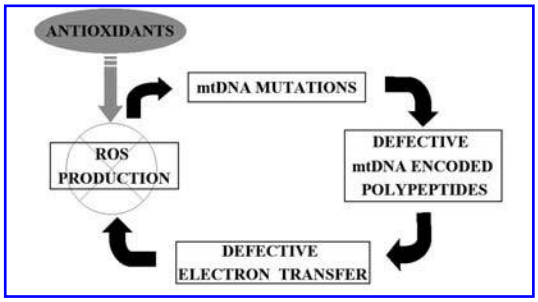

FIG. 6. A vicious circle of oxidative stress and mitochondrial DNA mutations in aging.

bolic rate and rate of ROS production of different species (10, 55, 226, 227) and within the same species under different activity conditions (257), and between lifespan and membrane lipid unsaturation (195). Furthermore the demonstration that sex differences in longevity are coherently correlated with differences in ROS production and detoxification (21) and that caloric restriction while prolonging lifespan in mammals reduces ROS production (9, 157) provides additional points in favor of the role of ROS in aging. The levels of antioxidant enzymes may also critically vary in aging: an increase of Mn-SOD not compensated by increased glutathione peroxidase activity may contribute to enhance oxidative stress by the Fenton reaction (255).

The negative correlation between metabolic rate and life expectancy (see above) is apparently in contrast with the findings that uncoupling protects from oxidative stress by reducing ROS generation (Section 4): indeed individual mice with high metabolic rate and oxygen consumption have greater mitochondrial uncoupling and live longer (230). However, the apparent contradiction may be easily explained by considering that *total* ROS production in a cell is necessarily higher *if* the mitochondrial mass or respiratory surface is larger, even if the *specific activity* of ROS production is not different; in addition to that, however, individual variations in energy dissipation within the same species or the same individual may clearly modulate the production of ROS through variations of membrane potential.

All biomolecules in the cell are targets of ROS and undergo chemical modifications that accumulate with age (cf. 67, 138): protein carbonylation and methionine oxidation (219, 233), advanced glycation end-products (AGE) (59, 223), lipid peroxidation (4), and nucleotide modifications (68, 121). However, it is not completely understood which species are responsible for the damage *in vivo* and how the increased availability of ROS translates into the accumulation of specific oxidative damage (218): for instance some proteins are better targets than others for oxidative damage (218).

Mitochondrial DNA polymorphisms, aging, and longevity

It is likely that aging is the result of fixation of many alleles that confer a deleterious phenotype in the postreproductive phase of life and are essentially evolutionarily neutral, that is, they do not undergo positive selection being beneficial in early life (53). It is also likely that mutations in the Y chromosome and in the mitochondrial genome are much more likely to be fixed than mutations in the autosomic genome and X chromosome since the former lack germline recombination and have a smaller population size (53). This is the reason of the higher rate of evolution of the mitochondrial genome (29). Thus the mitochondrial genome is more likely to contain deleterious mutations that can accumulate in post-mitotic tissues where mitochondria continue to divide hundreds of times during life (54).

For the same reason it is also likely that mtDNA polymorphisms, by changing some features of the oxidative phosphorylation system, may influence the mutational rate of mtDNA itself and thereby the lifespan of individuals (237). These polymorphisms are clustered in haplogroups each sharing some distinct mutations. De Benedictis *et al.* (57) have shown that male centenarians had a significantly higher frequency of haplogroup J than sex-matched younger subjects. Paradoxically it was also found (241) that mutations causing LHON were much more likely to cause the disease (see Section 5a) if they occurred in haplogroup J. The complete sequencing of haplogroup J in centenarians (207) revealed that mtDNA contains mutations falling in ND1 and ND5 subunits of Complex I, among others. Thus the same mutations can induce either longevity or disease: Rose *et al*. (208) have postulated as an example that a scarce performance of Complex I may confer a disadvantage in case of concomitant pathogenic LHON mutations but may be useful in inducing an increased ROS generation on its hand inducing over-expression of nuclear genes coding for detoxifying enzymes. In another study (259) it was found that a homoplasmic C150T transition in the D-loop is much more frequent in centenarians than in younger subjects; interestingly it was found that the mutations induced a new replication origin, presumably conferring a survival advantage.

Significance of mtDNA mutations/deletions

A large number of large-scale deletions, point mutations, and tandem duplications have been observed in tissues of aged individuals (134, 155, 193, 250, 255). Attardi's group (175) reported that a T414G transversion in the D-loop of mtDNA is accumulated in skin fibroblasts from old subjects; on the other hand, two different point mutations (A189G and T408A) accumulated in the D-loop in muscle of old individuals (254). These mutations are located in the control region of mtDNA and may impair its replication and transcription.

A minimal threshold level of 50 to 95% mutated mtDNA is usually necessary to impair respiratory chain function, depending on the type of mutation and the tissue affected (209, 216). Since the proportions of mutated mtDNA within aging human tissues (except the D-loop mutations) rarely exceed 1%, it has been questioned how these levels may cause significant bioenergetic effects. Hayakawa *et al.* (101) using 180 kinds of PCR primers detected all the deletions over 500 bp and found that mtDNA in elderly subjects is extensively fragmented in minicircles with different sizes. As a result, the amount of mtDNA mutations may reach such a high level that

it could cause significant impairment of oxidative phosphorylation (184). Such a high level of mtDNA fragmentation may be at first sight thought to be inconsistent with the less striking loss of bioenergetic capacity (58), but is in line with the high threshold required to impair oxidative phosphorylation. Furthermore, mutated mtDNA molecules may be distributed unevenly among the cells of affected tissues in a mosaic pattern of mtDNA segregation (152).

Exponential accumulation of mutated mtDNA during life suggests that there is a preferential replication of the mutant genomes. Such a preferential replication can be caused by the smaller size of deleted DNA circles (158) but also on some replicative advantage provided by the D-loop control region mutations (73, 203).

Are mtDNA mutations the cause of aging or do they just accompany the aging process as interesting biomarkers of senescence? In a recent study Trifunovic *et al.* (242) showed that expression of a proof-reading deficient mtDNA polymerase in a homozygous knock-in mouse strain leads to increased levels of somatic mtDNA mutations causing progressive respiratory chain deficiency; the mice develop symptoms strikingly reminiscent of aging. This is the most striking demonstration that mtDNA mutations can cause aging.

The natural way to induce mtDNA mutations is represented by ROS: it has been demonstrated that mtDNA is more sensitive than nuclear DNA to oxidative stress and is repaired less efficiently (163, 206, 256). The content of 8-hydroxy-2′-deoxyguanosine, an index of oxidative DNA damage, is higher in mtDNA than in nDNA and increases with aging (100). Laderman *et al.* (131) constructed a series of cybrids by fusion of mtDNA-less osteosarcoma cells with human fibroblasts from donors of different ages and showed that the cybrids obtained from older donors had lower mtDNA content and lower respiratory activity, strongly suggesting that mtDNA mutations in aging were the cause of the respiratory deficiency.

The compensatory mechanisms occurring in aging also appear to involve mtDNA transcript levels. Since the D-loop controls transcription, we have investigated the steady-state level of mitochondrial RNA transcripts of subunits ND1 and ND5 of Complex I and COX I and COX III of Complex IV in human platelets using Real Time RT PCR (173). Several surprising results were apparent from the study. First, the level of transcripts was not the same, and in particular the level of COX I was double that of COX III: this is unexpected since mtDNA transcription is polycystronic. Second, the levels of Complex I transcripts, but not of Complex IV transcripts, were increased in aging. It must be noted that in rat tissues, mtDNA transcription was unchanged or decreased in aging; however, Complex I transcripts were not investigated (8, 83). In a recent study (162) it was found that mtRNA transcripts increased in mouse brain slices from 2 up to 18 months of age but then strikingly decreased at 24 months. It may well be that a compensatory mechanism exists but is lost at extreme ages; this would explain the variability of literature data.

The different levels of different transcripts can only be explained by different stability of the RNAs once transcribed. The unexpected increase of Complex I transcripts in aging may be considered as a compensatory mechanism to cope with the functional alteration of the enzyme (109) due to its rate control over the oxidative phosphorylation system. It is not known whether the protein expression of these genes is also increased.

Functional impact of respiratory chain damage

There is overwhelming evidence that the bioenergetic function of mitochondria declines with aging, especially in postmitotic tissues (71, 92, 138, 145, 221, 255) and this decline may be induced by ROS, as demonstrated by the decrease of respiratory activities in MnSOD-deficient mice (171). The absent or scarce decrease of activity of individual respiratory complexes observed in many studies (1, 98, 178) may reflect the problem encountered for mitochondrial diseases (cf. Section 5), that is, electron transfer activity alone does not reflect the whole bioenergetic capacity of the mitochondria, since also membrane potential and ATP synthesis should be taken in account. To this respect the paper of Greco *et al.* (92) exploiting fibroblasts from donors of different ages and carefully exploring the P:O ratios is very informative in detecting a significant fall of phosphorylation efficiency in the elder. However, tissue differences also exist in the susceptibility of mitochondria to ROS-induced damage (70): for example the ATP content and production declined to 50% with age in the skeletal muscle (gastrocnemius) but underwent no change in the heart of Fischer-344 rats (71).

If mtDNA mutations/deletions are at the basis of human aging, the existence of a biochemical threshold complementing mtDNA mutations is critical, as discussed above. The notion that the respiratory chain is mainly controlled at the level of Complex I (see above) suggests that the main alterations due to aging must be found at the level of this enzyme (13, 145).

We have applied flux control analysis to Complex I in aerobic respiration in coupled liver mitochondria from young and aged rats of 30 months (246). In this system, in comparison with open SMP as cited above, there are other steps that partly control total respiration, such as the substrate carriers and the ATP synthesis, so that the control coefficients for Complex I are lower than in SMP. The plots in Figure 7 show that Complex I has little control in young rats but very high control in the old animals. This means that aging induces a profound alteration of Complex I that is reflected on the entire oxidative phosphorylation system.

In agreement with this observation, we found that aerobic State 3 respiration is decreased in aged rats (143, 145, 246). The alteration of Complex I is also documented by the small albeit significant decrease of rotenone sensitivity of the enzyme and of whole respiration, documented by the increase of I_{50}, the inhibitor concentration inducing half inhibition of the activities**.** A similar increase in I_{50} for rotenone was also found in the brain cortex of aged rats (86).

We have also investigated Complex I activity in human platelets from young and aged individuals. In this case the most striking result was the decrease of rotenone sensitivity (172); the class distribution of rotenone titers for half-inhibition was within a narrow range in the young, but was highly scattered in the old, with many individuals having high rotenone titers for half-inhibition. This study demonstrates that bioenergetic alterations may exist not only in cells of postmitotic tissues but also in cells directly deriving from mitotic divisions: in mitotic cells it is usually assumed that se-

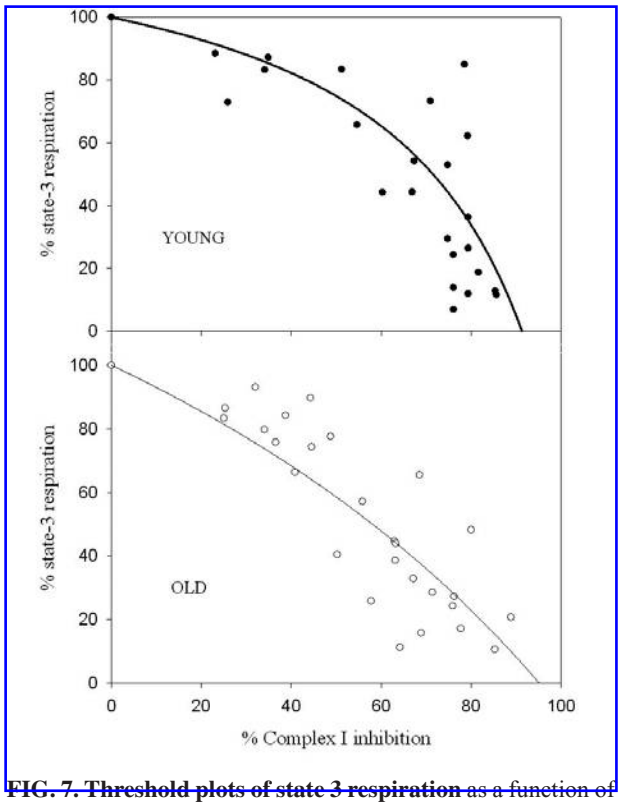

FIG. 7. Threshold plots of state 3 respiration as a function of percent inhibition of Complex I after rotenone titration in phosphorylating (state 3) liver mitochondria from adult (4 months) and aged (30 months) rats.

lection washes away the damaged cells (32); nevertheless, if cells can supply to their energy needs by glycolysis, they will survive and maintain the mitochondrial alterations.

Cross-talk between mitochondrial and nuclear genome

Recent investigations attempt more and more to relate the mitochondrial changes with the cellular environment, and the cross-talk between mitochondrial and nuclear genome is receiving increased attention. The availability of novel techniques as serial analysis of gene expression (245) has been applied to aging studies in *C. elegans* (118). Also the yeast model has provided some important clues: yeast is characterized by the retrograde response triggered by mitochondrial dysfunction (30, 31) and activating specific signaling proteins that migrate to the nucleus and induce numerous nuclear genes coding for metabolic enzymes and stress proteins (117). The result is an extension of yeast longevity.

Changes in the redox status of cellular components by oxidative stress during aging is considered the cause of the observed increased contents or DNA-binding activities of such transcription factors as NF-κB, AP-1 and HIF-1 (34, 122, 132), of heat-shock proteins (35), and of heme oxygenase (33); their increased activity is considered a compensatory mechanism for cellular protection (35) and may depend either upon direct alteration of the factor or indirectly through activation of related transduction pathways (122) by means of mechanisms still scarcely understood. In accordance with the importance of oxidative stress in activation of redox-sensitive transcription factors, caloric restriction, the main known factor recognized to delay aging (9, 157, 228, 258), was found to prevent their activation (122). Similarly, hepatocytes from old mice (148) and rats (116) showed reduced activation of ERK by H_2O_2, and the effect was suppressed by caloric restriction (116). Available data on redox-responsive transcription factors suggest that their uncontrolled activation in aging could lead to serious chronic pathogenic conditions characterized by what has been called "molecular inflammation" (50).

The mtDNA copy number per cell is often increased in tissues from aged human subjects and experimental animals (12, 84, 133, 198, however, cf. 92, 131 for opposite results), as a likely result of a feedback mechanism compensating for defective mitochondria having an altered respiratory chain. Accordingly, Lee *et al.* (135) found that hydrogen peroxide causes an increase of mitochondrial mass and mtDNA copy number in the human lung cell. In the lung fibroblast cell line MRC-5 undergoing replicative senescence, an increase in mitochondrial mass with cellular aging was found to parallel the increase in ROS production revealed by DCF fluorescence (136); the same study showed that the transcript levels of NRF-1 and PGC-1 were increased in replicative senescent cells; in addition, both protein and mRNA levels of mitochondrial transcription factor A and NRF-1 were found to be increased in skeletal muscle biopsies from aged humans (147). These factors are known to be involved in the control of mitochondrial biogenesis (180, 204).

CONCLUSION

Progress in bioenergetics and molecular biology has contributed to advance our understanding of the aging process. The increasing knowledge of the intensive cross-talk existing between mitochondrial and nuclear DNA and of its redox regulation enlarges the horizons of the intricate processes at the basis of senescence and contributes to unify stochastic and genetic theories of aging (cf. 138), but at the same time opens new challenges to aging research.

The organization of the respiratory chain and the regulation of oxygen radical production by the mitochondrial respiratory chain and other sources are still surprisingly uncertain, and their comprehension will certainly help the understanding of the molecular mechanisms leading to aging of cells. In this review we have attempted to describe the present state-of-the-art of this research area. The study of genetic mitochondrial diseases, due to known specific mutations but still the subject of intensive biochemical research, is believed to represent a useful model to understand the much more complex scenario of the alterations that occur during cell senescence.

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ABBREVIATIONS

AGE, advanced glycation end-products; CoQ, coenzyme Q; COX, cytochrome *c* oxidase (Complex IV); DCF, dichlorofluorescin diacetate; EPR, electron paramagnetic resonance; ERK, extracellular receptor activated protein kinase; FBSN, familial bilateral striatal necrosis; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; HIF, hypoxia-induced factor; LHON, Leber's hereditary optic neuropathy; MELAS, mitochondrial encephalopathy, lactic acidosis and stroke-like episodes; MILS, maternally inherited Leigh's syndrome; mtDNA, mitochondrial DNA; NARP, neurogenic weakness, ataxia, and retinitis pigmentosa; NF-κB, nuclear factor κB; NRF-1, nuclear respiratory factor 1; OXPHOS, oxidative phosphorylation; PCR, polymerase chain reaction; PGC-1, peroxisome proliferator activated receptor gamma coactivator 1; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; SAM, senescence-accelerated mice; SMP, submitochondrial particles; SOD, superoxide dismutase; UCP, uncoupling proteins; VDAC, voltage-dependent anion channel.

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Address reprint requests to:
Professor Giorgio Lenaz
Dipartimento di Biochimica "G. Moruzzi"
Via Irnerio 48
40126 Bologna, Italy

E-mail: lenaz@biocfarm.unibo.it

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