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Review

Mitochondrial oxidative phosphorylation changes in the life span. Molecular aspects and physiopathological implications

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Contents

1. Introduction

Mitochondrial oxidative phosphorylation (OXPHOS) in adult humans, under normal physiological conditions, covers more than 80% of the energy needs, the remaining being met by glycolysis. An adult man with daily energy needs of some 3000 kcal turns over 400 mol of ATP. OXPHOS capacity varies from tissue to tissue and so do the ATP needs. The brain is the organ with the highest demand for aerobic ATP production, accounting, with only 2% of the body mass, for 20% of total oxygen consumption at rest [1], but also heart, muscle, kidney and endocrine systems rely highly on OXPHOS capacity [2]. OXPHOS capacity of mammalian cells changes in the

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life-span. In fetal tissues anaerobic glycolysis represents the major source of cellular ATP, the capacity of oxidative phosphorylation being relatively low [3,4]. At birth a clear onset of oxidative phosphorylation occurs as a result of the rapid induction of mitochondrial biogenesis [5–7]. The capacity of OXPHOS attains a maximum level in young and adult mammals. The capacity of OXPHOS can, however, vary in response to changes in energy demand through adaptive response of mitochondrial activity and biogenesis to physical activity [8,9].

Morphological [10,11] and biochemical observations [12–18] indicate a progressive decline with age of the mitochondrial respiratory systems in human tissues. This, together with the finding of defective OXPHOS and the occurrence of deletion and point mutations of mitochondrial DNA in neuromuscular disorders and other degenerative diseases [2], suggests that progressive decrease with age of mitochondrial function is critical for the functional decline of brain, heart, muscle, endocrine systems and other post-mitotic tissues, which is associated with aging [2,19,20]. This review aims to provide a concerted picture of the different changes in OXPHOS taking place in the life span, from their genetic and molecular basis, to in vivo 'monitoring' of OXPHOS and physiopathological implications of OXPHOS changes.

2. The mitochondrial OXPHOS system

The mitochondrial enzyme-system of OXPHOS consists of five oligomeric protein complexes (Table 1) [2,21–33]. Complexes I [22–24] and II [25] collect electrons from NADH and succinate, respectively, and pass them to ubiquinone which is oxidized by ubiquinone cytochrome-*c* oxidoreductase (complex III) [26,27] and cytochrome-*c* oxidase (complex IV) [26,28–30]. With the exception of

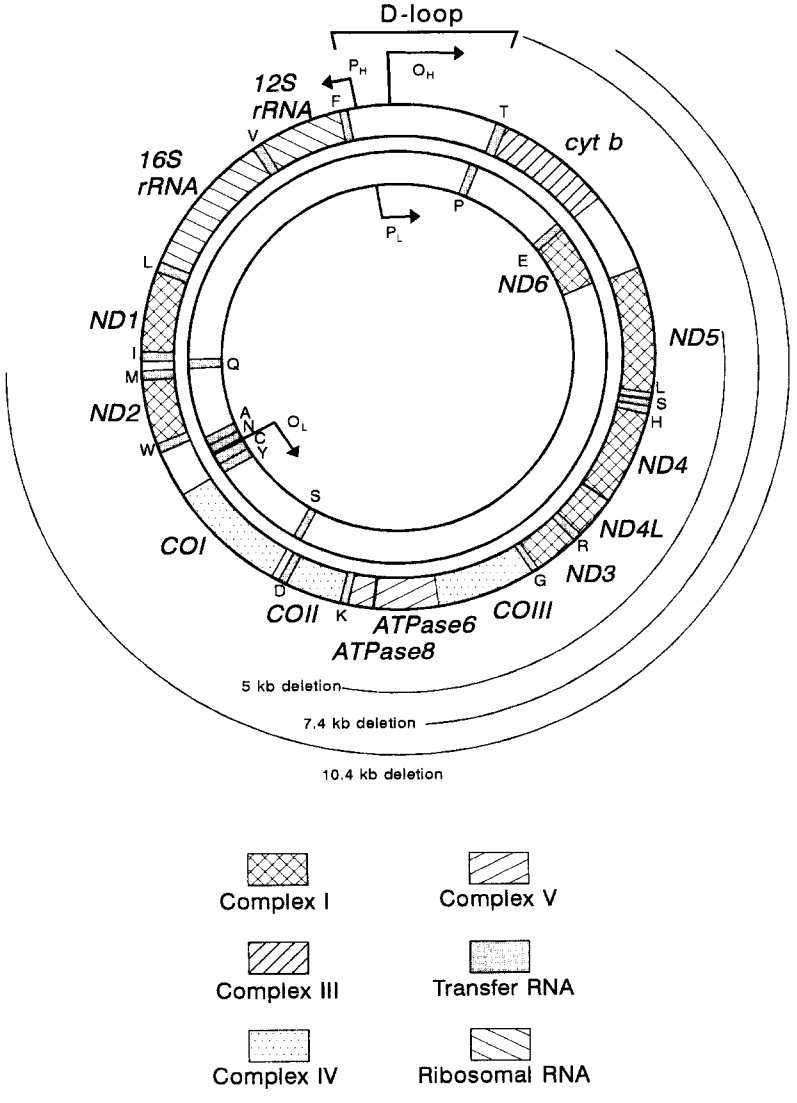

Fig. 1. The mitochondrial genome of mammalian cells. For details see text, Table 1 and Refs. [2] and [36]. The external lines indicate mtDNA deletions detected in human tissues in diseases (see Table 2).

Table 1
Redox centres and polypeptide composition, with respective molecular weights, of mammalian mitochondrial respiratory complexes and F_oF_1 ATP synthase

	NADH dehydrogenase complex I [22–24]				Succ. quinone reductase complex II [25]		Cytochromec *c* reductase complex III [26,27]		Cytochrome-*c* oxidase complex IV [26,28–30]		H^+-ATP synthase complex V [31–33]	
Redox centres	N-1:	2Fe-2S			FAD		b_{562} b_{566} c_1	2Fe2S	a	a_3		
	N-2,3,4:	4Fe-4S			S-1:	2Fe-2S	$\dot{Q}^-$		Cu_A	Cu_B		
	FMN				S-2:	3Fe-4S			Cu_A			
	$\dot{Q}^-$(?)				S-3: (cyt.b)	4Fe-4S						
Polypeptides (kDa)	*Nuclear*		15 kDa(IP)	12.5								
	encoded		B8	11.0							F_1	
			B12	11.0							3-α	55.2
	75kDa(IP)	77.0	13kDa(IP)	10.5	*Water-soluble part*		Core I	47.0	I(a,a_3Cu_B)	53.6 *mt*	3-β	51.6
	51kDa(FP)	48.4	SDAP	10.1			Core II	44.0	II(Cu_A,Cu_A)	26.0 *mt*	1-γ	30.1
	49kDa(IP)	49.2	MLRQ	9.3	A(Fp)	70	b	33.0 *mt*	III	29.9 *mt*	1-δ	15.1
	42kDa	36.7	B9	9.2	B(Ip)	27	c	30.0	IV	17.1	1-ϵ	56
	39kDa	39.1	10kDa(FP)	8.4			FeS	27.0	Va	12.4		
	30kDa(IP)	26.4	AGGG	8.5			VI *Uq binding*	14.0	Vb	10.6		
	24kDa(FP)	23.8	MWFE	8.1			VII	11.0	VIa(L/H)	9.4	IF_1	9.6
	B22	21.7	MNLL	7.0	*Anchor*		VIII	9.2	VIb	10.0		
	TYKY	20.2	KFYI	5.8	*proteins*		IX	8.0	VIc	8.4		
	PDSW	20.8			C	15	X	7.2	VIIa(L/H)	6.2	F_0	
	PSST	20.1			D *Uq binding*	13	XI	6.4	VIIb	6.0	a($ATP_{ase}6$)	24.8 *mt*
	PGIV	20.0							VIIc	5.4	b(F_0I-PVP)	24.7
	ASHI	18.7							VIII(L/H)	4.9	c(6-10)	8.0
	SGDH	16.7	*mt encoded*								d	18.6
	B18	16.5									F6	8.9
	18kDa(IP)	15.3	ND5	67.0							A6L	8.0 *mt*
	B17	15.4	ND4	52.0							e	8.2
	B15	15.1	ND2	39.0							f	10.2
	B14	15.0	ND1	36.0					L, liver		g	11.3
	B14.5b	14.1	ND6	19.0					H, heart		OSCP	22.9
	B13	13.2	ND3	13.0								
	B14.5a	12.6	ND4L	11.0								

complex II, each of these complexes couples electron flow to proton pumping and the ensuing protonmotive force is utilized by complex V (F_0F_1 H^+ ATP synthase) [31–33] to form ATP from ADP and P_i [34,35]. Each of the five complexes is made up of different subunits and complexes I, II, III and IV have multiple redox-active prosthetic groups (Table 1). Thirteen of the subunits of complexes I, III, IV and V are encoded by the mitochondrial genome (mtDNA), all the remaining by nuclear genes [2,36]. mtDNA also encodes for two rRNAs and all the tRNAs involved in mitochondrial protein synthesis [2,36] (Fig. 1).

Recent developments in understanding the mechanism of mtDNA replication and transcription have led to the identification of essential nucleus-encoded components [37–39]. The mitochondrial proteins synthesized in the cytoplasm are imported in mitochondria, processed and assembled in the mature enzymes by complex systems [40,41].

Mitochondrial biogenesis, involving the concerted expression of two different genomes [37,39,42], is likely to be particularly susceptible to mutagenic and other harmful agents. Since all the structural mitochondrial genes code for subunits of the OXPHOS enzymes, the protein factors controlling mitochondrial biogenesis at the level of replication, transcription and translation of nuclear and mitochondrial genes [37,39] and the proteins responsible for recognition by mitochondria, import, processing and assembly into the functional complexes of cytosolic precursor proteins [40,41], have to be coded by nuclear genes. Nuclear-encoded activating factors have been identified which are involved in the coordinated expression of the nuclear and mitochondrial genes of OXPHOS enzymes [39,42]. There are, however, observations indicating that in human cells, at least, the nuclear and mitochondrial transcription factors do not share regulatory polynucleotide sequence elements [43].

A human cell contains several hundred mitochondria and each mitochondria contains 5–10 mtDNA genomes [2]. This relative abundance of mtDNA copies implies that rearrangement and base substitution mutations in an mt gene in germline and/or somatic cells have to affect a substantial proportion of its copies so as to result in a phenotypic decline of OXPHOS and, when more pronounced, in an overt pathological condition [20].

Integration of mitochondrial OXPHOS with cytosolic energy metabolism and ATP utilizing processes is maintained by a series of nuclear-encoded substrate translocators in the inner mitochondrial membrane [44,45], among which the adenine [46], phosphate [47] and pyruvate [48] translocators play a central role. A defect in one of these translocators can also result in a decline of the OXPHOS capacity and in a pathological situation.

3. Developmental changes of OXPHOS

In mammalian tissues the enzymes of OXPHOS undergo definite changes in the transition from fetal to post-natal life. Fetal tissues are characterized by high levels of glycolytic enzymes [3] and a high rate of glycolysis [3,4,49]. At birth, mammalian tissues adapt themselves to the aerobic extrauterine environment with a switch from fetal anaerobic glycolysis to neonatal OXPHOS [4–7,50]. This change is associated with post-natal induction of

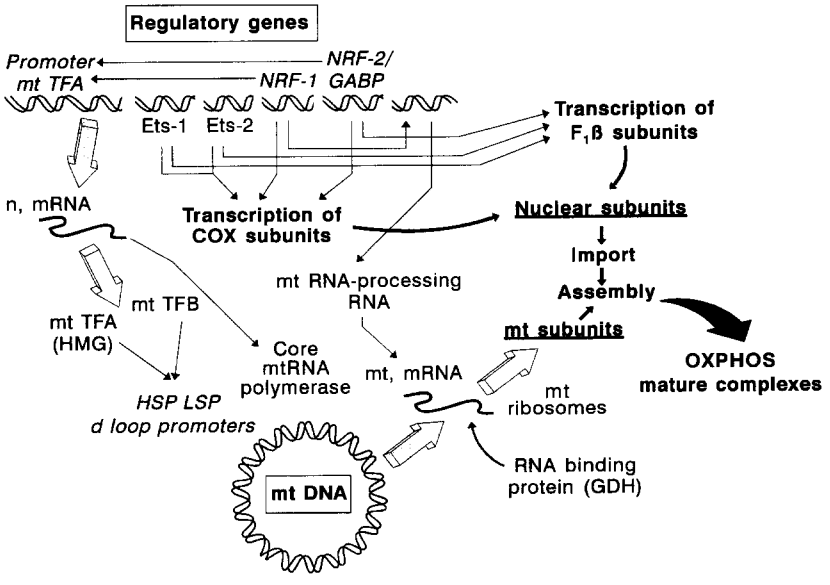

Fig. 2. Scheme describing the interplay of regulatory genes coding for transcription factors of nuclear and mitochondrial OXPHOS genes. mtTFA (HMG, high mobility group) and mtTFB, nuclear transcription factors of mt genome [37], HSP and LSP heavy and light strand promoters in the mt genome [36,37]. Ets-1 and Ets-2, nuclear genes of the Ets family [68] coding for transcription factors of cytochrome-*c* oxidase (COX) [71] and $F_1\beta$ subunit of F_0F_1 ATP synthase [70]. NRF-1 and NRF-2/GABP [73,74] nuclear genes coding for transcription factors of COX, other respiratory enzymes, $F_1\beta$ subunit [56,73,84] and mTFA [72].

mitochondrial biogenesis (mitochondrial differentiation and mitochondrial proliferation) [6,7]. During fetal rat-liver development, nuclear-encoded mitochondrial mRNAs accumulate in a translationally repressed state [51] while the amount and activity of the corresponding enzymes are particularly low [52]. Within one hour after birth, an activation of translation initiation of nuclear encoded mRNA occurs in the liver without changes in the amount of the translational machinery [50]. A marker of the translational activation of these mRNAs is represented by the β-subunit of F_1 ATPase whose level increases in the first hour after birth [50]. It has been proposed that the perinatal enhancement of translational efficiency of the mRNA for the βF_1 subunit could be due to changes in the poly(A)$^+$RNA fraction or in the reporter template itself [52]. The translationally-repressed βF_1 mRNA which accumulates in fetal liver is relatively stable, the post-natal translationally-activated βF_1 mRNA is, instead, more readily susceptible to decay [53]. In fact, there seems to be a direct relationship between the stability of this mRNA and protein synthesis [53].

A role in the translational efficiency and stability of mRNAs seems to be played by matrix dehydrogenases which contain RNA binding sites [54,55].

At variance with *mitochondrial differentiation*, which is associated with rapid post-natal translational activation of mRNAs already accumulated in the fetal liver, *mitochondrial proliferation*, which is a more prolonged phenomenon, depends on the transcriptional activity of genes [53]. The βF_1 nuclear gene is under complex transcriptional regulation. This includes an NRF-2 responsive element [56] (Fig. 2), an enhancer element shared by cytochrome-c_1 and subunit $E_1\alpha$ of pyruvate dehydrogenase [57], nuclear factors interacting with overlapping promoter elements [58] and thyroid hormone responsive elements [59].

The dramatic activation at birth of the expression in liver of nuclear genes coding for subunits of OXPHOS enzymes, as well as of mitochondrial genes [60], which is exerted at both transcriptional and post-transcriptional level, makes perinatal development a most useful model for the study of regulation of mitochondrial biogenesis.

It is worth mentioning here that rapidly growing tumour cells have a reduced number of mitochondria and exhibit a decrease in OXPHOS activity and enhancement of anaerobic and aerobic glycolysis [61,62]. Contrary to the expectation, this reduction in the number of mitochondria has been found to be associated, both in neoplastic tissues [63,64] and in viral and cellular oncogene transformed cell cultures [65–67], with enhanced levels of mRNA transcripts of nuclear and mitochondrial OXPHOS genes. Interestingly enough, among the OXPHOS genes induced in tumour cells there is the βF_1 gene whose transcript level is particularly high in fetal liver [7]. Thus, in neoplastic transformation the expression of OXPHOS genes seems to experience a fetal shift. Induction of OXPHOS genes in transformed cells could represent an abortive compensatory mechanism in response to events which ultimately lead to a reduction in the number of mitochondria and in the overall OXPHOS capacity of neoplastic cells.

It is tempting to speculate that certain critical genetic elements and/or systems involved in the regulation of mitochondrial biogenesis are affected, though in the opposite way, in both development and transformation. A role in this respect could be played by ets proteins [68]. These proteins, which resemble the V-ets oncogenes [69], with which they share an ets-domain interacting specifically with purine-rich DNA sequences, have been found in different species and are involved in the regulation of gene expression in a variety of biological processes [68]. The ets proteins regulate the expression of the βF_1 gene [70] and subunits 4 and 5a of cytochrome-*c* oxidase [71] and are considered to belong to the elements involved in the coordinate enhanced expression of OXPHOS genes in response to proliferative signals [39,70–74] (Fig. 2). It has also been found that a mitochondrial elongation factor similar to the *E. coli* EF-Tu and the mitochondrial EF-Tu of *S. cerevisiae* is overexpressed in a variety of tumours of human and animal origin [75].

4. Adaptation to exercise and OXPHOS

The number and volume of mitochondria relative to other cell constituents and the activity of OXPHOS enzymes are not fixed in adult animals and humans but appear to vary with the pattern of activity performed by the organs. This is clearly exemplified by the skeletal muscle where the number of mitochondria per cell and the specific activity of respiratory enzymes increase with exercise [8,76,77] and the same pattern has been observed in the heart [78]. No information is available for other organs but it is likely that adaptive response of mitochondria to the functional activity load is a general phenomenon in higher eukaryote cells. The content of mitochondria per cell varies also from type to type of striated muscle. Cardiac myocites contain up to 40% of their cell volume as mitochondria in small mammals, whilst type I skeletal fibers of humans contain as low as 1–2% of their cell volume as mitochondria and the mitochondrial content varies even within the same general type of muscle [79]. All this shows that vertebrate organisms have mechanisms which regulate the number of mitochondria and the content and activity of OXPHOS enzymes, at least in contractile tissues, in response to molecular signals generated in mesenchymal cells, as they differentiate into mature tissue cells, or in differentiated muscle and other tissues in response to changes in the functional activity.

A prolonged increase in the contractile activity of skeletal muscle in laboratory animals, as that produced for weeks by daily exercise, induces a dramatic (nearly one order) enhancement in the level and activity of OXPHOS

enzymes and reduction in glycolytic enzymes [8,76,77]. Similar and more rapid and even larger increases in OXPHOS enzymes are observed upon chronic electrical stimulation of specific muscles via the motor nerves in rabbits [80,81]. It should be noted that the induction of functional OXPHOS enzymes in the muscle requires the activation of the coordinate expression of nuclear and mitochondrial OXPHOS genes in the absence of cell division.

Increased contractile activity has been found in rabbit [82,83] and men [84] to enhance the level of mitochondrially encoded RNAs (cytochrome *b*, subunit 1 of complex IV, subunit ND6 of complex I, 16S rRNA), as well as of nuclear encoded RNAs (subunit 4 of complex IV, βF_1 subunit, succinate dehydrogenase) (see also Ref. [85]). The increase in the mRNA transcripts of the nuclear genes encoding mitochondrial proteins was, however, smaller than the observed enhancement of the mRNA transcripts of the mitochondrial genes. Prolonged exercise was also found to be associated with enhancement of the concentration of mtDNA, whereas nuclear DNA content was not changed [82,84,86]. This observation suggests that amplification of the mitochondrial genome relative to nuclear DNA is an important feature of the enhanced expression of OXPHOS enzymes in highly oxidative tissues [84,86].

Critical elements for concerted induction of expression of nuclear and mitochondrial genes encoding for OXPHOS enzymes could be represented by the nuclear respiratory factors (NRF) 1 and 2 which have been found to activate [72] the human mitochondrial transcription factor A gene (see Fig. 2). In fact, mtTFA seems to provide an important control point for both mitochondrial copy number and transcriptional activity [37]. NRF-1 and NRF-2 also activate, on the other hand, the expression of a number of nuclear genes which encode cytochrome-*c*, subunits for three of the five respiratory complexes and the mitochondrial RNA processing RNA [73,74] (Fig. 2). NRF-2 was identified as an activator of cytochrome oxidase gene expression in Hela cells, from which it has been purified to near homogeneity [71]. This multisubunit activator shares homologous subunits with the mouse GA binding protein, a member of the family of the ets protein [68], involved in the expression of Herpes simplex virus immediate early genes [87]. It is likely that ets proteins are involved in the concerted expression of OXPHOS enzymes in skeletal muscle in response to a work load.

5. Aging and mitochondrial DNA mutations

Germline and somatic human cells each contain thousands of mtDNA molecules. mtDNA is maternally inherited; somatic cells of an individual can receive from the mother a certain number of mutated mtDNA molecules mixed with normal mtDNA molecules. When these heteroplasmic cells divide, the normal and mutant mtDNA molecules get randomly distributed into daughter cells and the mtDNA genotype drifts towards pure, normal or mutated (genetic segregation and tissue genetic mosaicism) [2,10,19,20,88,89]. Mutations of mtDNA, occurring in germline or somatic cells, can consist of rearrangements (insertions and/or deletions) and base substitutions [2,20,90].

The mtDNA shows a much higher level of mutation than the nuclear genome [90,91]. This, in addition to reflecting the fact that cells contain thousands of copies of mitochondrial genes compared to only two copies of nuclear genes, it could also be explained by the following observations: (1) mtDNA is located at the matrix surface

Table 2
mtDNA mutations in mitochondrial diseases

Disease	mtDNA mutations
Chronic progressive external ophtalmoplegia, CPEO; Kearn Sayre Syndrome, KSS; Pearson's syndrome	Deletions between np 16 805 and 5786 (4997 bp deletion > 7436 bp deletion) [2]
Late onset diabetes mellitus	10 423 bp deletion [112]
Alzheimer's disease and Parkinson's disease, ADPD	np 3196 in 16S rRNA [113]
	np 721 in 12S rRNA [108]
Aminoglycoside-induced deafness, AID	np 1555 in 12S rRNA [114]
Leber's hereditary optic neuropathy, LHON	np 11 778 in ND4 gene (R to H) [115]
	np 14 484 in ND6 gene (M to V) [116]
	np 14 459 in ND6 gene (A to V) (LDYT) [117]
	np 3460 in ND1 gene (A to T) [118]
	np 15 257 in cytb gene (D to N) [119]
Neuropathy, ataxia and retinitis pigmentosa, NARP	np 8993 in ATP6 gene (L to R or [120,121]
Mitochondrial encephalomyopathy lactic acidosis and stroke-like simptoms, MELAS	np 3243 in $tRNA^L$ [122]
Myoclonic epilepsy and ragged red fibers, MERRF	np 8344 in $tRNA^K$ [123]
Hypertrophyc cardiomyopathy and myopaty, HCM	np 3260 in $tRNA^L$ [124]
Lethal infantile mitochondrial myopathy, LIMM	np 15 923 in $tRNA^T$ [125]
Fatal infantile cardiomyopathy plus, FICP	np 4269 in $tRNA^I$ [126]

Compiled on the basis of data from Refs. given in the table. Additional diseases with different mtDNA mutations have been reported in Refs. [2,108–110]. For details, see Fig. 1 and text. For some of the diseases (LHON, MELAS, MERRF), in addition to those reported in the table, other mutations have also been found [108–111].

of the inner mitochondrial membrane where reactive oxygen species (ROS), causing oxidative damage of DNA [92–94], can be generated by the respiratory chain [95–97]. (2) Apparent absence in mitochondria of DNA-protecting proteins like histones [98]. (3) Mitochondria lack the capacity to repair UV-induced pyrimidine dimers [99]; they seem, however, capable of repairing abasic sites [100], alkali-labile sites [101] and DNA oxidative damage [102]. The capacity to eliminate O^6-ethyl de-oxiguanosine appears to be active in a replicating tissue like liver but not in a post-mitotic tissue like brain [103]. (4) High amounts in mtDNA of direct repeats [104]. Finally there is debate on the fidelity of the mitochondrial DNA-polimerase which is reported to be poor by some authors [105,106] and high by others [107].

The mtDNA undergoes a continuous turnover both in mitotic (epithelial cells, bone marrow cells, hepatocytes, etc.) and post-mitotic cells (brain, heart, skeletal muscle, endocrine systems, etc.) and this increases the chance of mutations [92,108]. In this way mutated mtDNA molecules, maternally inherited or produced by a mutational event in somatic cells, can accumulate with age in post-mitotic tissues. Cells harboring mutated mtDNA molecules, which can be few at birth, with age reach an amount which can impair the overall OXPHOS capacity of the tissue and with it the functional activity of the organ [2,19,20,108].

Some maternally inherited neuromuscular diseases (see Table 2) are associated with mtDNA rearrangements [2,112]. These rearrangements, resulting from a mutational event in the female germline cells, consist of inserted (duplicated) and/or deleted mtDNA molecules (see Table 2) which are transmitted into the somatic cells together with normal molecules (trimolecular mtDNA heteroplasmy). It has been proposed [108] that duplicated mtDNA molecules may be the prevailing maternally transmitted form of mutated mtDNA, which could be converted to deleted molecules [127], these undergoing clonal expansion in various tissues and zones of the same tissue [108]. The extent and time course by which cells harboring a certain percentage of deleted mtDNA molecules replace those with normal mtDNA with age, determines the onset, progression and severity of a mitochondrial disease [2,20,108].

Other neuromuscular diseases are associated with mtDNA base substitutions (Table 2). These base-substituted mtDNA molecules are also maternally transmitted together with normal mtDNA (heteroplasmy) and expand clonally in various tissues and tissue zones with age. Some of the base substitutions can occur in structural mtDNA genes affecting the corresponding OXPHOS complex [128,129], others occur in rRNA genes [113,114] and in tRNA genes [122–126] and consequently have a more general detrimental impact on the biogenesis of the proteins encoded by the mt genome [2,20].

There is evidence showing that maternally inherited and somatic mutant mtDNA molecules are preferentially amplified in post-mitotic tissues [11,20]. Whilst this explains why mutated mtDNA molecules accumulate with age and can determine OXPHOS decline, the molecular mechanism by which their preferential amplification takes place is unknown. It has been proposed that the respiratory and OXPHOS deficiency caused by mutated mtDNA is sensed by nuclei which, in response to this, try to compensate by upregulating replication and expression of the mutated mt genes [20]. Again, a critical point is represented by the nuclear-mitochondrial genome interaction. A role can be played here by the emerging myriad of protein factors, which encoded by the nucleus, regulate the replication and expression of the mitochondrial genome [37,71,130].

The same mutations found in the mtDNA in overt neuromuscular diseases can also occur in somatic cells of normal subjects and, for the reasons described above, they can accumulate with age in post-mitotic tissues. Studies from different laboratories show that aging is associated in humans, exempt from mitochondrial disease, with accumulation of different deletions of mtDNA, in particular in post-mitotic tissues [131–136]. The common 5 kb mtDNA deletion, characteristic of the Kearns-Sayre syndrome, and the 7436 bp mtDNA deletion [2] were found in tissues of old subjects. The percentage of the mtDNA deletions accumulating with age varies substantially from tissue to tissue [2,131]. In brain basal ganglia over 10% of mt DNA contains the 5 kb mtDNA deletion, by the age of 80 years, but cerebellum accumulates a relatively small amount of this deletion [108]. In a study of Lezza et al. [134], the 5 kb mtDNA deletion in human skeletal muscle was found to increase exponentially from the age of 40 to 80 years, when it reached only a plateau of 0.3% of total mtDNA (Fig. 3). In this study a large individual variability in the percentage of the deletion was observed. Although the 5 kb deletion never exceeded the value of 0.3%, a direct relationship was always found between its abundance and the extent of cytochrome-*c* oxidase deficiency [134]. The percentage of the 5 kb deletion appeared to be too low to

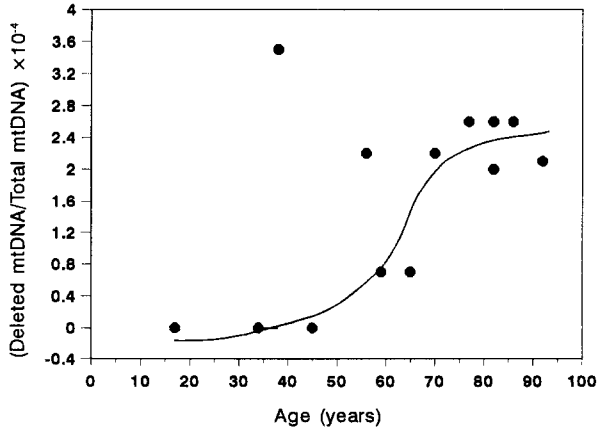

Fig. 3. Accumulation of common 5 kb deletion of mtDNA in human skeletal muscle. Linear plot of deleted mtDNA fraction in biopsy samples of human skeletal muscle vs. age. Reproduced from Ref. [134].

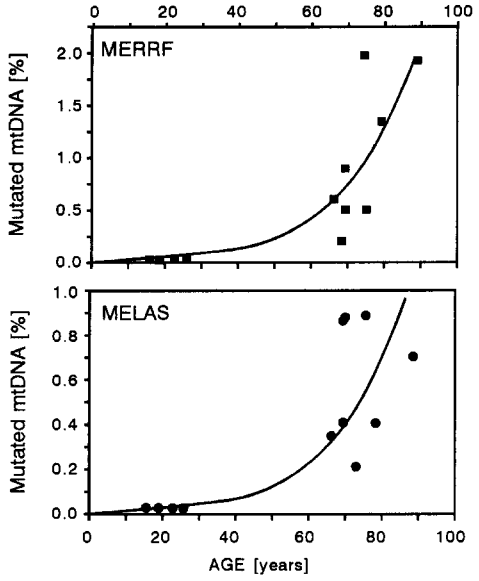

Fig. 4. Quantitative analysis of point mutations characteristic for MERRF and MELAS disease (see Table 2) by PCR-cycle titration in extraocular muscles from healthy humans of various age. Reproduced from Ref. [111].

be considered by itself a cause of the deficiency of cytochrome-*c* oxidase. The 5 kb mtDNA deletion could represent the tip of the iceberg, being indicative of multiple accompanying mutations. An involvement of defective nuclear genes might, however, be possible, as supported, in fact, by observations of Hayashi et al. [137]. This aspect will be further dealt with in Section 8.

Analyses of Kadenbach et al. [111,138,139] (see also [140]) have shown, using a PCR method which detects 0.1% mutated out of total mtDNA [141], that base substitution mutations, characteristic of mitochondrial diseases, are also present in old control subjects (Fig. 4). These studies reveal the following aspects: (1) Specific base substitutions which are absent in young subjects accumulate exponentially with age, reaching values of 1–2% around the age of 80 years. (2) The abundance of base substitutions shows a large individual variability in agreement with the multifactorial characteristic of their propagation [2]. (3) The base substitutions characteristic of MERF (np 8344 in $tRNA^{Lys}$), MELAS (np 3243 in $tRNA^{Leu}$) (Fig. 4) and chronic intestinal pseudo-obstruction (CIPO, np 10006 in $tRNA^{Gly}$) were found in all extraocular muscles of old control subjects [139]. In contrast, different substitution mutations related to other mitochondrial diseases were not generally found in muscles of old control subjects [139]. This would suggest that there are sites in the mt genome which are particularly susceptible to mutations.

What has been discussed leads us to conclude that aging is associated with accumulation in animal tissues of rearrangement and base substitution mutations of mtDNA. In post-mitotic tissues these age-linked mutations of mtDNA become particularly significant as compared to replicating tissues, evidently due to the fact that in mitotic cells normal mitochondria substitute for those which are damaged [142].

In senescent rats the accumulation of mutated mtDNA has also been found to be associated in brain and heart mitochondria, but not in liver mitochondria, with a reduced level of both the 12S rRNA and the mRNA for subunit 1 of complex IV as compared to adult tissues [143]. Contrary to what was expected, the reduction in the level of these transcripts of mtDNA was associated with an enhanced, rather than decreased, copy content of mtDNA [144] (see also Ref. [145]). The decrease in the mtRNA content observed in senescent rat-brain was, in fact, linked to a lower efficiency of the mitochondrial transcription [146]. These observations would suggest that as a compensatory response to defective mitochondrial biogenesis and OXPHOS, senescent cells produce more copies of mtDNA, as it occurs in mitochondrial disease [147]. As already discussed, mutant mtDNA molecules seem to be preferentially amplified as a possible feedback response of the nucleus to defective mitochondrial function. Altered $[ATP]/[ADP] \cdot [P_i]$ ratio, redox state of NAD(P) and/or Ca^{2+} cellular homeostasis could be involved in these compensatory cellular loops.

6. Aging and decline of OXPHOS enzymes

From several studies carried out in various laboratories, it is now clearly established that in tissues of animals and humans there occurs an age-linked decrease in the activity of mitochondrial respiratory enzymes [12–20,135,148] and F_0F_1 ATP synthase [149,150]. The general decline of the OXPHOS capacity is associated with the appearance in senescent tissues of marked structural changes in mitochondria, like enlargement, matrix vacuolization, shortened cristae, etc. [151]. As only part of these damaged mitochondria (light mitochondria as compared to normal heavy mitochondria [152]) can be recovered in the isolation procedure, it is possible that differences in the respiratory and OXPHOS capacity of mitochondria isolated from old versus young animals are underestimated by selective loss of damaged organelles. This may be one reason [153] for the apparent lack of age-associated biochemical changes in mitochondria reported by some authors [154,155].

In humans the most extensively screened tissue is skeletal muscle due to the relative ease of obtaining biopsy samples from subjects exempt from clinical symptoms of mitochondrial diseases. The biochemical analysis of these samples shows that, in skeletal human muscle, aging is associated with decline of mitochondrial respiration [12,14–18]. An extensive epidemiological study, covering more than 200 control human subjects of ages varying from 10 to 90 years, carried out in the author's laboratory [17,18], has demonstrated a statistically significant age-lin-

ked decline of mitochondrial respiration in skeletal muscle, which is associated with decrease of the activity of cytochrome-*c* oxidase (complex IV). No significant decrease with age was apparent for the activity of the ubiquinone-cytochrome-*c* oxidoreductase (bc_1, complex III), when the measurements for females and males were pooled together [17]. Later extension of the number of subjects screened allowed us to examine the sex dependence of age-linked decline of respiratory rates, specific activities of respiratory chain complexes and cytochrome content in mitochondria from human skeletal muscle [18]. It seems particularly instructive to examine in more detail this large collection

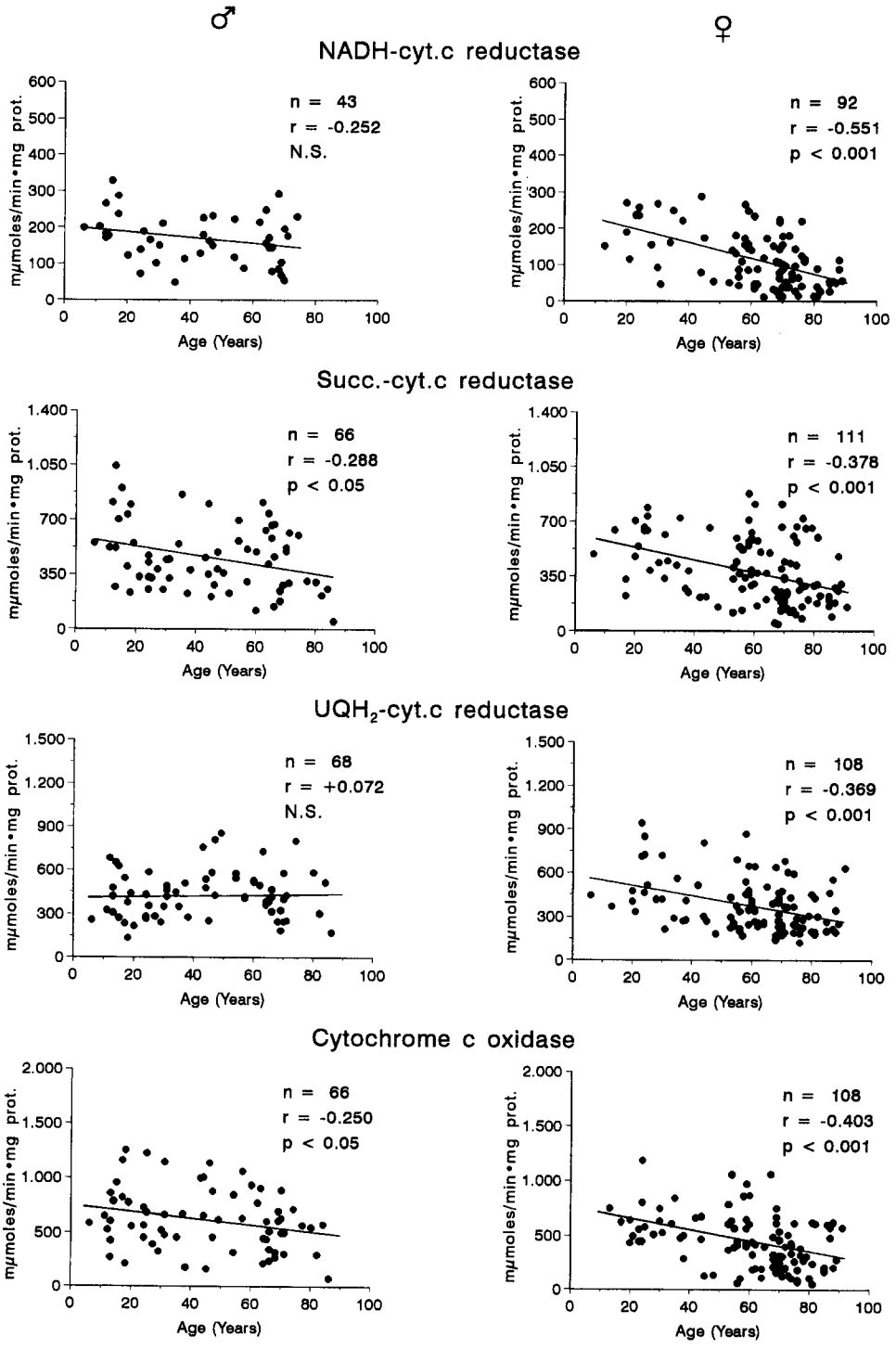

Fig. 5. Statistical analysis of the activities of respiratory chain complexes in human muscle mitochondria as a function of age in males and females. Compiled from Refs. [17,18] and additional data from the author's laboratory (D. Boffoli, S.C. Scacco, R. Vergari, T. Persio, A. Gaballo and S. Papa, manuscript in preparation). For experimental procedure and details see Refs. [17,18].

of data. Plots of the activity of respiratory enzymes as a function of age showed the measured values to be quite scattered, for both sexes, and at each age (Fig. 5). There is evidently a large variability in the genotype and phenotype of humans. This large variability can encompass cases of mitochondrial diseases which could have escaped standard clinical inspection. The possibility, emerging from these observations, is that individuals with capacities of OXPHOS enzymes which are borderline with overt mitochondrial disease, are relatively frequent and these cases should be seriously considered. These situations, which might be without pathological consequences under normal conditions, could result in acute crisis in the energetic capacity of the tissue and sudden organ failure when the individual attempts an intense physical effort or is put under stress. The variability in mitochondrial respiratory capacity can also reflect differences in life style, such as dietary habits, smoking, physical activity, exposure to infective agents and to toxic substances. Statistical analysis of the data, carried out by plotting the respiratory rates as a function of age, however, showed a statistically significant negative correlation [17,18].

Comparison of the data from males and females reveals a number of interesting aspects. (1) The age-linked decline of respiratory rates with pyruvate plus malate, succinate and ascorbate plus TMPD is practically equivalent in males and females [17]. (2) The activity of complex IV, when measured by following spectrophotometrically the oxidation of ferro-cytochrome-*c*, appears to decline more markedly in females than in males (Fig. 5). Both in females and males, the decline in the activity of the oxidase is not accompanied by any significant decrease in the content of hemes $a + a_3$ [17,18]. Thus it cannot be ascribed to a depressed expression of subunit 1 of the oxidase, which binds the hemes and is encoded, together with subunits 2 and 3, by the mitochondrial genome [2,36]. (3) The activity of the bc_1 complex, (Fig. 5), as well as the content of cytochrome *b* [18] in the skeletal muscle of females, exhibit a marked decrease with age that is not observed in males.

Cytochrome *b* is the first largest protein of the three conserved subunits of bc_1 complexes [156]. In the mammalian enzyme, which is made up of 11 subunits [157], cytochrome *b* is the only one to be encoded by the mitochondrial genome [26,158]. The structural gene coding for cytochrome *b* is located in the heavy (H) strand of mtDNA, near the major control region delineated by the displacement D-loop and is affected in some mtDNA deletions observed in human neuromuscular diseases (see Fig. 1 and Table 2). The decline observed in females with aging, together with the observation that the activity of the bc_1 complex is in young females significantly higher than in young males, would indicate that the content of cytochrome *b* in mitochondria and of an active bc_1 complex is directly or indirectly controlled by females sex hormones. Related to these observations seems to be the finding that the physiologically altered function of the human ovary during menopause is accompanied by an accumulation of deleted mtDNA in the ovary [159].

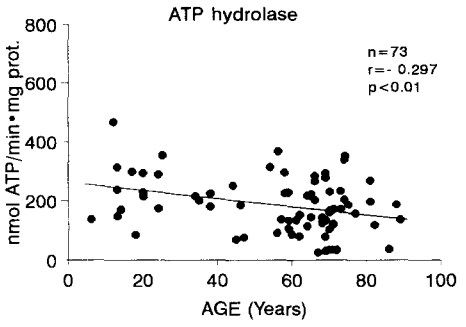

Fig. 6. Statistical analysis of the ATP hydrolase activity in human muscle mitochondria as a function of age. Compiled from data from the author's laboratory (D. Boffoli, S.C. Scacco, R. Vergari, T. Persio, A. Gaballo and S. Papa, manuscript in preparation). For experimental procedure see Ref. [168].

As mentioned in Section 1, the central nervous system derives its energy almost exclusively from OXPHOS, and deficiency of this process is likely to have a severe pathological effect on this tissue. It has been proposed that CNS degenerative diseases, like Alzheimer's disease [108,113], Parkinson's disease, Huntington's disease, heart degenerative disease, late onset diabetes mellitus, which affect humans in their late years, are associated with OXPHOS decline [2,108,160,161]. 'In vivo' analysis of OXPHOS enzymes in these tissues, in the course of aging, is practically impossible. The difficulty can be circumvented at least in some cases by analysis of respiratory activities in blood cells like platelets and by in vivo spectroscopic ^{31}P-NMR (see Section 9).

More recently, the author's group has initiated a screening of the activity of the mitochondrial F_oF_1 ATP synthase (complex V) in human skeletal muscle in control subjects of different ages. The data collected show that the activity of the ATP synthase, measured as ATP hydrolase (Fig. 6), decreases significantly with aging.

The measurement of the activities of the OXPHOS complexes is human skeletal muscle is now being extended, in our laboratory, to a simultaneous semiquantitative analysis of their content and polypeptide composition using the blue-native, non-denaturating, two-dimensional electrophoresis procedure developed by Schägger et al. [162–164].

Studies of Guerrieri et al. [149,150,165] have shown that in rat-heart and rat-brain mitochondria, the content and the activity of the F_oF_1 ATP synthase first increases, going from young to adult animals, and then sharply declines with aging. Age-linked changes in the content and activity of the ATP synthase were also found in rat-liver mitochondria [166,167], although these were much less marked than those observed in heart and brain mitochondria [149,150]. The content and activity of the ATP syn-

thase change also in the course of liver regeneration. Both decrease in the first hours after hepatectomy, than return to control levels when the liver regains the initial mass [166–168]. The mRNA transcript of the βF_1 subunit, on the contrary, shows only some increase in the latter phase of liver-regeneration [167]. The initial decline of the ATP synthase, rather than being due to a depressed transcription, could be associated with enhanced proteolytic degradation triggered by reactive oxygen species [169].

7. OXPHOS and oxygen free-radical balance

Sustained oxidative damage of nucleic acids, proteins and lipids caused by reactive oxygen species (ROS), is considered to be a major factor in the general functional decline of tissues associated with aging and age-associated degenerative diseases like cardiovascular diseases, CNS dysfunctions, immune-system decline [142,153,159,160,170–172] and cancer [173]. It has been calculated that ROS are responsible for around 10000 DNA base oxidative modifications per cell per day and it is quite conceivable that a finite fraction of these damages escapes repair by the array of cellular defense mechanisms [171,172].

The respiratory chain, particularly under certain physio-pathological conditions, is responsible for most of the ROS produced in human tissues [95]. Mitochondrial respiration accounts for about 90% of cellular oxygen uptake and 1–2% of the oxygen consumed is converted to ROS [95]. ROS are also produced by cytochrome P450 and cytosolic soluble oxidases particularly in the presence of oxidant producing drugs [174]. Oxygen superoxide, $\dot{O}_2^-$ is generated by complexes, I, II and III of the respiratory chain [95–97,175] but not by cytochrome-*c* oxidase which seems, on the other hand, to display SOD activity itself [176] (Fig. 7). $\dot{O}_2^-$ production by complexes I, II and III is promoted when the supply of respiratory substrates, derived from food, exceeds the energy demand. Under these conditions the phosphate potential ([ATP]/[ADP] [P_i]) is high, aerobic $\Delta\mu H^+$ in mitochondria depresses respiration (State 4), the electronegativity of autooxidizable redox carriers in complexes I, II and III and cellular oxygen tension rise with consequent enhanced ROS production [95,177]. The autoxidizable redox intermediates in complexes I and II are flavin radicals, nonheme iron and semiquinone radicals, the latter two species are also formed in complex III [96,178]. The level of these reactive reductants and hence of ROS can be enhanced by defects in respiratory complexes or by inhibitors of complex III, like antimycin [96,178], or of complex I like rotenone [175] or neurotoxic meperidine analog derivatives MPTP and MPP^+ [179–181] (see, however, Ref. [182]) which cause Parkinsonism-like symptoms [183]. $\dot{O}_2^-$ produced at the cytosolic surface of the inner mitochondrial membrane can be oxidized by cytochrome *c*, cytochrome-*c* oxidase or converted to H_2O_2 by the cytosolic Zn-SOD. $\dot{O}_2^-$ produced at the matrix side is essentially converted to H_2O_2 by the Mn-SOD of the matrix [184] (Fig. 7). In both the matrix and cytosol H_2O_2 is reduced to H_2O by glutathione peroxidase [185]. H_2O_2 which diffuses to peroxisomes is cleaved there by catalase to H_2O and O_2 [186]. Further-

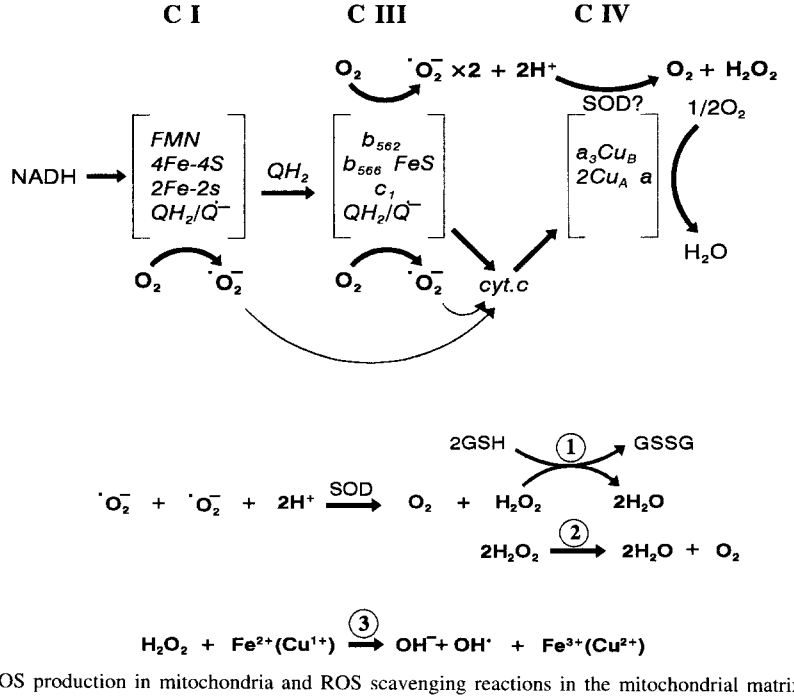

Fig. 7. Sites and reactions of ROS production in mitochondria and ROS scavenging reactions in the mitochondrial matrix, cytosol and peroxisomes. 1, Glutathione peroxidase; 2, catalase; 3, Fenton reaction.

more, ROS are scavenged by vitamins C and E and ubiquinone. The excess H_2O_2 is converted in the presence of Fe^{2+} to OH· by the Fenton reaction [187]. The OH· radical is extremely reactive and produces oxidative damage of lipids, nucleic acid and proteins [171].

With age, the fluidity of cell membranes, including the mitochondrial one, decreases and this is associated with enhanced lipid peroxidation [188]. The linolenic acid content of mitochondrial lipids, in particular of cardiolipin, decreases with increase in more polyunsaturated fatty acids which are more sensitive to oxidation reactions [188]. Cardiolipin oxidative damage is particularly deleterious as this phospholipid seems to be required for the activity of mitochondrial enzymes [189,190] and metabolite translocators [191]. Membrane damage caused by lipid peroxidation can be repaired by phospholipase A_2. This enzyme removes oxidized lipids from membranes [192] and its activity increases under conditions of oxidative stress [193].

Recent availability of adequate analytical methods has made it possible to determine oxidative damage of nucleic acids [92,94,153,194–196]. It has been found that levels of oxidative damage of mtDNA from rat-liver and human brain are several fold higher than those of nuclear DNA [92,196]. A direct marker of oxidative damage of DNA is represented by the conversion of dG to 8-OH-dG [196]. Analysis on mtDNA hydrolysate from human myocardium autoptic samples showed that in normal subjects the content of 8-OH-dG was undetectable up to the age of 40, then started to increase exponentially with age to reach 1.5% of dG residues at 90 years [94] (Fig. 8). Accumulation of 8-OH-dG in DNA could trigger double-strand separation which favors DNA deletions. A clear correlation between the accumulation of 8-OH-dG in mtDNA and mtDNA deletion was, in fact, observed in the myocardium of aging human subjects (Fig. 8) [94]. 8-OH-dG in mtDNA has also been detected in the diaphragm muscle (0.5% of dG residues) [195] and in brain (0.8%) of 85- to 90-year-old human subjects [196].

Oxidative damage of mitochondrial proteins with formation of reactive carbonyl groups has been found to increase with age [171]. These oxidized proteins could undergo cross-linking, proteolysis and loss of functional activity. As previously mentioned in rat heart and brain, aging has been found to be associated with decrease in mitochondria of the content of the βF_1 subunit and depression of the catalytic activity of the F_oF_1 ATP synthase [149,150]. The same decrease in content and activity of the ATP synthase was observed when the formation of the OH· radical was promoted by exposing the isolated mitochondrial membrane to Co irradiation [165].

The enhanced oxidative damage of lipids, proteins and nucleic acids in mitochondria observed in aged humans (and animals) indicates that the balance between ROS production and scavenging is altered in the course of aging so as to result in a progressive age-linked enhancement in the steady-state level of ROS in the cell, particularly in

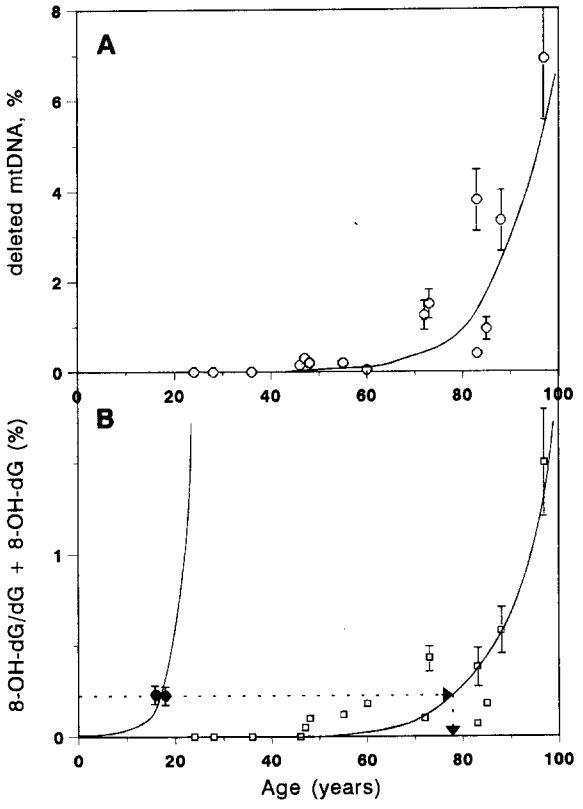

Fig. 8. Correlation in the deletion (A) and oxygen damage (B) in mtDNA associated with age and mitochondrial cardiomyopathy. In (B) the black circles refer to the 8-OH-dG content of mitochondrial cardiomyopathy patients and the arrows with dashed lines indicate their equivalence to that of the normal subjects of age 78. The assay was carried out on autoptic miocardium. Reproduced from Ref. [94].

mitochondria, and/or enhanced sensitivity to ROS of DNA, proteins and lipids. Oxidative damage of mtDNA can lead to mtDNA mutations and this to defective OXPHOS enzymes. Defects in respiratory enzymes deriving from mtDNA mutations and/or nuclear DNA mutations, or directly from oxidative damage of the enzyme proteins and associated lipids can, on their part, lead to enhanced

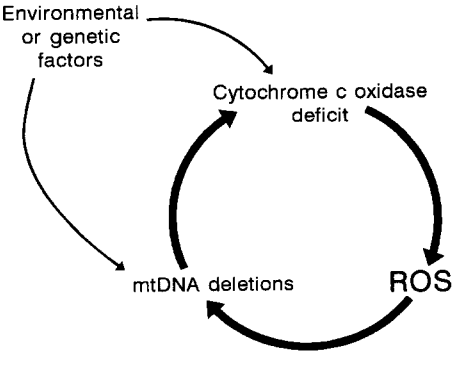

Fig. 9. Vicious cycle for ROS production, mtDNA deletion and cytochrome-*c* oxidase defect in aging.

ROS production. In particular, a defect of cytochrome-*c* oxidase (this enzyme contributes to lower the level of ROS [176]), relative to complexes I, II and III which produce ROS, can contribute to enhance ROS level, which in turn will cause more mtDNA damage. Thus, a vicious cycle can set up which will produce progressive damage of the OXPHOS system (Fig. 9).

8. OXPHOS decline, bioenergetic threshold and apoptosis

Aging in humans is characterized by decrease in the mass and functional capacity of brain, heart, skeletal muscle and other post-mitotic tissues, in particular those having a high demand for OXPHOS [2]. Evidence was reviewed in Section 7 indicating that a major effector of tissue aging is given by oxidative damage of lipids, proteins and DNA. mtDNA is particularly sensitive to oxidative damage, this contributing to age-linked accumulation of mtDNA mutations. We have seen in Section 6 how OXPHOS capacity declines with age, particularly in post-mitotic tissues. However, these observations raise a number of questions which, at present, can only be partially answered. (1) To what extent mtDNA mutations determine the age-linked decrease of OXPHOS; (2) to what extent this decrease is involved in the decline of post-mitotic tissues; (3) are there other events triggered by ROS which can play a determining role in the age-linked decline of these tissues?

Rearrangement and base substitution deletions accumulate exponentially with age particularly in post-mitotic tissues [2,88,94,111]. Studies on cell cultures show that an impairment of energy metabolism is observed only at above 60–90% of mutated mtDNA [111,197–199]. The onset of clinical symptoms in MERRF patients was observed above 90% of mutated mtDNA [200,201]. The percentage of the single mtDNA mutations which accumulate with age, as for example the 5 kb deletion seem to reach significant values in brain, basal ganglia and cortex by the age of 80 [108]. If the 5 kb deletion is accompanied by other mutations, then the total level of mtDNA mutations could reach the critical threshold with impairment of OXPHOS. Under these conditions, it is quite likely that in the brain tissues, which can present a mosaicism of cells with different abundance of mutated mtDNA, those cells which reach the critical threshold of mtDNA mutations start to be functionally compromised and there will occur a progressive reduction in the overall number of cells and functional brain decline. This situation, which is likely to arise from extensive oxidative damage of mtDNA, might in fact be peculiar to dopaminergic neurons which have a high content of monoamine oxidase, an enzyme producing hydrogen peroxide [181,182].

The situation seems to be different in the skeletal muscle. The studies on human biopsies of human subjects have shown that the 5 kb deletion accumulates exponentially with age but it levels off at the age of 80–90 years at a level of only 0.3% (see Section 5) [134]. The respiratory rates with different substrates and the activities of redox complexes and ATPase decline, on the other hand, linearly and markedly with age [17,18]. This would suggest that other factors can be involved in the age-dependent decline of mitochondrial respiratory enzymes. Direct oxidative damage of the protein subunits of respiratory enzymes and of ATP synthase might play a role.

Cytochemical and in situ hybridization studies on extraocular muscle fibers in old subjects show that most of the fibers which presented focal-segmental defects of cytochrome-*c* oxidase, exhibited a normal hybridization pattern for mtDNA, this indicating the contribution in the deficiency of the oxidase of nuclear factors [202]. By means of intercellular transfer of mtDNA and nuclear genome in cultures of human fibroblasts and mt-DNA less Hela cells, Hayashi et al. [137] have produced results indicating that the nuclear genome is involved in the age-linked decline of cytochrome-*c* oxidase in human fibroblasts. Another possible difference between brain cortex and muscle in their response to mtDNA deletions and OXPHOS decline could be represented by a different critical OXPHOS threshold, higher in brain cortex and other nerve cells as compared to muscle cells, so that, due to the age-linked decline, OXPHOS capacity falls below the threshold earlier in nerve cells as compared to muscle.

Normal development and differentiation of multicellular organisms require controlled cell proliferation and death. Alterations in the controlled balance of cell proliferation and/or death appear to be critical in neoplastic proliferation [203–205]. Alteration in the control of cell death may also be involved, on the other hand, in age-linked cell mass reduction and degeneration of post-mitotic tissues. Gene directed programs of cell death (PCD) have been identified in different tissues [206,207]. PCD can be induced by diverse stimuli. However, these appear to activate a common program of biochemical and morphological events, which is apoptosis [206–208]. In the apoptotic death, cells undergo transition from a normal state into successive shrunken forms with reduction of their volume without detectable release of their constituents [206]. The shrunken apoptotic remnants are then phagocytosed by macrophages and their constituents recycled [209].

Apoptosis is promoted by activation of endogenous factors like the cytokine tumour necrosis factor (TNF), the anti-FAS antibody/FAS antigen, sulphated glycoprotein-2, glucocorticoid receptor [210–213] and by exogenous stimuli like cortico-steroid treatment, exposure to ionizing radiation or to exogenous ROS or ROS producing substances [205,214].

Apoptosis is blocked by overexpression of the Bcl-2 protein, whose gene (protooncogene) was discovered at the t (14,18) chromosomal breakpoint in human follicular lymphomas [215]. The Bcl-2 protein is primarily localized

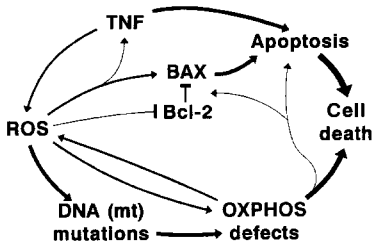

Fig. 10. Possible mechanisms for involvement of ROS, apoptosis, DNA mutations and OXPHOS defects in age-linked cell death. The thin lines indicate additional hypothetical pathways.

in the inner mitochondrial membrane [216–218], but it has also been found in endoplasmic reticulum and nuclear membrane [219,220]. The Bcl-2 protein can form homodimers or heterodimers in the membrane with another protein, BAX (Bcl-2 associated x protein) [218]. Evidence has been obtained indicating that in normal conditions Bcl-2 and BAX are present in the membrane as heterodimer [218]. When Bcl-2 is overexpressed, Bcl-2 homodimers dominate and cells are protected against death. When BAX is in excess, BAX homodimers dominate and cells can undergo apoptosis [205,218].

A possible role of apoptosis in the age-linked reduction of the mass of post-mitotic tissues [181] can, in principle, be reconciled with the proposed role of OXPHOS deficiency in tissue aging [2,108]. Various observations point to a relationship among apoptosis, mitochondrial damage and tissue aging. A link among these processes can in fact be provided by ROS [205,208,214,221]. The role of ROS in mitochondrial damage has already been discussed (Section 7. Continuous exposure of cells to low levels of oxidants seems to trigger apoptosis rather than necrosis [214]. Increasing doses of oxidants progressively result in cell proliferation, apoptosis and necrosis [214,221,222]. Antioxidants have been found to prevent apoptosis induced by TNF, antiFAS/FAS and drugs [221–225].

From what has been discussed, the following picture can be outlined (Fig. 10). As age advances, progressive imbalance between ROS production and scavenging results in enhanced cellular ROS levels with continuous exposure of cell structures to oxidative damage. This seems to result in activation of apoptosis factors, which can take place at gene structure/expression and/or protein level. Apoptosis can be counteracted by enhanced expression of the Bcl-2 protein. The action of Bcl-2 could, however, be itself impaired by progressive oxidative damage. Activation of apoptosis promoters like TNF can contribute to further increase in ROS production [225–227]. Enhancement of ROS cellular concentrations above a critical level and consequent significant oxidative damage of DNA (primarily mtDNA) lead to progressive accumulation of DNA deletions and impairment of the activity of respiratory chain complexes and ATP synthase. Cells undergoing apoptosis exhibit a decrease in the mitochondrial aerobic $\Delta\mu H^+$ [228], indicative of OXPHOS deficiency, and depression of the translation of subunits of cytochrome oxidase and F_0F_1 ATP synthase encoded by mtDNA [229]. This depression in mitochondrial translation is of nuclear origin, since the level of mtDNA is apparently unaffected [229]. Deficiency of respiratory complexes will contribute to further enhance ROS production and thus the vicious cycle already discussed is set up (Fig. 9). Decrease of the respiratory rate results in enhancement of cellular oxygen tension which, in combination with enhanced levels of autooxidizable reductants, enhances ROS production, with accumulation of DNA oxidative damage and mutation, OXPHOS deficiency, activation of apoptosis [230]. Thus, with aging, activation of apoptosis and decline of OXPHOS can converge in causing cell death. This cyclic combination of events can be inhibited at various points by the array of cellular defense systems involved in protein, lipid and DNA repair. Oxidative damages can also be counteracted by exogenous antioxidants like, vitamin E, vitamin C, lipoic acid, etc. These substances are already taken up in good amounts with a balanced diet and can be administered in appropriate doses and combinations when their needs are increased.

Severe prolonged deficiency of vitamin E in man gives rise to a well-characterized neurological syndrome [231] and, although less studied, to a myopathy [232]. Vitamin E deficiency also causes a neuromuscular syndrome in rats [232,233]. This has been shown to be associated with muscle fibre necrosis, reduced mitochondrial membrane fluidity, possibly secondary to lipid peroxidation, and significant decrease of the activities of complexes I and IV [234].

9. In vivo monitoring of OXPHOS capacity

Non-invasive spectroscopic techniques have been developed in the last years and are now used for in 'vivo' monitoring of the bioenergetics of human organs [235–238]. The most widely used of these techniques, and the one giving the more relevant information, is the ^{31}P-nuclear magnetic resonance spectroscopy (NMRS) which provides direct 'in vivo' measurement of the concentrations of P_i, phosphocreatine, ATP, hexose-mono and diphosphates and intracellular (cytosolic) pH in human organs, skeletal muscle in the first place, but also brain, heart, liver, etc. [235].

^{31}P-NMR spectra of skeletal muscle can be taken at rest, during exercise and in the recovery phase [235,236]. At rest, steady-state concentrations of P_i, Pcr, ATP and hexose phosphates reflect the basal energy expenditure for hormonally controlled transport processes and can be of use in exploring the function of endocrine systems and kidney [239,240].

In the resting human skeletal muscle, typical concentrations of phosphoryl compounds are 4 mM [ATP], 0.01 mM [ADP], 25 mM [Phosphocreatine] and 13 mM [Creatine] [235]. Phosphocreatine represents the major reservoir of

high potential phosphoryl groups in skeletal muscle, due to the high concentration of creatine and the high activity of creatine kinase which transfers the phosphoryl group from ATP, produced in excess of the energy demand, to creatine. During exercise, the concentration of phosphocreatine drops dramatically to rise again in the recovery phase. This recovery is directly relevant for 'in vivo' evaluation of the OXPHOS capacity since the contribution of glycogenolysis for ATP production, which can be significant during exercise, is shut during the recovery and ATP is entirely produced by OXPHOS [236]. The post-exercise initial rate of phosphocreatine recovery provides a measure of the rate of ATP production 'in vivo' by OXPHOS. The rate of mitochondrial ATP synthesis by OXPHOS (Q), obtained from the measured rate of phosphocreatine recovery, shows a saturation dependence on the ADP concentration at the end of exercise. This relationship is described by the equation:

$$Q = Q_{max}/(1 + K_m/[ADP])$$

which can be used to calculate Q at half maximal [ADP] and the maximum rate value (Q_{max}) which amounts to about 40 mmol/l cell water per min. The concentration of ADP and hence the rate of aerobic ATP synthesis and the phosphorylation potential ([ATP]/[P_i][ADP]) can easily be calculated from the equilibrium constant of the creatine kinase reaction once the concentrations of P_i, phosphocreatine and ATP are determined by ^{31}P-NMR spectra [236].

Using these procedures, the 'in vivo' phosphorylation potential in the resting human skeletal muscle and maximal capacity (Q_{max}) of OXPHOS in the post-exercise recovery, together with H^+ clearance, have been measured by ^{31}P-NMR spectroscopy in a large number of normal subjects and patients diagnosed or suspected of mitochondrial myopathies [235,236]. These studies have contributed to define a number of abnormalities of energy metabolism which characterize OXPHOS deficiencies [235,236,241]. In nearly all patients affected by mitochondrial myopathies, human skeletal muscles (flexor digitorum superficialis, gastrocnemius), showed a low phosphorylation potential at rest and, more significantly, a slow recovery of phosphocreatine and decreased Q_{max} of OXPHOS. This was accompanied by faster H^+ clearance (recovery from cytosolic acidosis) and increase in [ADP], which in part compensates in defective mitochondria for the decreased Q_{max} [235].

Examination of these parameters by ^{31}P-NMR spectroscopy in healthy subjects over wide age ranges has produced apparently contrasting results. While a study showed a decrease of 'in vivo' OXPHOS capacity in skeletal muscle of aging human subjects [242], another study did not reveal any age-dependent impaired 'in vivo' OXPHOS function in old subjects, as judged from practically unchanged values of Q_{max} of OXPHOS [243]. Whilst the causes for these different findings are not clear at present, it would be important to standardize the measuring conditions. ^{31}P-NMR spectroscopy in combination with other 'non-invasive' spectroscopic techniques (like near-infrared spectroscopy, NIRS [238]), morphometric and ergometric analysis of muscle mass and strength, inspection of heart and CNS function, and imaging NMR for less accessible organs like brain, liver, etc., can provide an ultimate 'in vivo' assessment of the impact of OXPHOS deficiency on the functional activity of tissues, life quality and life expectancy in aged subjects.

10. Conclusions

OXPHOS undergoes definite changes in the life span of animals and humans. The first is observed in the transition from fetal to neonatal life (adaptation to aerobic life). This change involves complex up-regulation of co-ordinate mitochondrial biogenesis. Opposite changes in the pattern and activity of OXPHOS enzymes seem to occur in the malignant transformation of cells.

The biogenetic and catalytic activity of mitochondria are able to adjust the capacity of OXPHOS to the continuously changing energy demand, as shown by up-regulation of mitochondrial biogenesis and function in response to exercise in muscle.

With aging DNA mutations, particularly in the mitochondrial genome, accumulate. mtDNA mutations seem to be primarily caused by ROS whose cellular level increases as a consequence of imbalance between their production, mainly in mitochondria and scavenging. Accumulation of DNA mutations, possibly in combination with direct oxidative damage of protein subunits of redox complexes and ATP synthase, result in the age-linked general decline of the OXPHOS capacity of post-mitotic tissues. Age-linked enhancement of ROS cellular level can induce apoptosis. Apoptotic cell death involves activation of the mitochondrial BAX protein, which can be blocked by the mitochondrial Bcl-2 protein. A study of the effect of ROS on the expression and activity of these two proteins would be highly desirable. The age-linked impairment of OXPHOS can activate apoptosis through enhancement of the cellular level of ROS or by some other mechanism. OXPHOS deficiency can thus lead to apoptotic or different forms of cell death.

Development, physiological adaptation, transformation and aging appear to rely on the 'switching on and off' of certain critical nuclear genes (and protooncogenes) which also affect mitochondrial biogenesis. A particular role can be played, in this respect, by the ets proteins, NRF-1 and NRF-2, BAX and Bcl-2 and TNF. The relationships between these factors, oxidant/antioxidant balance, amplification of mtDNA mutations, OXPHOS defects, apoptosis and their impact in the age-related decline of human beings, will also attract much interest, especially in consideration of the prolonged life-expectancy of humans.

Acknowledgements

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