MINIREVIEW

Oxidative Stress and Mitochondrial DNA Mutations in Human Aging (44205)

YAU-HUEI WEI[1]

Department of Biochemistry and Center for Cellular and Molecular Biology, National Yang-Ming University, Taipei, Taiwan 112, Republic of China

Abstract. **The mitochondrial respiratory system is the major intracellular source of the reactive oxygen species (ROS) and free radicals, which are generated as byproducts during the transfer of electrons from NADH or $FADH_2$ to molecular oxygen under normal physiological conditions. An age-dependent increase in the fraction of these toxic byproducts that may escape the defense mechanism of human and animal cells can induce a broad spectrum of oxidative damage to the biomolecules in the mitochondria and the cell as a whole. Abundant evidence has been gathered to suggest that an elevation of oxidative stress and associated oxidative damages gradually occur in the mitochondria of tissue cells during aging. The mitochondrial DNA (mtDNA), while not protected by histones or DNA-binding proteins, is continually exposed to a high steady-state level of ROS and free radicals in the matrix of the mitochondria. Thus, oxidative modification and mutation of mtDNA occur with great ease, and the extent of such alterations of mtDNA increases exponentially with age. The concurrent enhancement of lipid peroxidation and oxidative modification of proteins in mitochondria elicited by the ever-increasing amount of the ROS further aggravate the mutation and oxidative damage to mtDNA in the aging process. The respiratory enzymes containing the defective mtDNA-encoded protein subunits exhibit impaired electron transport function and thereby increase the electron leak and ROS production, which in turn elevate the oxidative stress and oxidative damage to mitochondria. This vicious cycle operates in various tissue cells at different rate and leads to differential accumulation of oxidatively modified and mutant mtDNAs. This may explain the difference in functional decline and structural deterioration of different organs and tissues in human aging. The central role that alterations of the mitochondria and mtDNA may play in aging and age-related degenerative diseases is discussed in relation to the "Mitochondrial theory of aging."** [P.S.E.B.M. 1998, Vol 217]

Aging is a complex biological process accompanied by a progressive decline in the biochemical and physiological functions of various organs in an individual. A large body of evidence has been accumulated to support the free radical theory of aging as proposed by Harman about 40 years ago (1, 2). It proposes that aging is caused by the accumulation of oxidative damage to various biomolecules brought about by free radicals during a human's life time. It is generally accepted that mitochondria are the major intracellular source and target of reactive oxygen species (ROS) and free radicals (3, 4). Although human cells have developed various enzymatic and nonenzymatic systems to cope with the ROS and free radicals generated in the respiratory chain, a fraction of them may escape these cellular defense mechanisms and cause transient or permanent damage to cellular constituents including nucleic acids, proteins, lipids and membranes (5–7). Recently, many investigators have demonstrated that the rate of superoxide anion and hydrogen peroxide production in mitochondria

[1] To whom requests for reprints should be addressed at Director, Center for Cellular and Molecular Biology, National Yang-Ming University, Taipei, Taiwan 112, Republic of China.

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increases with age in the tissues of many mammals (8–10). Thus, the level of oxidative stress in mitochondria is increased during the aging process (11, 12).

In the past few years, a considerable amount of research has shown that a broad spectrum of oxidative damages caused by elevated oxidative stress is increased in an age-dependent manner (5–8, 13, 14). It is conceivable that the mechanism by which oxidative stress affects the aging process is effected by its infliction of oxidative damage on various vital biomolecules of the cell. Among the aging-related oxidative damages, base modification and mutation of the mitochondrial DNA (mtDNA) have received increasing attention in recent years. It is widely accepted that mtDNA is 10–20 times more vulnerable to mutation and oxidative damage than nuclear DNA (13, 15, 16). To date, more than two dozen large-scale deletions and two point mutations of mtDNA have been reported to occur and accumulate in aging human tissues (17–24). Although the number of aging-associated mtDNA mutations keeps increasing at a very quick pace, the mechanism(s) by which these mutations occur and accumulate in human tissues has remained unclear. This review updates the aging-associated oxidative damage and mutation of mtDNA and discusses the roles that ROS and free radicals may play in eliciting qualitative and quantitative changes of mtDNA in the aging process. In addition, the mitochondrial theory of aging is discussed on the basis of the data obtained from several lines of experimental work gathered in the past few years.

Mitochondrial Respiratory Function Decline in Aging

It is well recognized that physiological functions of various human organs decline with age. The bioenergetic basis underlying this phenomenon has been investigated extensively in the past decade. Müller-Höcker first discovered that a loss of cytochrome *c* oxidase activity occurs in single cells or muscle fibers with increasing frequency in human heart (25), limb muscle, and diaphragm (26) with advancing age. The density of cytochrome *c* oxidase-negative defects in the heart increases from about 3 defects/cm^2 in the third decade of life to 50 defects/cm^2 in individuals over 70 years of age (27). These defects are distributed in the muscle in a random manner that results in a tissue mosaic pattern, which may account for the age-dependent decline of contractile force of the muscle. On the other hand, Yen *et al.* (28) and Trounce *et al.* (29) concurrently demonstrated that mitochondrial respiratory functions decline with age in human liver and skeletal muscle, respectively. Both the respiratory control and oxidative phosphorylation efficiency of the mitochondria are decreased in an age-dependent manner (28). Moreover, the resting (State 4) and ADP-stimulated (State 3) respiration rates and the activities of the respiratory enzyme complexes all decline with age in various tissues (28–33). These findings suggest that the structure of mitochondria is impaired, and thereby the respiration is gradually uncoupled from oxidative phosphorylation in the aging process. It is noteworthy that among the five respiratory enzyme complexes, Complexes I and IV are often found to be the most severely impaired in aged human tissues (30–33). This observation and the fact that there exist seven and three mtDNA-encoded protein subunits, respectively, in these two respiratory complexes led us to search for mtDNA mutations in aging human tissues. Results supported the hypothesis that mtDNA mutations are associated with and a contributory factor of the age-dependent decline of the respiratory functions of mitochondria (30–35).

There is another implication of the observation that mitochondrial function declines with age. Since it has been well documented that the production of ROS and free radicals in mitochondria is enhanced during defective respiration caused by ischemia-reperfusion or specific inhibitors such as rotenone and antimycin A (36–38), we conjectured that the age-dependent decline in respiratory enzyme activities may have some consequences other than lowering the production of ATP in the tissue cells. When the electron transfer function is impaired, the electron leak of the respiratory chain is elevated and thereby enhances the univalent reduction of molecular oxygen to generate superoxide anion and hydrogen peroxide in the mitochondria (39). Recently, Sohal (40) demonstrated that cytochrome *c* oxidase activity declines in the latter part of the life span of the housefly and that a submaximal inhibition of the terminal step of the electron transport chain causes an increase in the rate of hydrogen peroxide production in the mitochondria. Moreover, the age-dependent decline or inhibition of cytochrome *c* oxidase may also decrease the endogenous peroxidase activity of this enzyme (41, 42) and affect the self-removal of hydrogen peroxide by the mitochondria (Fig. 1). In addition, the inefficient or ill-coordinated electron transfer resulting from defective respiratory enzyme complex(es) may alter the reduction potentials of some electron carriers (e.g., ubiquinone) and thereby elicit one-electron reduction of molecular oxygen (39, 43, 44).

Oxidative Stress of Mitochondria

Mitochondria serve as the power plant of human and animal cells by further oxidation of intermediary metabolites (e.g., pyruvate, fatty acids and α-keto acids) to supply most of the ATP needed for various cellular functions. In this energy conservation process, ROS and other free radicals such as ubisemiquinone and flavosemiquinone are generated as byproducts of the electron transport in the respiratory chain (8–10, 43, 44). Normally, these toxic waste products are disposed of by antioxidants and free radical scavenging enzymes, which include Mn-superoxide dismutase (Mn-SOD), catalase, and glutathione peroxidase inside the mitochondria. However, during aging or in some disease states the activities and/or quantities of some of these free radical scavengers are decreased (45–48) so that an increasing proportion of the ROS and free radicals are not efficiently disposed of and thereby elevate the oxidative stress of the mitochondria. In fact, the levels of superoxide anions

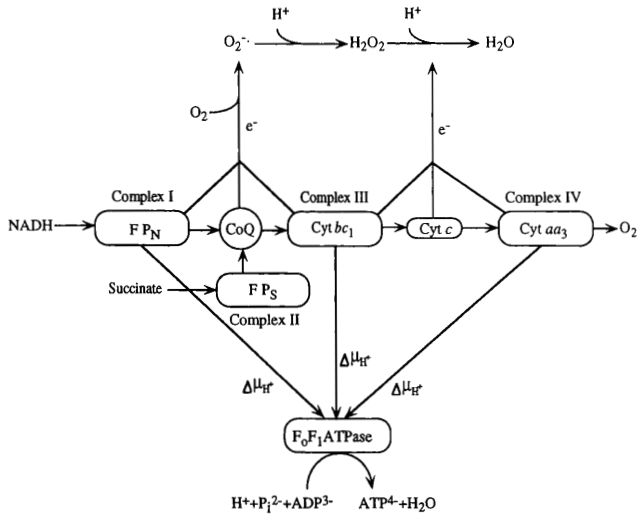

Figure 1. The generation and self-removal of the reactive oxygen species (ROS) by the electron transport chain of mitochondria. A small fraction (1%–5%) of the oxygen consumed in the mitochondria reacts with electrons leaked out from the Complex I and Complex III segments of the respiratory chain to generate superoxide anions, which are effectively removed by Mn-superoxide dismutase, catalase, and glutathione peroxidase under normal physiological conditions. Cytochrome *c* oxidase (Complex IV) can also decompose some of the H_2O_2 in the inner membrane by its peroxidase activity before H_2O_2 is released to the matrix or the cytoplasm. However, the enzyme activities of some of the free radical scavengers and cytochrome *c* oxidase decline with age and the removal of ROS tends to be less efficient and the steady-state level of the ROS in the mitochondria and the whole cell is thus increased upon aging. The increase of oxidative stress will inevitably lead to oxidative damage to proteins, lipids, and DNA of the mitochondria and impair the respiration and oxidative phosphorylation function of the mitochondria. The electron leak and ROS generation by the respiratory chain are thus further increased to elevate the mitochondrial oxidative stress. FP_N and FP_S represent NADH:coenzyme Q oxidoreductase (Complex I) and succinate:coenzyme Q oxidoreductase (Complex II), respectively. CoQ represents coenzyme Q (ubiquinone).

and hydrogen peroxide have been shown to increase significantly with age in various tissues of the animals (9, 10, 49, 50). This in turn may cause oxidative damage to mtDNA (51, 52) and lipid peroxidation of mitochondrial membranes, which contain abundant unsaturated fatty acids (8–10). Moreover, the age-dependent increase in the *in vivo* exposure of tissue cells to lipid peroxides and their derivatives can lead to further elevation of the endogenous level of oxidative stress in the mitochondria and the cell as a whole (6–9, 52, 53).

The notion that oxidative stress plays an important role in the aging process is strongly supported by the recent findings that amelioration of oxidative stress by the overexpression of Cu, Zn superoxide dismutase (Cu, Zn-SOD) and catalase significantly lowers the level of oxidative damage and extends the life span of transgenic *Drosophila melanogaster* (54). Furthermore, it was recently shown that the rates of production of superoxide anion and hydrogen peroxide are well correlated to the rate of mitochondrial respiration, which corresponds to the specific metabolic rate and inversely correlates with the life span of the animal (55, 56). Ames and coworkers first reported that oxidative damage to mtDNA is much more extensive than nuclear DNA, and both damages are dramatically increased in aged animals (13, 16). Shortly after, Sohal and colleagues substantiated those original findings by showing that the specific content of 8-hydroxy-2′-deoxyguanosine (8-OH-dG) in the mtDNA is about 10 times higher than that of nuclear DNA and that both increase with age in mouse liver (57). Similar findings were reported in the whole-body tissues of houseflies (58) and various tissues including brain, heart, liver, kidney, and skeletal muscle of the Mongolian gerbil (12). On the other hand, protein carbonyls and oxidized amino acids, derived from the oxidatively damaged proteins, were found to increase markedly with age (6, 59). Agarwal and Sohal (14) further demonstrated an aging-associated increase of oxidative damage to mitochondrial proteins in the thoracic flight muscle of houseflies. Sohal *et al.* (60) concurrently showed that the specific protein carbonyl content of the flight muscle mitochondria of dipteran flies is inversely related to the average life span potential of the animals. Furthermore, it has long been established that lipid peroxidation is enhanced in aging animal tissues (7, 53, 61). This is caused, at least in part, by an imbalance between the prooxidants and antioxidants in the mitochondria and the cell as a whole (9–14). The mitochondrial inner membrane contains large amounts of unsaturated fatty acids, which are proximal to the ROS generation site and are thus extremely prone to oxidative damage. We have recently demonstrated that the specific contents of lipid peroxides in the submitochondrial particles of human liver, muscle, and testis increase concurrently with age (Table I) (see also ref. 62, 63). All of these oxidatively damaged mitochondrial constituents will further elevate the oxidative stress (8, 11) and aggravate the deterioration of the structure and function of the mitochondria and mtDNA during the aging process (64).

Mitochondrial Genetics

In addition to the nuclear genome, human and animal cells contain a second genome in the mitochondria. The existence of this extra-chromosomal genetic system confers mitochondria the capability to synthesize proteins in a semi-autonomous manner (65). Human mtDNA is a 16,569-bp double-stranded circular DNA molecule that encodes 13 polypeptides, which are required for the assembly of 4 respiratory enzyme complexes, and 2 rRNAs and 22 tRNAs that constitute the mitochondrial translation machinery (66). Each human cell contains a few hundred to more than one thousand mitochondria, and each mitochondrion has 2–10 copies of mtDNA (67). Human mtDNA is strictly maternally inherited (68) and is randomly distributed at the tissue level and randomly segregated during cell division at the cellular level (69). It is possible that more than one type (e.g., wild-type and mutant type) of mtDNA coexist in the same cell, a condition termed "heteroplasmy." Moreover, the distribution of the mutant mtDNA molecules differs widely from tissue to tissue and results in either "focal" or "mosaic" defects upon histochemical or biochemical ex-

Table I. Comparison of the Proportion of the 4977-bp dmtDNA and Lipid Peroxide Content of Mitochondrial Membranes in Different Human Tissues from Subjects of Different Age Groups

Age group (year)	Proportion of 4977-bp dmtDNA ($\times 10^{-3}$%)			Lipid peroxides content (nmol/mg protein)		
	Muscle	Testis	Liver	Muscle	Testis	Liver
1–10	ND (1)	NA	NA	NA	NA	NA
11–20	0.23 ± 0.01 (2)	ND (2)	NA	1.29 ± 0.28 (2)	NA	NA
21–30	1.53 (1)	ND (3)	ND (1)	1.10 ± 0.06 (2)	0.88 ± 0.10 (3)	NA
31–40	10.56 ± 7.89 (6)	ND (2)	ND (3)	1.70 ± 0.25 (7)	NA	NA
41–50	20.25 ± 9.15 (6)	ND (2)	2.67 ± 1.41 (3)	1.93 ± 0.19 (9)	1.22 ± 0.01 (2)	1.34 ± 0.19 (3)
51–60	25.18 ± 9.36 (10)	ND (1)	2.08 (1)	2.01 ± 0.23 (12)	1.40 (1)	2.33 (1)
61–70	34.57 ± 9.21 (7)	14.11 ± 5.29 (7)	3.24 ± 1.48 (3)	3.06 ± 0.32 (14)	2.77 ± 0.32 (9)	4.18 ± 0.38 (3)
71–80	60.02 ± 14.53 (3)	27.47 ± 1.72 (6)	7.63 ± 2.19 (3)	2.26 ± 0.30 (8)	3.53 ± 0.41 (11)	5.08 ± 0.39 (3)
81–90	NA	52.89 ± 3.23 (2)	NA	4.57 ± 1.07 (4)	6.42 ± 1.29 (2)	4.90 (1)

The data are expressed as the mean ± SEM of the results obtained in the specified tissue from the number of subjects indicated in the parenthesis for that age group. NA, not available; ND, not detectable.

aminations of the subjects harboring such an mtDNA mutation. The organization of the human mitochondrial genome is very compact. It has no introns, and both strands are transcribed to generate three types of RNAs for the assembly of a functionally competent protein synthesis machinery in the mitochondria (66). Human mtDNA lacks the protection of histones and other DNA-binding proteins, and is replicated without an efficient proof-reading and DNA repair system (70–73). Moreover, mtDNA is at least transiently attached to the inner membrane of mitochondria (74) and is highly exposed to the ROS and other free radicals that are continually generated by the respiratory chain (3, 8, 9, 12). The random hit of the naked mtDNA by ROS or free radicals will inevitably cause oxidative damage or mutation with serious consequences (12, 16, 52).

On the other hand, mtDNA contains an unusually high amount of direct repeats of the size from 4 bp to 17 bp, which may induce large-scale deletions by slippage mispairing during DNA replication via the so-called D-loop mechanism (75). These peculiar properties of mtDNA and the unfriendly environment in which it is located have rendered it to evolve and mutate at a rate 10 to 20 times higher than that of nuclear DNA (15, 16, 76). Since the concurrent discovery in 1988 of the first disease-associated large-scale deletions (77) and point mutation (78) of mtDNA, more than 50 pathogenic mtDNA mutations have been found in each type of the genes in mtDNA that are associated with or responsible for specific human diseases (76, 79–81). Some of these disease-associated mtDNA mutations and many others have been demonstrated to be also present in various tissues of old humans (22–24, 63, 79–83). We believe that a broad spectrum of mutations of mtDNA occur spontaneously and accumulate in various human tissues in the aging process.

Mitochondrial DNA Mutations in Human Aging

Beginning in the late 1970s, Piko and coworkers conducted a series of studies on the structural alterations of mtDNA in leukocytes of old humans (84) and in senescent rat and mouse tissues (85). They first observed a significant increase in the frequency of rearranged (unicircular dimer) mtDNA molecules in senescent animal tissues. The proportion of the altered mtDNAs was increased from about 0.1% in young adult tissues to approximately 2% in old mouse brain, 1.5% in old rat kidney, 0.6% in heart tissues of old mice and rats, and 4.5% in the leukocytes of an elderly human subject (84). In a later study, they allowed the *Eco* RI-digested rat and mouse mtDNA molecules to denature and reanneal, respectively, and then analyzed the DNA duplexes formed by the 14-kb *Eco* RI-restricted DNA fragment by electron microscopy in search of the mismatched segments. They found that the proportions of the mtDNA molecules with deletions and/or insertions in the liver tissues increased from approximately 1% in adult mice to about 5% in senescent mice (86). They further observed that these aging-related deletions/insertions of mtDNA occur at variable sites, but their distribution is nonrandom. On the basis of these observations together with earlier findings, Piko *et al.* (86) suggested that sequence rearrangements of mtDNA occur and accumulate with aging in animal tissues. Interestingly, the first specific aging-associated mtDNA mutation was demonstrated by Ikebe *et al.* (17) in late 1990 in a study of mtDNA mutation in brain tissues of patients with Parkinson's disease. They detected with PCR techniques a 4977-bp deletion of mtDNA in the nigrostriatum of the brian of the patients and some elderly normal subjects. Many investigators have confirmed this finding (18–21, 82).

In addition to this so-called common deletion, many different types of mtDNA mutations (Fig. 2) have been found to exist alone or together in various tissues of old humans (22–24, 63, 76, 82). At least five different types of mtDNA mutations have been found to be age-related: (1) large-scale deletion, (2) point mutation, (3) insertion, (4) small tandem duplication in the D-loop region, and (5) DNA rearrangement. Many of these mtDNA mutations were originally detected in the target tissues of patients with specific mitochondrial diseases. However, the proportions of the disease-associated mutant mtDNAs in the target organs or affected tissues are generally higher than 80%, but those of the aging-associated mutant mtDNAs are usually no

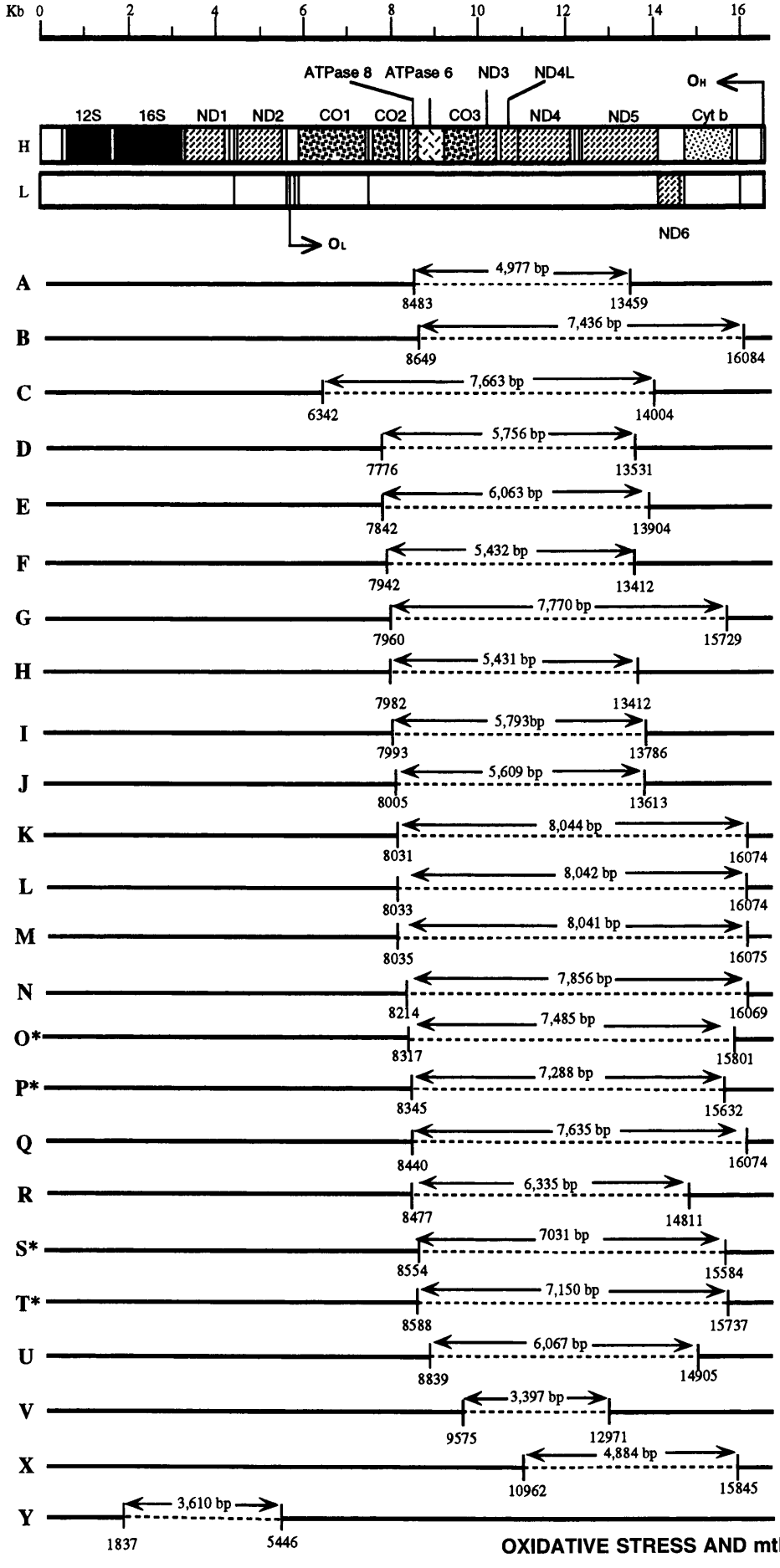

Figure 2. Mitochondrial DNA deletions that have been identified in various tissues of old humans. The shaded or stippled boxes on the heavy (H) and light (L) strands of mtDNA represent the coding regions for protein subunits constituting the respiratory enzyme complexes. O_H and O_L represent the replication origins of heavy and light strands of mtDNA, respectively. ND indicates NADH dehydrogenase, CO indicates cytochrome *c* oxidase, Cyt *b* indicates cytochrome *b*, ATPase represents F_0,F_1 ATPase, and 12S and 16S indicate 12S rRNA and 16S rRNA, respectively. From this figure one can see that large-scale deletions of mtDNA occur most frequently inside the large arc between O_H and O_L and that some structural genes are removed or truncated by these deletions, which will inevitably impair the function of mitochondrial respiratory enzymes that contain defective mtDNA-encoded protein subunits. The mtDNA deletions marked with an asterisk (O, P, S, and T) were detected in the cheek and flank skin of an elderly human subject (ref. 128).

more than 1% (87–90). They tend to occur more frequently and accumulate in the postmitotic tissues with high energy demand, including muscle, brain and heart (87, 88, 91). The aging-associated large-scale deletions seen most often are the 4977-bp and 7436-bp deletions, which are among the many mtDNA deletions first reported to exist in the muscle of patients with KSS or CPEO syndrome (75–77, 79, 92).

In addition to the large-scale deletions, point mutations at np 3243 (A3243G) and np 8344 (A8344G) of mtDNA were also found in the muscle of aged individuals (23, 24). This has lent support to the notion that point mutations of mtDNA also occur during human aging. This was recently re-investigated by Pallotti *et al.* (93), and the results did not support the earlier claim. They found that the A3243G transition and the T8993G transversion of mtDNA occurred in the skeletal muscle of 13 subjects of different ages, but the proportions of the mutant mtDNAs did not increase with age. Kadenbach *et al.* (94) further examined the A3243G and A8344G mutations together with five additional mutations at np 12220, np 12229, np 12230, np 4293, and np 4433 of mtDNA, respectively, in the extraocular muscle of elderly subjects. They found that the proportions of both A3243G and A8344G mutant mtDNAs increase exponentially with age. However, among the five new mutations, only one was found to exist in the aging human muscle. The mutation at np 12220 in the $tRNA^{Ser}$ gene was only detected in two out of seven individuals, and the proportion of the mutant mtDNA was 0.5% in the muscle of an 85-year-old woman (94). They suggested that some unknown point mutations other than the A3243G and A8344G transitions of mtDNA may contribute to human aging. The results from these studies indicate that point mutations of mtDNA occur more frequently in some ''hot spots'' and that not all nucleotide positions are amenable to somatic point mutations. It is possible that the ''hot spots'' are the nucleotides located in specific regions of mtDNA with labile or unusual DNA structures, which have recently been determined to be prone to large-scale deletions (95).

We have found that insertion of nucleotides and small tandem duplications in the D-loop region of mtDNA also occur in the postmitotic tissues (notably muscle) in human aging (96, 97). Recently, Howell and coworkers (98) reported that the mutations in the D-loop of human mtDNA occur more rapidly than previously thought and that new mutations may arise in just one generation time. They detected two germ-line mtDNA mutations in the members of one branch of a large matrilineal Leber's hereditary optic neuropathy (LHON) pedigree leading to descendents with triplasmic mtDNA genotypes. These findings suggest that mutations in the D-loop region of human mtDNA may occur spontaneously at a very high rate. By use of PCR and oligonucleotides directed to the COI and COIII genes of mtDNA, Juretic (99) detected a plethora of mtDNA fragments with rearrangements including deletions and insertions in human liver. Recently, a variety of rearrangements of mtDNA were reported to occur and accumulate in the skeletal muscle of aged individuals (100). Although the amount of each of these five types of age-related mutant mtDNAs seldom reached 1%, some of them were found to coexist in the same tissues in old humans (32, 101–103). Multiple deletions of mtDNA were detected in the skeletal muscle of elderly subjects (32, 102, 103) and in aged rats (104) and mice (105, 106).

Somatic mtDNA mutations have been found also in the tissues of patients with age-related degenerative disorders, which include Parkinson's disease (17, 107), Alzheimer's disease (107–109), and Hungtington's disease (110). When these mutant mtDNAs coexist in any combination in tissue cells, they may exert synergistic deleterious effects on the respiratory function of mitochondria. Recently, Laderman *et al.* (111) investigated the respiratory function alterations of cybrids made by fusion of mtDNA-less human osteosarcoma cells (termed ρ^0 cells) with the enucleated fibroblasts of human subjects with different ages. They showed that both the growth rate and oxygen consumption rate of the cybrids decrease with the donor age of the fibroblasts. Moreover, they found that the oxygen-consumption rate of the 11 respiratory-deficient cybrid clones after extended culture is significantly lower for those derived from the fibroblasts of older donors. They conjectured that the accumulation of age-dependent mtDNA mutations resulting from free radical damage may underlie the age-related respiratory deficiency of the cybrids. This is the first report to show a direct link between mtDNA alterations and the decline of bioenergetic function of the mitochondria in the human aging process.

Oxidative Damage to Mitochondrial DNA

Since mtDNA is located in the vicinity of the ROS generation site in the mitochondrial inner membrane, oxidative damage to this genome has been shown to be very extensive (8, 13, 16). The most widely studied oxidative damage to mtDNA is the formation of 8-OH-dG, which can be readily determined by HPLC/Mass or HPLC equipped with an ECD and UV detector (112–114). Richter *et al.* (16) first reported that the content of 8-OH-dG in mtDNA is about 16 times higher than that in nuclear DNA in 3-month-old rats. Ames and coworkers (13, 112, 113) further demonstrated that the content of 8-OH-dG in the liver mtDNA of 24-month-old rats is 3 times higher than that of 3-month-old rats and that this increase in nuclear DNA is only about two-fold. Hayakawa *et al.* (114, 115) measured the content of oxidatively modified nucleobases in mtDNA of human diaphragm and heart muscle and found that the 8-OH-dG content in mtDNA of both tissues increases with age in an exponential manner. The amount of 8-OH-dG in mtDNA of the diaphragm muscle in an 85-year-old individual was found to be about 0.5% of total deoxyguanosine (114) and that of the heart muscle mtDNA of a 97-year-old subject was determined to be approximately 1.5% (115). Moreover, they found a positive linear correlation between the propor-

tion of the 7436-bp deleted mtDNA and the 8-OH-dG content in the heart muscle.

It is noteworthy that the oxidative damage to mtDNA is much more dramatic in the brain tissues. Mecocci and coworkers (116) measured the 8-OH-dG content of mtDNA and nuclear DNA in three regions of cerebellum and cerebral cortex from 10 human subjects, ages 42 to 97. They found that the 8-OH-dG content was gradually increased in both mtDNA and nuclear DNA, but the extent of increase was greater in mtDNA. The specific content of 8-OH-dG in mtDNA was about 10-fold higher than that in nuclear DNA from various regions of the brain. This correlates with the previous findings of the same group that the amounts of 4977-bp and 7436-bp deleted mtDNAs of human brain tissues increase with advanced age (91). These findings and related evidence have been accumulated to indicate a direct link between oxidative modification of nucleotides and large-scale deletions in mtDNA during human aging. It is argued that oxidative damages to mtDNA can lead to mutations in this genome that have been seen in aging and age-related degenerative diseases. Adachi *et al.* (117) provided the first direct evidence to show that ROS are responsible for the occurrence of mtDNA deletions. They clearly showed that a four-kb deletion of mtDNA was generated in Balb/c mice that had received chronic intraperitoneal injection of doxorubicin, an anticancer drug that has been known to induce cardiomyopathy and elicit profound lipid peroxidation in mitochondria. Administration of coenzyme Q, a free radical scavenger, effectively prevented the mtDNA deletion and decreased the content of lipid peroxides in the heart mitochondria. This finding suggests that ROS is involved in the formation of the deleted mtDNA molecules. However, the mechanism by which ROS and free radicals elicit large-scale deletion of mtDNA remains to be elucidated. On the other hand, oxidative damage to mtDNA may result in quantitative change of mtDNA. Hruszkewycz (118, 119) described that lipid peroxidation can induce the formation of 8-OH-dG and mtDNA-protein adducts and thereby facilitate the degradation of mtDNA, which may lead to a decrease in the content of mtDNA in aging human tissues.

Age-related Depletion of Mitochondrial DNA

In addition to the qualitative changes mentioned above, the copy number of mtDNA in animal tissues has been shown to change also with age. Pertruzzella and coworkers (120) used a DNA probe specific to the D-loop region of mtDNA to measure the content of mtDNA of rat brain tissues by Southern hybridization. The results showed that the content of the D-loop region of mtDNA is significantly decreased with age. By use of the nuclear repetitive LINE sequence as an internal standard, Asano and coworkers (121, 122) demonstrated that the mtDNA/nuclear DNA ratio decreases in the liver and heart muscle of the rat. They found that nearly 50% of mtDNA is depleted in both liver and heart in the 12-month-old rats. In the cybrid study mentioned above (111), Attardi's group also found that the mtDNA/nuclear DNA ratio of the cybrids decreases with the donor age of the fibroblasts. When the oxygen consumption rate was plotted against the mtDNA/nuclear DNA ratio for all 198 cybrid clones, they found a positive relationship between the two indices. However, the statistical significance of this correlation was found to be decreased in the respiratory-deficient cybrids. This observation may imply that age-dependent mtDNA mutation(s) exert more deleterious effect on the respiratory function of these defective cybrids, and the effect of mtDNA depletion is somehow overshadowed under that experimental condition. In a study of the putative role of mtDNA mutations in the development of noninsulin-dependent diabetes mellitus, Serradas *et al.* (123) discovered that the contents of both mtDNA and mtRNA of the islets from adult Goto-Kakizaki diabetic rats are markedly decreased as compared with those of the control islets. However, no such changes were found in the fetal islets of the diabetic rats. They suggested that the long-lasting metabolic dysfunction of the adult diabetic rats may exert environmental insult to the islet mtDNA and thereby impair the replication and transcription of mtDNA. A paucity of mtDNA was also reported in patients with some mitochondrial diseases (124, 125) and in AIDS patients receiving treatment of dideoxynucleosides or azidothymidine (AZT), which are specific inhibitors of viral and mitochondrial DNA polymerases (126).

Concluding Remarks

The past decade has witnessed the remarkable advances leading to our understanding of the important roles that mtDNA play in the maintenance of normal bioenergetic functions of mitochondria and the involvement of oxidative damage and mutation of mtDNA in the pathogenesis of various neuromuscular diseases and human aging (76–81, 97, 127). As the major energy supplier in human and animal cells, mitochondria are actively involved in biological oxidation by channeling the catabolism-generated reduced coenzymes (NADH, and $FADH_2$) into the membrane-located respiratory chain where oxygen is consumed under delicate control. However, a small fraction of the oxygen consumed in mitochondria is not fully reduced by the electron transport chain to form water but results in the ROS. Furthermore, the rate of production of superoxide anions and hydrogen peroxide in mitochondria increases with age. Unfortunately, the activities and quantities of free radical scavengers tend to decrease with age. Thus, the ever-increasing ROS and other free radicals in the mitochondria will inevitably lead to enhanced production of the more vicious hydroxyl radicals in the presence of divalent metal ions (e.g., Fe^{2+} and Cu^{2+}). The ROS and the lipid peroxides thereby generated in the mitochondrial inner membrane will gradually elevate the oxidative stress of the mitochondria in aging tissues. The naked inner-membrane-associated

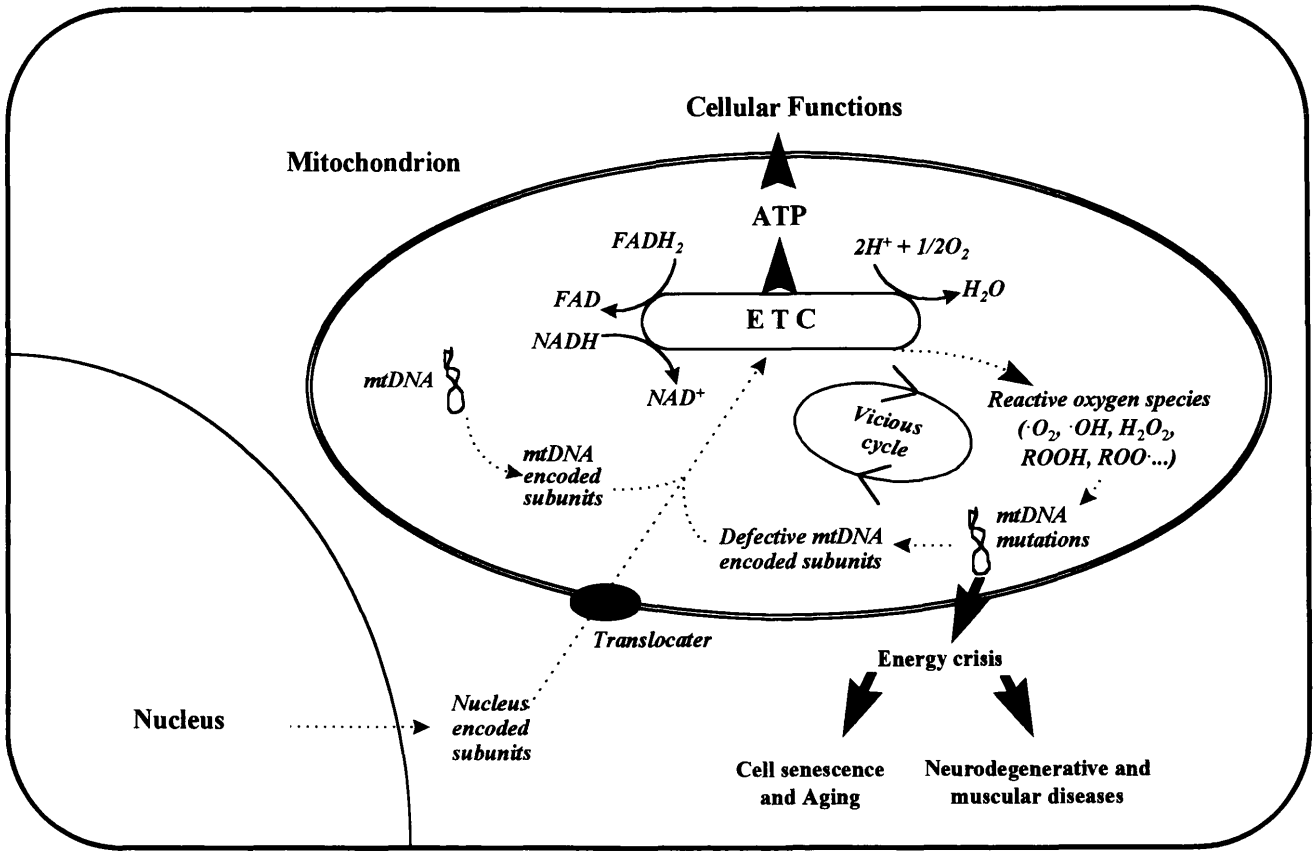

Figure 3. Mitochondrial theory of aging and age-related degenerative diseases. The electron transport chain (ETC) in the mitochondrial inner membrane, which is composedof mtDNA-encoded and nuclear DNA-encoded protein subunits, is actively involved in ATP synthesis-coupled respiration that consumes about 95% of the oxygen utilized by the tissue cells. A small fraction of the oxygen are incompletely reduced by one-electron transfer (mostly via ubisemiquinone) to generate the ROS and organic free radicals (ROO ·), which may cause oxidative damage and mutation of the nearby mtDNA molecules that are attached, at least transiently, to the inner membrane. The oxidatively modified and/or mutant mtDNAs are transcribed and translated to produce defective protein subunits that are assembled to form defective respiratory enzymes. The impaired ETC system not only works less efficiently in ATP synthesis but also generates more ROS, which will further enhance the oxidative damage to various biomolecules in mitochondria. This vicious cycle is operated in an age-dependent manner, and results in the widely observed age-related occurrence and accumulation of oxidative damage and mutation of mtDNA, which ultimately leads to a progressive decline in the bioenergetic function of tissue cells in the aging process.

mtDNA is thus increasingly vulnerable to oxidative damage and mutations. The frequency of occurrence and relative proportion of the mutated or modified mtDNA have been clearly shown to increase exponentially with human age (87, 97, 112–114). As the mutant mtDNAs accumulate during aging in the mitochondria, the functions of the respiratory enzymes containing the mutant mtDNA-encoded protein subunits gradually decline in the tissue cells. The defective respiratory chain not only works less efficiently in the energy conservation process but also generates more ROS and free radicals, which further increase the oxidative stress and oxidative damages to mtDNA and other vital biomolecules in the mitochondria and the cell as a whole. This vicious cycle of oxidative damage to mtDNA by ROS and free radicals (Fig. 3) may be considered the molecular basis for the so-called "Mitochondrial theory of aging." The abundant evidence accumulated in the past decade has lent great support to this novel variant of the free radical theory of aging.

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