MINIREVIEW

Oxidative Stress, Mitochondrial DNA Mutation, and Impairment of Antioxidant Enzymes in Aging[1]

YAU-HUEI WEI[2,*] AND HSIN-CHEN LEE†

Department of Biochemistry and Center for Cellular and Molecular Biology, National Yang-Ming University, Taipei 112, Taiwan; and †Institute of Biochemistry, Chung Shan Medical University, Taichung 402, Taiwan, Republic of China

Mitochondria do not only produce less ATP, but they also increase the production of reactive oxygen species (ROS) as by-products of aerobic metabolism in the aging tissues of the human and animals. It is now generally accepted that aging-associated respiratory function decline can result in enhanced production of ROS in mitochondria. Moreover, the activities of free radical-scavenging enzymes are altered in the aging process. The concurrent age-related changes of these two systems result in the elevation of oxidative stress in aging tissues. Within a certain concentration range, ROS may induce stress response of the cells by altering expression of respiratory genes to uphold the energy metabolism to rescue the cell. However, beyond the threshold, ROS may cause a wide spectrum of oxidative damage to various cellular components to result in cell death or elicit apoptosis by induction of mitochondrial membrane permeability transition and release of apoptogenic factors such as cytochrome *c*. Moreover, oxidative damage and large-scale deletion and duplication of mitochondrial DNA (mtDNA) have been found to increase with age in various tissues of the human. Mitochondria act like a biosensor of oxidative stress and they enable cell to undergo changes in aging and age-related diseases. On the other hand, it has recently been demonstrated that impairment in mitochondrial respiration and oxidative phosphorylation elicits an increase in oxidative stress and causes a host of mtDNA rearrangements and deletions. Here, we review work done in the past few years to support our view that oxidative stress and oxidative damage are a result of concurrent accumulation of mtDNA mutations and defective antioxidant enzymes in human aging.** Exp Biol Med 227: 671–682, 2002.

Key words: aging; mtDNA; mitochondria; ROS; oxidative stress; apoptosis.

Human cells rely on ATP for growth, differentiation, and response to physiological stimuli and environmental challenge. It has been established that mitochondria make ATP by the coupling of respiration-generated proton gradient with the proton-driven phosphorylation of ADP by F_o,F_1ATPase. On the other hand, mitochondria are the major intracellular source of reactive oxygen species (ROS) and free radicals due to electron leakage from the respiratory chain.

It was proposed 45 years ago that free radicals are the major factor involved in the aging process (1). The main idea was that aging is caused by the accumulation of free radical-elicited oxidative damage to various biological molecules in tissue cells. Subsequently, Harman (2, 3) refined the hypothesis and suggested that mitochondria are the major target of free radical attack that leads to human aging. In the last two decades, this "free radical theory of aging" has been widely examined and has gained substantial support from research at the molecular and cellular levels (4). It was Miquel and co-workers (5, 6) who provided first experimental support to this theory in early years by showing that oxidative damage to mitochondrial DNA (mtDNA) and li-

[1] Part of the content of this article is derived from information gathered from work supported by research grants from the National Science Council (NSC90-2320-B010-079) and the National Health Research Institutes (NHRI-EX91-9120BN), Republic of China. One of the authors, Y.-H.W., wishes to express his appreciation to the National Science Council for the long-term financial support in the study of mitochondrial role in human aging. This manuscript is an update of a previously published minireview entitled, "'Oxidative Stress and Mitochondrial DNA Mutations in Human Aging,'" Proc Soc Exp Biol Med **217**:53–63, 1998.

[2] To whom requests for reprints should be addressed at Professor Yau-Huei Wei Department of Biochemistry, School of Life Science National Yang-Ming University Taipei, Taiwan 112, Republic of China. E-mail: joeman@ym.edu.tw

1535-3702/02/2279-0671$15.00

pofuscin pigment formation in animal tissues are concurrently increased during aging. On the basis of the fact that mitochondria are the major intracellular source and vulnerable target of ROS, Linnane *et al.* (7) put forward a timely hypothesis in 1989 to campaign that accumulation of somatic mutations in mtDNA is a major contributor to human aging and degenerative diseases. This so-called "mitochondrial theory of aging" emphasized that enhanced production of ROS and accumulation of mtDNA mutations in mitochondria of postmitotic cells are a contributory factor to human aging.

In recent years, convincing data have been accumulated to suggest that mitochondria act like a timer that ticks all the way through the aging process. Under normal physiological conditions, a small fraction of the oxygen consumed by mitochondria is constantly converted to superoxide anions, hydrogen peroxide, hydroxyl radicals, and other ROS. Although within a certain local concentration range, ROS play important roles in regulating many cellular functions and acting as a secondary messenger to activate specific transcription factors such as NF-κB and AP-1 (8), an excess production of ROS is harmful to cells. To cope with the ROS, animal and human cells express an array of antioxidant enzymes, including Mn^{2+}-dependent superoxide dismutase (MnSOD), copper/zinc (Cu/Zn) SOD, glutathione peroxidase (GPx), glutathione reductase (GR), and catalase (CAT). MnSOD and Cu/ZnSOD convert superoxide anions to hydrogen peroxide, which is then transformed to water by GPx or by CAT. However, the activities of these antioxidant enzymes and the concentrations of small-molecular-weight antioxidants in blood and tissue cells are altered (mostly declined) in the aging process. Thus, there is an age-dependent increase in the fraction of ROS and free radicals that may escape these cellular defense mechanisms and exert damage to cellular constituents, including DNA, RNA, lipid, and proteins. Any signal or stimulus that triggers overproduction of ROS may induce the opening of the membrane permeability transition pore in mitochondria and release of cytochrome *c* and other apoptogenic factors, which ultimately lead the cell into apoptosis (9).

In the past decade, investigators in this and other laboratories have established that respiratory function of mitochondria declines with age (10–12). In addition, it has been shown that impairment of electron transport chain, elicited by respiratory inhibitors, mtDNA mutation, or gene knockout results in enhanced production of ROS in mitochondria due to incomplete reduction of oxygen (13–15). Thus, it is reasonable to conjecture that the aging tissues harboring mtDNA mutations exhibit defective respiratory function and are exposed to higher oxidative stress elicited by enhanced production of ROS in mitochondria. In this article, we review recent advances in molecular and cellular biology research of aging pertaining to the "mitochondrial theory of aging" on the basis of research work done in this and other laboratories.

mtDNA MUTATION IN AGING

In the past 15 years, a large number of large-scale deletions, point mutations, and tandem duplications of mtDNA have been found in various tissues of aged individuals (Table I) (12, 16, 17). Each mammalian cell contains several hundred to more than one thousand mitochondria, each of which carries two to 10 copies of mtDNA (18). The mutant mtDNA(s) usually co-exist with the wild-type mtDNA within a cell (a condition termed heteroplasmy), and the degree of heteroplasmy often varies in different tissues of the same individual (19, 20). The random segregation of mtDNA during cell division often results in the mosaic pattern of distribution of mutant mtDNA molecules in the affected tissues. It has been established that many of these mtDNA mutations start to occur after the mid-thirties and they accumulate with age in postmitotic tissues of the human body (16, 21–32). Some of these aging-associated mtDNA mutations were originally observed in the affected tissues of patients with mitochondrial diseases. Among them, the most common one is the 4,977-bp deletion with a 13-bp direct repeat flanking the 5′– and 3′–end breakpoints at nucleotide position (np) 8,470/8,482 and np 13,447/13,459, respectively (33, 34). This mtDNA deletion was first observed in the muscle of patients with chronic progressive external ophthalmoplegia, Kearns-Sayre syndrome, and Pearsons' syndrome (33, 35). Multiple large-scale deletions of mtDNA can be easily detected, but at different levels in a number of tissues of elderly subjects (16, 26, 31). Two point mutations at np 8,344 and np 3,243 of mtDNA, which are respectively associated with myoclonic epilepsy and ragged-red fibers (MERRF) and mitochondrial encephalomyopathy, lactic acidosis and stroke like episodes (MELAS) syndromes (36), have been also found to accumulate in the muscle of aged individuals (37–39). Recently, it was reported that a T414G transversion in the D-loop of mtDNA is accumulated at 25%–50% of the total mtDNA in the skin fibroblasts from elderly subjects (40). This mutation is located at the control region of mtDNA and may impair the replication and transcription of mtDNA in tissue cells of the elderly human subjects. Additionally, we found that six different tandem duplications occur in the D-loop region of mtDNA from the brain, muscle, liver, and skin tissues of normal subjects and that the incidence and abundance of mtDNAs with these tandem duplications increased with age (16, 29). It is noteworthy that there is a high degree of heteroplasmy in the D-loop region of mtDNA in the human brain, and the sequence variations are increased in aged individuals (41).

It is important to note that the proportions of mtDNA with these mutations in aging human tissues rarely exceed 1% (16, 26, 28, 29, 31, 32). It has been questioned as to how can such low levels of mtDNA mutation cause significant effects on the bioenergetic function of mitochondria in aging. One of the possibilities is that the mutated mtDNA molecules in aging tissues may be underestimated due to the

Table I. Aging-Associated mtDNA Mutation in Human Tissues

	Nucleotide position	Tissues harboring the deletion	References
Deletions			
4977 bp	8483 to 13459	Liver, muscle, brain, heart, lung, spleen, testis, diaphragm, kidney, adrenal gland, and skin	21, 24–26, 28, 30, 32
7436 bp	8649 to 16084	Heart, muscle, liver, and skin	22, 31, 53
6063 bp	7842 to 13904	Muscle and liver	27, 53
3610 bp	1837 to 5446	Muscle	23
5827 bp	7993 to 13786	Muscle	26
6335 bp	8477 to 14811	Muscle	26
7635 bp	8440 to 16074	Muscle	26
8041 bp	8035 to 16075	Brain	26
Point mutations			
A3243G	3243	Muscle	38
A8344G	8344	Extraocular muscle	37
Duplications			
260 bp	–567/301	Muscle, skin, and liver	16, 29, 31
200 bp	–493/301	Muscle and skin	16, 29, 31

Note. The A3243G transition of mtDNA was found to be not associated with aging in human lung (67).

limitation of available analytic methods. To tackle this question, a comprehensive detection system was designed for screening of all possible large-scale deletions of mtDNA in human tissues (42). By using 180 kinds of PCR primer pairs, this system has made it possible to detect all the deletions in mtDNA over 500 bp. Hayakawa *et al.* (42) applied this system to analyze mtDNAs from normal hearts of human subjects of different ages. They found that mtDNA molecules in the heart muscle of elderly subjects were extensively fragmented into minicircles with different sizes. As a result, the total amount of mtDNA mutations, including point mutations and length mutations, may reach such a high level that it could cause significant impairment on mitochondrial respiration and oxidative phosphorylation (43). The other possibility is that the mutated mtDNA molecules may be distributed unevenly among the cells of affected tissues (a mosaic pattern of mtDNA segregation). It is equally plausible that mutated mtDNA molecules are distributed in the affected muscle fibers in a segmental manner. Indeed, the respiratory function of skeletal muscle mitochondria in the fiber segments harboring high proportions of mutated mtDNAs is severely impaired (44). These observations are supported by the recent reports that mtDNA rearrangements are extensive and the levels of full-length mtDNA are reduced in the cytochrome *c* oxidase-negative fibers of the skeletal muscle of aged individuals (45, 46).

Functional Decline of Mitochondria in Aging

It is generally accepted that accumulation of mutated mtDNA is a contributory factor for the age-dependent decline of the respiratory function, especially in postmitotic cells (7, 12, 19). The five enzyme complexes in the respiratory chain are composed of more than 100 polypeptides, most of which are encoded by genes in the nuclear genome. However, 13 polypeptides involved in the respiration and oxidative phosphorylation are encoded by mtDNA. The respiratory function and number of mitochondria are delicately regulated by a coordinated expression of mitochondrial proteins encoded by the nuclear and mitochondrial genomes (47, 48). It was recently demonstrated that mitochondrial biogenesis and respiration of the human cells are vigorously controlled by the nuclear respiratory factors NRF-1 and NRF-2, mitochondrial transcription factor A (mtTFA), and the thermogenic coactivator PGC-1 (a 90-kDa nuclear protein) (48, 49). Thus, any molecular defect that leads to altered expression of the mtDNA-encoded genes or impairment in the biogenesis of mitochondria would cause a deficiency in energy metabolism of the affected tissue cells.

There are three lines of research that lead to the same conclusion that bioenergetic function of mitochondria declines with age. First, it was found that cytochrome *c* oxidase-negative fibers in human heart, ocular muscle, and diaphragm are increased with age (50–52). Second, it was demonstrated in late 1980s that the respiratory function of isolated mitochondria and electron transfer activities of respiratory enzyme complexes gradually decline with age in human liver (10) and skeletal muscle (11, 53), respectively. Because the age-dependent decline of the glutamate-malate-supported respiration was found to be more dramatic than that of the succinate-supported respiration, it was suggested that mutation(s) in the seven genes of NADH dehydrogenase encoded by mtDNA may be involved in this aging-associated respiratory function decline. Beside this, it was observed that the extent of mtDNA mutation strongly correlates with the progressive decrease of cytochrome *c* oxidase activity in aging human muscle (52). These findings have been confirmed in later studies on various human tissues (11, 54, 55).

On the other hand, a reduction in the steady-state levels of mtRNA was reported in *Drosophila melanogaster* (56)

and in the aging tissues of the human and rats (57–60). The decrease in the levels of mitochondrial transcripts may result in the decline of protein biosynthesis and mitochondrial respiration. The age-related decrease in the transcripts of mtDNA may result from a decline in the efficiency of mitochondrial transcription or reduction in the copy number of mtDNA in tissue cells. Indeed, it was demonstrated that the efficiency of mitochondrial transcription is decreased in aged tissues of the rat (57, 58). Oxidative stress may cause incomplete processing of the transcripts of the mitochondrial genome encoding ATPase subunits 6 and 8 plus the adjacent gene for cytochrome *c* oxidase subunit III (61). However, the mtDNA copy number was found to increase with age in *D. melanogaster* (62) and in various tissues of the rat (63) and humans (59, 60, 64, 65), respectively. It was argued that the age-related increase in mtDNA content is not correlated with the lower level of transcripts and a decline in mitochondrial function. This can be rationalized by the fact that mutation and oxidative damage of mtDNA are increased with age in various tissues of the human (12, 16, 28, 29, 66–68) and animals (63). These observations suggest that both the decrease in the efficiency of mitochondrial transcription and decline in the quality of mtDNA contribute to the reduced steady-state levels of mitochondrial transcripts in aging tissues.

Last, it was found that the mitochondrial membrane potential, the driving force for oxidative phosphorylation, is decreased in tissue cells of old animals (44, 69–71). This was supported by the recent work of Harper and co-workers (72), who demonstrated quantitatively that the proton leakage of the respiratory chain (for heat production) is increased and ATP synthesis is decreased with age in the liver mitochondria of the mouse. Taken together, data accumulated from the above three lines of research strongly support the view that the bioenergetic function of mitochondria declines with age in the human and animals.

Eenhanced Oxidative Stress and Damage in Aging Tissues

Because mitochondria are not only the main producer of ATP but are also the major intracellular source of ROS in the human cell, it is expected that mitochondrial disorders be primarily manifested in the organs or tissues that have a high demand for energy. We demonstrated in early 1990s that oxidative damage and mutation of mtDNA are increased with age more conspicuously in those tissues with higher energy demand (16, 28). Under normal physiological conditions, ROS and free radicals (e.g., ubisemiquinone and flavosemiquinone) are generated and maintained at a relatively high steady-state level in mitochondria of tissue cells (73, 74). Respiratory enzyme Complex I and protonmotive Q cycle operating in Complex III are the major sites that generate ROS in the respiratory chain (74, 75). It was estimated that one normal rat liver mitochondrion can produce about 3×10^7 superoxide anions in a day (7).

It has been well documented that the rate of production of superoxide anions and hydrogen peroxide in mitochondria is increased with age in animal tissues (76–78). It was found that the increase in hydrogen peroxide production of *D. melanogaster* under oxidative stress is related to the oxidative damage to mtDNA and membrane lipids of mitochondria (79). Sohal *et al.* (80) further demonstrated that the average lifespan of dipteran flies is inversely correlated with the rate of production of superoxide anions and hydrogen peroxide in mitochondria and with the level of protein carbonyls in the tissue cells. Moreover, the age-related increase in the rate of generation of hydrogen peroxide in mitochondria was observed to decrease 40% in the fruit flies overexpressing Cu/ZnSOD and catalase as compared with the wild-type flies (81). Therefore, the rate and amount of hydrogen peroxide generated by mitochondria is an important determinant of the oxidative damage sustained by mitochondria. Richter *et al.* (82) first demonstrated that oxidative damage to mtDNA is much more extensive than that to nuclear DNA. The specific content of 8-hydroxy 2′-deoxyguanosine (8-OHdG), an index of oxidative damage to DNA, of mtDNA was about 16 times higher than that of nuclear DNA in the liver of 3-month-old rats. Furthermore, the 8-OHdG level in liver mtDNA of the 24-month-old rat was three times higher than that of the 3-month-old rat. Moreover, the levels of oxidative stress and proteins with oxidative modification and lipid peroxides in mitochondria have been shown to increase with age (83, 84). In addition, the 8-OHdG contents in the mtDNA of human diaphragm, heart muscle, and brain tissues were found to increase in an age-dependent manner (66, 85). These observations are consistent with the report that mitochondrial glutathione is markedly oxidized with aging in the rat and mouse (86). The ratio between the oxidized and reduced glutathione rises with age in the liver, kidney, and brain of these animals. In the same study, the 8-OHdG content of mtDNA was also found to increase with age of the rat and mouse. Moreover, these investigators showed that oral administration of antioxidants protected the animals from glutathione oxidation and mtDNA damage. Several recent studies also demonstrated that human cells harboring mutated mtDNA and/or defective mitochondria had lower respiratory function and exhibited higher rate of production of superoxide anions, hydroxyl radicals, and hydrogen peroxide (14, 71, 87). Laderman *et al.* (88) investigated the respiratory function of a series of cybrids constructed by fusion of mtDNA-less human osteosarcoma cells with human fibroblasts from donors of different ages. They showed that both growth potential and oxygen consumption rate of the cybrids with respiratory deficiency are significantly lower for those made from the fibroblasts of older donors. They suggested that accumulation of age-dependent mtDNA mutations resulting from damage by free radicals and ROS might be responsible for the age-dependent decline of respiratory function of the cybrids.

On the other hand, age-related increase in mitochondria may provide an extra source of oxidants. Overproliferation of abnormal mitochondria generally occurs in the muscle of aged individuals and of patients with mitochondrial myopathy. An increase in mtDNA copy number has been observed in aging human tissues (59, 60, 64, 65). In addition, both mitochondrial mass and mtDNA copy number are increased during *in vivo* aging process and *in vitro* cellular replicative senescence (89, 90). In a previous study, we demonstrated that sublethal levels of oxidative stress caused an increase in the mitochondrial mass and the mtDNA copy number of human cells (91). Moreover, we found that ROS production was elevated in the cells harboring higher density of mitochondria (90, 92). Therefore, these observations suggest that the age-dependent increase in the production of superoxide anions and hydrogen peroxide from the defective mitochondria is one of the factors involved in the decline of respiratory function during the aging process.

These findings and other evidence have been accumulated to support the notion that oxidative stress is an important contributor to the decline of mitochondrial respiratory function during aging (12). Mitochondrial function is sharply declined in cultured human cells after treatment with hydrogen peroxide (93). Beside exogenous oxidative insults, endogenous oxidative stress elicited by electron leakage of the mitochondrial electron transport chain was suggested to cause a loss of mitochondrial function (94). Moreover, it was recently shown that an increase in mitochondrial ROS production contributes to the decline in the activities of NADH dehydrogenase and succinate dehydrogenase in skeletal muscle and heart of the MnSOD-deficient mice (95). This is the first direct proof that mitochondrial respiratory function is impaired under oxidative stress. These observations suggest a close relationship between oxidative stress, indicated by glutathione oxidation and oxidative damage to mtDNA, and mitochondrial dysfunction in human and animal cells during the aging process (Fig. 1). We have viewed this mitochondrial role in aging in the scenario of a "vicious cycle" (12, 67, 92). However, it remains unclear whether the decline in mitochondrial respiratory function during aging mainly results from oxidative stress or is a consequence of synergistic effects of many factors associated with aging

The Impairment of Antioxidant Enzymes in Aging

It is generally accepted that the activities and capacities of antioxidant systems of tissue cells are declined with age, leading to the gradual loss of prooxidant/antioxidant balance and accumulation of oxidative damage in the aging process. Although many investigators have studied aged-related changes in antioxidant defenses, the results are controversial (96). It is worth mentioning that the activity of MnSOD located in the mitochondria is most significantly elevated during aging in various tissues of the human and animals. Recently, it was found that human cells overexpressing MnSOD resulted in cell cycle arrest, decrease in mitochondrial mass, accumulation of intracellular hydrogen peroxide, and induction of mRNA levels of matrix-degrading metalloprotease-1, which plays a major role in the process of carcinogenesis and aging (97). This indicates that superoxide anions produced by mitochondria may be scavenged by MnSOD, but hydrogen peroxide thus accumulated in mitochondria may increase oxidative stress during the aging process. Moreover, the recently developed MnSOD knockout mice exhibited dilated cardiomyopathy, neonatal lethality, severe defect in mitochondrial function, increased cortical damage in ischemic injury, and enhanced oxidative damage to mitochondria (98). The proper level of expression of MnSOD is important for cells to cope with oxygen radical-mediated chemical toxicity. On the other hand, alterations in the levels of Cu/ZnSOD, CAT, and GPx are also important contributory factors in the aging process. It has been observed that the fruit flies with homozygous mutations in either Cu/ZnSOD or CAT gene exhibit increased sensitivity to oxidative stress and have reduced viability and shorter lifespan (99, 100). Flies overexpressing Cu/ZnSOD alone or in combination with the overexpression of CAT exhibited higher resistance to oxidative stress, had significantly less oxidative damage to proteins, and lived longer (81, 101). The aforementioned results indicate that a balance between the free radical scavenging enzymes is important for the cellular resistance to oxidative stress.

Furthermore, caloric restriction was demonstrated to extend the average and the maximum lifespan, and to concurrently decrease the age-related accumulation of 8-OHdG in various tissues of the mouse (77, 102). It was recently found that the activities of antioxidant enzymes such as CAT were increased and the age-related deterioration of respiratory enzymes was retarded in the liver of the rats fed on a restricted diet with 60% calorie of the *ad libitum* diet (103). These observations imply that oxidative damage to DNA and other cellular components elicited by oxidative stress and free radicals generated by aerobic metabolism indeed play an important role in aging (104).

Common Cause of Oxidative Damage and mtDNA Mutation

Although the proportion of the mutant mtDNA was found to correlate with the 8-OHdG content of mtDNA (85, 104), it is poorly understood as to how oxidative stress or ROS causes mtDNA mutations. Human mtDNA is a naked compact DNA molecule without protection by histones or DNA-binding proteins, and it is replicated rapidly by a unique D-loop mechanism without proofreading (105). The presence of the D-loop during replication of mtDNA elicit many large-scale deletions that have occurred in the large arc between the replication origins of the heavy and light strands of mtDNA (19). Moreover, mtDNA is attached to the mitochondrial inner membrane in which a considerable

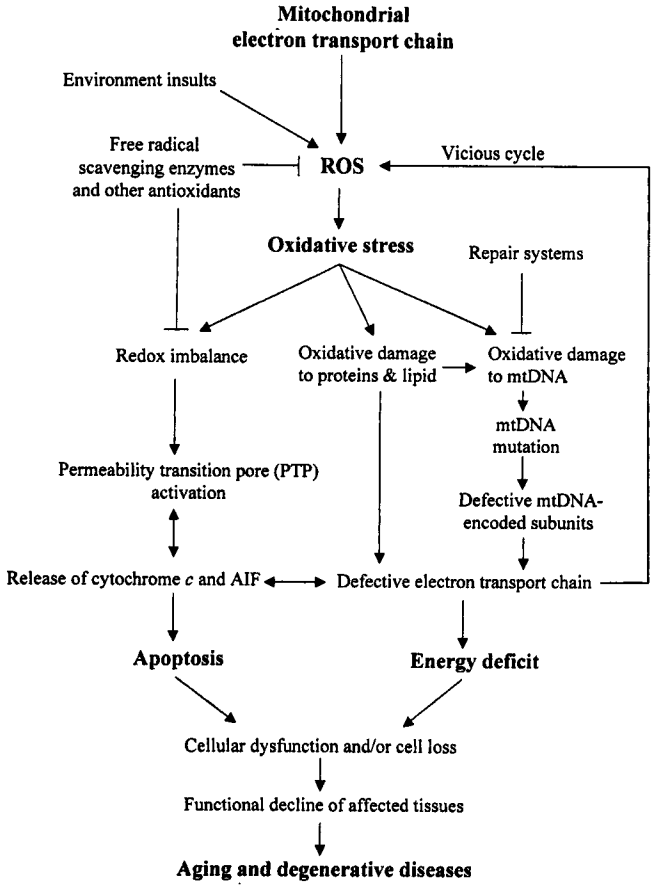

Figure 1. Mitochondrial role in human aging and age-related degenerative diseases. The electron transport system in the mitochondrial inner membrane, which is composed of protein subunits encoded by mtDNA and nuclear DNA, is actively involved in ATP synthesis through coupling with respiration that consumes about 90%of the oxygen uptake of the tissue cells. A fraction of the oxygen is incompletely reduced by one-electron transfer (mostly via ubisemiquinone) to generate the ROS and organic free radicals, which are usually disposed of by the coordinated function of antioxidant enzymes. If escaped, they may cause oxidative damage and mutation of the nearby mtDNA molecules that are attached, at least transiently, to the inner membrane. The mtDNA with oxidative modification and/or mutation are transcribed and translated to produce defective protein subunits that are assembled to form defective electron transport chain (ETC). The impaired ETC not only works less efficiently in ATP synthesis, but it also generates more ROS, which will further enhance the oxidative damage to various biomolecules in mitochondria. This "vicious cycle" is propagating in an age-dependent manner, and results in the widely observed age-related 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. On the other hand, free radical scavenger enzymes and DNA repair systems for removal of the oxidative damages by ROS and free radicals become less efficient in the aging process. In addition, the high levels of oxidants can indirectly induce apoptosis by changing cellular redox potentials, depleting reduced glutathione, reducing ATP levels, and decreasing reducing equivalents such as NADH and NADPH. These changes can facilitate lipid peroxidation and the opening of permeability transition pores, leading to the subsequent release of cytochrome *c* and apoptosis inducing factor (AIF). Aging-related overproduction of mitochondrial ROS may thus lead to activation of apoptotic pathways. Therefore, the accumulation of oxidatively damaged and mutated mtDNA molecules and defective mitochondria, together with the enhanced apoptosis, act synergistically to cause the general decline of biochemical and physiological functions of tissue cells in the aging process of the human.

amount of ROS is continually produced by the respiratory chain (106, 107). These characteristics have rendered mtDNA vulnerable to attack by ROS and free radicals generated by the electron leakage of the respiratory chain of mitochondria, particularly in the aging tissue cells (12, 19, 82, 108).

It is possible that exposure of mtDNA to elevated oxidative stress (increased ROS levels) causes base modifications at some nucleotides in mtDNA, which are more prone to large-scale deletions (Fig. 1). Adachi and co-workers (109) demonstrated that ROS may cause large-scale deletion of mtDNA in animals. They detected a 4-kb deletion of mtDNA in the heart of the Balb/c mice that had received chronic intraperitoneal injection of doxorubicin, which is known to induce cardiomyopathy and elicit profound lipid peroxidation of heart mitochondria. Moreover, they found that administration of coenzyme Q_{10} (a free radical scavenger) to the mice could effectively prevent the mtDNA deletion and could decrease the lipid peroxides content of the heart mitochondria. This and a later study from our laboratory (104) provided important data to support the notion that ROS and free radicals are involved in the mechanism underlying large-scale deletion of mtDNA. Ishii *et al.* (110) reported that a mutation in cytochrome *b* gene of *Caenorhabditis elegans* resulted in enhanced oxidative stress and displayed a phenotype of oxygen hypersensitivity and shortened lifespan. This observation indicates that a defect in mitochondrial respiratory system can lead to an increase of oxidative damage and premature aging or death. In the development of animal models for studies of mitochondrial diseases, Wallace and co-workers (95) succeeded in knocking-out *sod-2* and *ant1* genes, respectively. They found that *sod-2* knockout mice displayed elevated oxidative stress and enhanced oxidative damage to proteins and DNA in brain and heart tissues (95). Moreover, the MnSOD-deficient mice exhibited declined electron transport activities in respiratory enzyme Complexes I and II, a phenotype similar to that commonly found in patients with mitochondrial myopathy. In another model, they demonstrated that the mitochondria from skeletal muscle, heart, and brain of the mice lacking adenine nucleotide translocase (ANT) produced markedly increased amounts of ROS (14). Most importantly, a host of deletions and rearrangements of mtDNA were induced in the heart muscle of *ant1* knockout mice. Although it remains to be established as to how mtDNA mutations are initiated or promoted by ROS and free radicals, these molecular biological and biochemical studies have provided strong evidence to support that oxidative damage is generally accompanied by defects in mitochondrial respiration and oxidative phosphorylation.

Possible Mechanisms of mtDNA Mutation

Although mtDNA mutations have been detected in a wide range of aging human tissues, it is still poorly understood as to how they occur. Sequence analysis of the re-

ported deletions of human mtDNA revealed that they occurred more frequently between the origins of replication of the H and L strands (111). These deletions cause a loss or truncation of many genes encoding tRNAs and mRNAs that are essential for the proper functioning of mitochondria (15, 105). The breakpoints of many of these mtDNA deletions are flanked by direct repeat sequences. Slipped mispairing during DNA replication between these direct repeat sequences (34), homologous replication (112), and topoisomerase II cleavage (113) have been suggested to be the possible mechanisms for mtDNA deletions. Presumably due to their stem-loop structures, the mitochondrial tRNA genes have been found to be hot spots for point mutations of mtDNA (20). Indeed, 15 different point mutations have been found in the $tRNA^{Leu(UUR)}$ gene (113). On the other hand, the start sites and the insertion sites of the tandem duplications in the D-loop region of mtDNA have been found to be localized in the regions containing either a poly C run or a direct repeat sequence (16). Moreover, certain regions of human mtDNA have been demonstrated to be particularly sensitive to oxidative insult of ROS and are prone to mutation (114). The putative hot spots for oxidative modification and mutation of mtDNA could be near or at the unusual structures including bent, antibent, and non-B DNA sequences in human mtDNA. These observations suggest that the unusual structure and/or nucleotide sequences of some segments of human mtDNA are the important factors involved in aging-associated mtDNA mutations (114).

In addition, it was hypothesized that genotoxic intermediates of lipid peroxidation may play a role in eliciting age-associated DNA mutation (107). Recently, Esteve *et al.* (115) demonstrated that oxidative damage to mtDNA is directly related to the GSSG/GSH ratio in fibroblasts, an index of oxidative stress. Because mtDNA is attached to the ROS-generating sites in the mitochondrial inner membrane, it should be more susceptible to oxidative damage, strand breakage, and point mutation. Furthermore, ROS-induced mutagenesis has been observed to be DNA polymerase specific (116). Thus, it is possible that the frequency of occurrence and the type of mtDNA mutation are determined, at least in part, by the interaction between mitochondrial DNA polymerase and the DNA molecules that bear the ROS-induced oxidative damage during DNA replication.

It was demonstrated that treatment of human skin fibroblasts with a sublethal level of oxidative stress results in the formation and accumulation of the common 4977-bp deletion in mtDNA (117). Moreover, an environmental insult such as UV irradiation was reported to induce large-scale deletions in human skin (30, 31, 118) and in cultured human skin fibroblast (119). Fahn *et al.* (120) found that the frequency of occurrence and abundance of mtDNA deletions in the lung of smokers were higher than those of the nonsmokers. Moreover, the proportions of deleted mtDNAs were increased as a function of smoking index in the lung of smokers. Furthermore, Mansouri *et al.* (121) demonstrated that multiple mtDNA deletions are accumulated in the liver biopsies of alcoholic patients, especially those with microvesicular steatosis. Recently, we found that betel quid chewing enhances the occurrence and accumulation of mtDNA mutations in human oral tissue (122). These observations suggest that ROS and free radicals generated by the environmental insults (e.g., UV irradiation, cigarette smoke, and air pollutants) and xenobiotics (e.g., drugs and betal quid), and alcohol drinking may induce the occurrence and accumulation of mtDNA mutations in human tissues during the aging process. Moreover, exogenous oxidative stress may also impair the replication of mtDNA and result in a decrease of mtDNA copy number (mtDNA depletion). Indeed, Attardi and co-workers (88) found that the mtDNA/nuclear DNA ratios of the cybrids made from fibroblasts of old donors was significantly lower than those of the young donors. These molecular changes in the quality and quantity of mtDNA may affect, in a synergistic manner, the bioenergetic function of mitochondria in the aging process (123).

DNA Repair Systems in Mitochondria

As mentioned above, oxidative damage to mtDNA occurs at a frequency approximately 20 times greater than that for nuclear DNA (20, 82). It is expected that mitochondria would need efficient DNA repair mechanisms to remove oxidative damage for the damage to their own DNA. The base excision repair (BER) is an important mechanism for the removal of oxidative DNA damage. Recent studies revealed that mitochondria contain the BER system such as uracil- or 8-OHdG DNA glycosylase (124, 125), apurinic/apyrimidinic (AP) endonucleases (126–128), and 8-OHdGTPase (129). In the process of DNA repair by BER, DNA glycosylase recognizes a damaged base and then cleaves the N-glycosyl bond between the sugar and the base to generate an AP site. Some glycosylases have an associated AP lyase activity that cleaves the DNA phosphate backbone, whereas others rely on AP endonucleases for strand cleavage. A phosphodiesterase then excises the unsaturated sugar derivatives at the 3′ end of the DNA, and the gap of one nucleotide thus generated is bridged by a DNA polymerase and the ends are sealed by a DNA ligase. The 8-OHdG residues in oxidatively modified mtDNA molecules may be removed by mitochondrial 8-OHdGTPase. However, this enzyme does not remove the other modified nucleobases such as 2,6-diamino-4-hydroxy-5-formamidopyrimidine. Recombinational DNA repair has also been reported to exist in mammalian mitochondria (130, 131). However, UV damage cannot be repaired in mitochondria because nucleotide excision repair (NER) exists in the nucleus but does not appear to be present in mitochondria (132–134). Hirano *et al.* (135) examined the age-related change of the DNA repair activity for 8-OHdG in TIG-3 cells and found that the activity of 8-OHdG repair is indeed changed with age in cultured human fibroblasts. Driggers and co-workers (136) also reported that repair of oxidative damage to mtDNA is defective in a human cell line. However, Souza-Pinto *et al.* (128) demonstrated that

8-OHdG glycosylase/AP lyase activity was increased with age in rat heart and liver mitochondria. By contrast, the activity of endonuclease G, which is the major mitochondrial endonuclease, was found to decrease with age in the heart mitochondria of the rat (128). Thus, the DNA repair system in human mitochondria is not fully competent in the repair of various types of damage caused by ROS and free radicals. Even though the activities of some of the endonucleases capable of repairing oxidative damage are upregulated in the aging tissues, some others are unchanged (e.g., uracil DNA glycosylase) or even declined (e.g., endonuclease G). Because successful repair of damaged DNA relies on the coordinated action of many repair enzyme systems, age-dependent decline or imbalance of the activities of the aforementioned DNA repair enzymes will result in the compromise of the overall capacity of repair for the damaged DNA molecules in the aging process. This may account for the ever-increasing accumulation of oxidative damage and mutation to DNA in mitochondria of the aging tissues.

Mitochondrial Role in Apoptosis During Aging

Aging is characterized by an increased production of ROS in somatic tissues (4), and an increase in the production of ROS may promote the induction of apoptosis. Several lines of evidence suggest that aging is accompanied by alterations in the apoptotic process. It has been demonstrated that overproduction of oxidants can induce oxidative stress and cell death (4, 9). Mitochondria are the major intracellular source of ROS (4, 12, 73). Moreover, age-related increase in mitochondrial mass of tissue cells may generate higher levels of ROS (90, 92). Thus, the high levels of oxidants produced from mitochondria can induce apoptosis by changing cellular redox potentials, depleting reduced glutathione, reducing ATP levels, and decreasing reducing equivalents such as NADH and NADPH. These changes can facilitate lipid peroxidation and the opening of permeability transition pores, leading to the subsequent release of cytochrome *c*. Interestingly, it was observed that there is enhanced activation of the mitochondrial permeability transition pore (PTP) in the brain and liver of aging mice (137). This leads to a lower threshold for the release of apoptogenic proteins into the cytosol. Moreover, aged cells have been associated with increased mitochondrial oxidant production and elevated intracellular Ca^{2+} levels (138, 139). These events contribute to the enhanced activation of PTP and the release of proapoptotic proteins from the intermembrane space of mitochondria. Recently, Kokoszka *et al.* (140) demonstrated that chronic oxidative stress in the mice with a partial deficiency in MnSOD resulted in an increased sensitization of the mitochondrial PTP and premature induction of apoptosis. Therefore, respiratory functional decline, mitochondrial ROS production, oxidative stress, and the susceptibility to apoptosis are central events of the aging process.

Mitochondrial oxidative stress and a decline in mitochondrial energy production may lead to activation of apoptotic pathways. It has been shown that apoptosis is enhanced in the liver, brain, heart, skeletal muscle, splenocytes, thymocytes, and oocytes of aged animals (141–149). This loss in cells via apoptosis may be mediated by mitochondrial dysfunction caused by chronic exposure to oxidants, which increased the likelihood of the opening of mitochondrial permeability transition pores.

Interestingly, a reduction in the total number of myocytes has been associated with an accelerated decline in cardiac function in the aging heart (144, 145). In addition to necrosis, apoptosis may be a major factor contributing to the age-associated loss of cardiac myocytes. Moreover, it was observed that apoptosis is more prevalent during the late stages of aging (143). Recently, it was shown that cytosolic cytochrome *c* content was significantly elevated in the heart cells of 16- and 24-month-old male Fischer 344 rats when compared with that of the 6-month-old rats (146). Moreover, mitochondrial Bcl-2, an antiapoptotic protein, was found to show a strong tendency to decrease with age, but mitochondrial Bax, a proapoptotic protein, remained unchanged (146). Because Bcl-2 can prevent cytochrome *c* release, these observations have provided a potential mechanism underlying the increase in apoptosis observed in the aging heart.

Recently, it was found that splenocytes and thymocytes undergo apoptosis with aging in rats and the apoptosis was associated with enhanced expression of p53, Bax, and caspase-3 (148). Moreover, it was also demonstrated that there are differential expressions of Bcl-2 family proteins and caspase family proteins during aging in rat brain (150, 151). Bcl-2 protein level reached the highest level on embryonic day 19 (E19) and decreased after birth. Bax, Bak, and Bad levels were high from E19 to 2 weeks, and decreased significantly at 4 weeks, but Bcl-x levels remained high from early stage to 96 weeks of age (150). Moreover, the expression profile of caspase-3 and -7 is similar to that of Bax, Bak, and Bad, whereas that of caspase-8 and -10 is similar to that of Bcl-2. The constitutive expression of caspases 6 and 9 and Apaf-1 is similar to that of Bcl-x (151). However, the biological significance of the alterations of these enzymes and proteins involved in the execution and regulation of apoptosis during aging remains to be defined.

On the other hand, it was demonstrated that aging attenuates apoptosis in the colonic mucosa of Fischer 344 rats (152). Moreover, it was also shown that the livers of old rats are resistant to apoptosis in response to a moderate dose of genotoxic stress compared with the younger rat (153). These observations suggest that the age-related downregulation of apoptosis might be involved in the increase of DNA damage and mutations with age, and could partially explain the increase in the incidence of cancer during aging. However, it remains to be answered as to whether these results are tissue specific and characteristic of mitotic but not postmitotic tissues. Whether the alterations in the sensitivity to pro-

apoptotic conditions observed during aging are part of the consequences of aging, and whether apoptosis per se participates in the normal aging process warrant further investigation.

Concluding Remarks

Although the classical role of mitochondria in the generation of ATP by aerobic metabolism has been established for more than half a century, the other face of mitochondria in the participation of human aging and disease was not recognized until about 15 years ago. It has been appreciated only very recently that mitochondria are not only the major metabolic energy supplier, but are also the main intracellular source and target of ROS and free radicals generated by the respiratory chain. In the mammalian cell, the proper assembly and functioning of mitochondria are effected through the coordination between gene products encoded by the nuclear and mitochondrial genomes (47). Communication between the nucleus and mitochondria is essential for delicate regulation of synthesis of proteins in the cytoplasm and their subsequent import into mitochondria. ROS and some metabolites that regulate the activation of specific transcription factors, which may exert their functions in the nucleus, have been proposed to be among the signals for communication between mitochondria and the nucleus (49). Beside the effects of the nuclear genome on the expression of mitochondrial genes, the mitochondrial genome can also affect the expression of some nuclear DNA-encoded mitochondrial proteins (154, 155). Oxygen tension in tissue cells, exercise, and hormone levels have been shown to be able to regulate the mRNA levels of cytochrome *c* oxidase subunits in the mammal (156, 157). Therefore, mitochondrion may act as a sensor in regulating energy metabolism and release of ROS in response to extracellular stimuli. Under normal physiological conditions, the ROS generated by the respiratory chain can be scavenged by enzymatic and nonenzymatic antioxidant systems to prevent deleterious oxidative damage to the cell. However, as a result of aging-associated increase of ROS generation in the respiratory chain and decrease in the intracellular concentrations of antioxidants and activities of free radical scavenging enzymes, an elevation of ROS and oxidative stress is inevitable, and oxidative damage and apoptosis might just occur in the cell (158). As the major intracellular source of ROS, mitochondria and their constituents including mtDNA are particularly vulnerable to oxidative damage. Experimental data from this and other laboratories have provided ample evidence to support the notion that mutation and oxidative damage to mtDNA and mitochondrial respiratory function decline in tissue cells are important contributors to human aging (Fig. 1) (12, 92). Although a causal relationship between oxidative modification and mutation of mtDNA, mitochondrial dysfunction, and aging has emerged, the detailed mechanisms by which these molecular and biochemical events cause human aging remain to be established. Understanding of the age-related changes in the structure and function of mitochondria in the aging process is critical for the elucidation of the molecular basis of aging and for the better management of aging and age-related diseases in the new millennium.

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