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Role of Mitochondria in Human Aging

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Abstract
Mitochondria are the major intracellular source and target sites of reactive oxygen species (ROS) that are continually generated as by-products of aerobic metabolism in animal and human cells. It has been demonstrated that mitochondrial respiratory function declines with age in various human tissues and that a defective respiratory chain results in enhanced production of ROS and free radicals in mitochondria. On the other hand, accumulating evidence now indicates that lipid peroxidation, protein modification and mitochondrial DNA (mtDNA) mutation are concurrently increased during aging. On the basis of these observations and the fact that the rate of cellular production of superoxide anions and hydrogen peroxide increases with age, it has recently been postulated that oxidative stress is a major contributory factor in the aging process. A causal relationship between oxidative modification and mutation of mtDNA, mitochondrial dysfunction and aging has emerged, although some details have remained unsolved. In this article, the role of mitochondria in the human aging process is reviewed on the basis of recent findings gathered from our and other laboratories.

Introduction

Aging is a multifactorial biological process, which is accompanied by a general decline in biochemical and physiological functions that leads to the decreased ability of an individual to respond to a wide range of stresses or challenges and increased susceptibility to age-associated diseases and death.

In 1956, Harman [20] first proposed that free radicals are likely the key factor involved in the aging process. The main concept was that the accumulation of free-radical-elicited oxidative damage to macromolecules is a major contributor to aging. Subsequently, he extended the idea to suggest that mitochondria are the major target of free-radical attack that leads to human aging [19]. In the past two decades, the free-radical theory of aging has been widely tested and gained great support from molecular and cellular biological research of aging. Miquel et al. [38, 39] provided great support to this notion by showing that mitochondrial DNA (mtDNA) damage and lipofuscin pigment formation in animal tissues are concurrently increased during aging. In light of the fact that mitochondria are the major intracellular source and vulnerable target of reactive oxygen species (ROS) and free radicals [10, 43], Linnane et al. [37] further hypothesized that accumulation of somatic mutations in mtDNA is a major contributor to human aging and degenerative diseases. This modern hypothesis of aging focused on enhanced production of ROS and accumulation of mtDNA mutations in mitochondria of postmitotic cells upon human aging.

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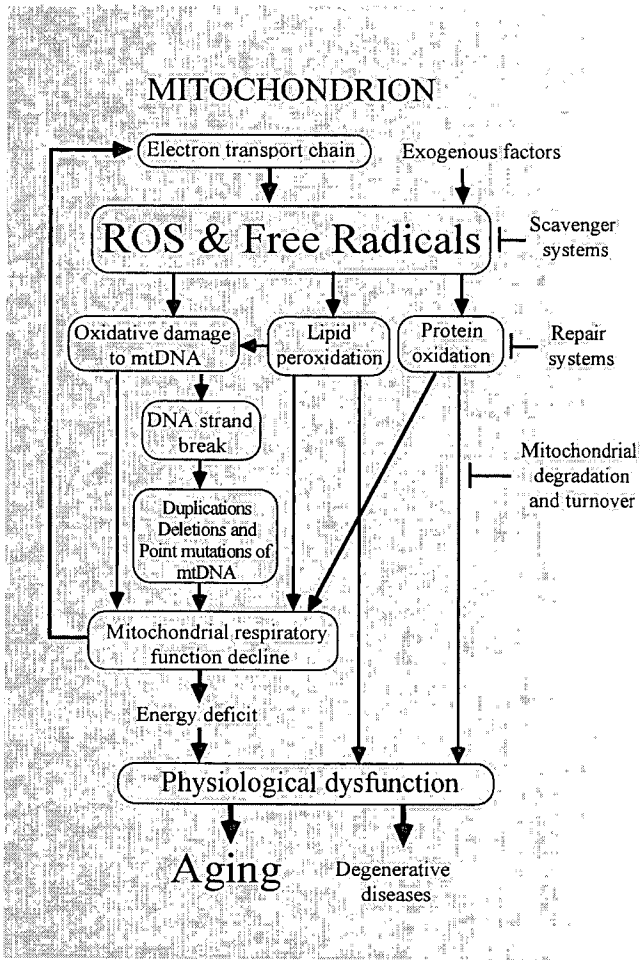

Fig. 1. Mitochondrial theory of aging and age-related degenerative diseases. The electron transport system in the mitochondrial inner membrane, which is composed of mtDNA-encoded protein subunits and nuclear DNA-encoded subunits, is actively involved in the adenosine-triphosphate-synthesis-coupled respiration that consumes about 95% of the oxygen uptake of the tissue cells. A fraction of the oxygen is incompletely reduced by 1-electron transfer (mostly via ubisemiquinone) to generate ROS and organic free radicals, 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 mutated mtDNA are transcribed and translated to produce defective protein subunits that are assembled to form defective respiratory enzymes. The impaired electron transport chain not only works less efficiently in adenosine triphosphate synthesis, but also generates more ROS, which will further enhance the oxidative damage to various biomolecules in mitochondria. This vicious cycle operates 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 systems, DNA repair systems and mitochondrial turnover for removal of the oxidative damages by ROS and free radicals become less efficient in the aging process. Therefore, the accumulation of oxidatively damaged and mutated mtDNA and defective mitochondria and perturbation of mitochondrial turnover act synergistically to cause the general decline of biochemical and physiological functions of tissue cells in the aging process of the human.

In this article, we review recent findings of the oxidative-stress-elicited oxidative damage and mutation of mtDNA and mitochondrial function decline during human aging and then discuss the pivotal role of mitochondria in the aging process.

Oxidative Stress and Aging

Mitochondria are the intracellular organelles responsible for adenosine triphosphate synthesis through the coupling of oxidative phosphorylation to respiration in human and animal cells [48, 50]. Under normal physiological conditions, about 1–5% of oxygen consumed by mitochondria is converted to superoxide anions, hydrogen peroxide and other ROS [10, 51]. It has been established that several sites of the respiratory chain are involved in the generation of ROS [61, 70], and that ROS and free radicals (e.g. ubisemiquinone and flavosemiquinone) are continually generated and maintained at a relatively high steady-state level in mitochondria. It was recently estimated that one normal rat liver mitochondrion can produce about 3×10^7 superoxide anions in a day [10]. In fact, within a certain concentration range, ROS assume important physiological functions such as oxygen burst of neutrophils and smooth muscle relaxation. In addition, ROS and free radicals have been demonstrated to act as a secondary messenger to activate the transcription factors including NF-κB and AP-1 [32, 54]. However, an excess of ROS is harmful to cells [51, 68]. To cope with the ROS, human cells express antioxidant enzymes including manganese superoxide dismutase (MnSOD), copper/zinc superoxide dismutase (Cu/ZnSOD), glutathione peroxidase and catalase. MnSOD and Cu/ZnSOD convert superoxide anions to hydrogen peroxide, which is then transformed to water by glutathione peroxidase or by catalase.

Although these enzymes, together with other antioxidants, can dispose of ROS and free radicals, a fraction of them may escape these cellular defense mechanisms and cause damage to cellular constituents including DNA, RNA, proteins and lipids [4, 51]. In fact, the concentration of 8-hydroxy-2′-deoxyguanosine (8-OH-dG), a specific product of oxidative damage to DNA, has been shown to increase with age in the tissues of mammals and insects [2, 17, 52, 57]. Agarwal and Sohal [2] demonstrated that hyperoxia and X-ray irradiation result in a substantial increase in the level of 8-OH-dG in the exposed body site of houseflies. Moreover, it was observed that the specific contents of 8-OH-dG in the flies overexpressing Cu/ZnSOD and catalase were much lower than those of the wild-type flies [56]. Furthermore, caloric restriction was demonstrated to extend the average and the maximum life spans and to concurrently decrease the age-related accumulation of 8-OH-dG in various tissues of the mice [57]. These observations suggest that oxidative damage to DNA plays an important role in aging.

On the other hand, aging-associated accumulation of inactivated or modified proteins, such as enzymes that are partially denatured, oxidized and catalytically inactive, has been demonstrated in living organisms including nematodes, flies and humans [58, 63, 64]. The accumulation of protein carbonyls and the loss of glucose-6-phosphate dehydrogenase activity, used as indicators of protein oxidative damage, have been observed to increase with age [56, 58, 64]. The intracellular levels of proteolytic enzymes that hydrolyze oxidatively modified proteins are insufficient for effective disposal of the aging-associated increase to aberrant proteins [64]. In the muscle of *Drosophila melanogaster,* the induction of heat shock protein (hsp) 70 was shown to be responsive to aging [71]. The same muscle-specific induction of hsp70 was also observed in young flies with mutations in the Cu/ZnSOD or catalase gene. hsp70 is normally induced in response to heat and other stresses, and apparently functions to promote renaturation and folding of proteins, prevent further protein aggregation and denaturation and facilitate proteolysis of degraded proteins [36, 71]. Several lines of research have suggested that aging-specific hsp70 expression may be a result of oxidative damage to proteins [36]. On the other hand, it was shown that the activities of proteases involved in the degradation of aberrant proteins is markedly decreased in aged animal tissues [33]. It has long been proposed that perturbation of proteolysis is involved in the formation of lipofuscin and possibly in the manifestation of animal aging [30, 31]. In fact, lipofuscin and lipofuscin-like secondary lysosomes are accumulated in various animal tissues with age [24, 30]. The fluorescent pigments are thought to result from cross-linking between oxidatively modified proteins and lipid peroxidation products, which are concurrently increased during aging [38, 39]. These observations, together with the recent findings of wide-spread aging-associated mtDNA mutations [34, 68, 74], have led us to propose that oxidative damage to cellular constituents and their accumulation with age are the major contributors to the aging process (fig. 1).

It has also been observed that the fruit flies with homozygous mutations in either the Cu/ZnSOD or catalase gene exhibit increased sensitivity to oxidative stress and have a reduced viability and life span [18, 49]. Because glutathione peroxidase is absent in *D. melanogaster,* Cu/ZnSOD and catalase thus provide the major enzymatic antioxidant defenses [59]. Flies that overexpress Cu/ZnSOD alone or in combination with the overexpression of catalase were found to exhibit increased resistance to oxidative stress and have significantly less oxidative damage to proteins and a longer life span [44, 45, 56]. It is generally accepted that the activities and capacities of antioxidant systems of tissue cells are declining with age, leading to the gradual loss of pro-oxidant/antioxidant balance and accumulation of oxidative damage in the aging process. These observations provide further support of the notion that oxidative stress plays an important role in the aging process.

Oxidative Damage to Mitochondria in Aging

Since mitochondria are the major intracellular source of ROS, they are thus subjected to direct attacks of ROS in animal and human cells. It has been recently demonstrated that the rate of production of superoxide anions and hydrogen peroxide in mitochondria increases with age in several mammalian and insect tissues [51, 60–63]. The increase in hydrogen peroxide production of *D. melanogaster* under aging-elicited oxidative stress was demonstrated to be related to the oxidative damage to mtDNA and membrane lipids of mitochondria [60]. Sohal et al. [63] further demonstrated that the average life span of dipteran flies is inversely correlated with the rate of mitochondrial production of superoxide anions and hydrogen peroxide and with the level of protein carbonyls in the tissue cells. Moreover, the age-related increase in the rate of generation of mitochondrial hydrogen peroxide was observed to decrease by 40% in the fruit flies overexpressing Cu/ZnSOD and catalase as compared with the wild-

type flies [56]. Therefore, the rate of hydrogen peroxide release by mitochondria is an important determinant of the oxidative damage sustained by mitochondria. Ames et al. [4] first demonstrated that oxidative damage to mtDNA is much more extensive than that to nuclear DNA. The specific content of 8-OH-dG of mtDNA was about 16 times higher than that of nuclear DNA in the liver of 3-month-old rats. Furthermore, the 8-OH-dG content in liver mtDNA of the 24-month-old rat was 3 times higher than that of the 3-month-old rat [17]. Moreover, the levels of oxidative stress and oxidatively modified proteins and lipid peroxides in mitochondria have been shown to increase with age [3, 7, 28, 58]. In addition, the 8-OH-dG contents in the mtDNA of human diaphragm, heart muscle and brain tissues were found to increase in an age-dependent manner [21, 22]. It has also been found that mitochondrial glutathione is markedly oxidized with aging in the rat and mouse [6]. 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-OH-dG content of mtDNA was also found to increase with age in the rat and mouse. Additionally, oral administration of antioxidants protected against both glutathione oxidation and mtDNA damage in rats and mice. These observations suggest a close relationship between oxidative stress, indicated by glutathione oxidation, and mtDNA damage during the aging process.

Mitochondrial DNA Mutations in Human Aging

Each human and animal cell contains several hundred to more than a thousand mitochondria, each carrying 2–10 copies of mtDNA. Human mtDNA is a 16,569-bp circular double-stranded DNA molecule [5]. This extrachromosomal genome contains genes coding for 13 polypeptides involved in respiration and oxidative phosphorylation and 2 ribosomal RNA and a set of 22 transfer RNA (tRNA) that are essential for protein synthesis in mitochondria [5, 50]. mtDNA is a naked compact DNA molecule without protective histones and replicates rapidly without efficient proofreading and DNA repair systems [12, 14]. It is transiently attached to the mitochondrial inner membrane, in which a considerable amount of ROS is continually produced by the respiratory chain [27, 28]. These characteristics have rendered mtDNA vulnerable to attacks by ROS and free radicals generated by the electron leak of the respiratory chain of mitochondria [46, 67, 68].

In the past 8 years, a number of point mutations, deletions and tandem duplications of mtDNA have been found in various tissues of aged individuals [13, 15, 29, 34, 35, 42, 69, 72, 74, 77, 78]. These mutant mtDNAs usually coexist with the wild-type mtDNA within a cell (heteroplasmy), and the degree of heteroplasmy often varies in different tissues of the same individual [34, 77, 79]. It has been well established that many of these mtDNAs-mutations accumulate with age in postmitotic tissues of the human [15, 34, 35, 68, 72, 77]. Some of these aging-associated mtDNA mutations were originally observed in the affected tissues of patients with mitochondrial diseases. The most common mtDNA mutation 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 [55, 74]. This mtDNA deletion was first observed in the muscle of patients with mitochondrial myopathies, including chronic progressive external ophthalmoplegia, Kearns-Sayre syndrome and Pearsons' syndrome [25, 55]. Multiple large-scale deletions of mtDNA have also been found in various tissues of elderly subjects [67, 68, 72, 77, 79]. Two point mutations at np 3,243 and np 8,344 of mtDNA, which are, respectively, associated with mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) syndrome and myoclonic epilepsy and ragged red fibers (MERRF) syndrome [66] have been also detected in the muscle of aged individuals [42, 78]. Additionally, 6 different types of tandem duplications were found in the D loop region of mtDNA from the brain, muscle, liver and skin tissues of normal subjects; the incidence and abundance of these tandemly duplicated mtDNA are increased with age [35, 69].

It is important to note that the proportions of these mutant mtDNAs in aging human tissues rarely exeed 1% [15, 34, 35, 68, 72, 77]. The type and relative proportion of the aging-associated mutant mtDNA are usually determined by the choice of primers and PCR conditions. Recently, a more detailed detection system was designed for extensive screening of mtDNA deletions in human tissues [23, 75]. By using 180 kinds of PCR primer pairs, this system enables one to detect all the possible mtDNA with deletions over 500 bp. Hayakawa et al. [23] applied this system to analyze mtDNA from normal hearts of human subjects of various ages. They observed an extensive fragmentation of mtDNA into minicircles with different sizes. The incidence and abundance of the mutant mtDNA were found to increase with age and to correlate well with the oxidative damage to mtDNA [23, 75]. It is worth mentioning that the aforementioned mutation and

oxidative damage of mtDNA represent only the tip of the iceberg of all the mtDNA damages occurring in the aging process [68].

Although the proportion of the mutant mtDNA was found to correlate with the 8-OH-dG content of mtDNA [22, 75], it is poorly understood how oxidative stress or ROS cause mtDNA mutations. Adachi et al. [1] have recently demonstrated that ROS may cause large-scale deletion of mtDNA in animals. They detected a 4-kb deletion of mtDNA in the heart of 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 [1]. Moreover, they found that administration of coenzyme Q_{10} (a free-radical scavenger) to the mice could effectively preprevent the mtDNA deletion and decrease the lipid peroxide contents of the heart mitochondria. This finding provides first direct evidence to support the notion that ROS and free radicals are involved in the large-scale deletion of mtDNA. Although it remains to be established how mtDNA mutations are initiated or promoted by ROS and free radicals, recent molecular biological studies have provided useful information to better understand the mechanisms of mtDNA mutations.

Sequence analysis of the reported deletions of human mtDNA revealed that they occurred more frequently between the origins of replication of the H and L strands [55, 69] and caused a loss or truncation of genes encoding tRNA and mRNA that are essential for the proper functioning of mitochondria [68]. The breakpoints of many of these mtDNA deletions are flanked by direct-repeat sequences. Slipped mispairing during DNA replication between direct repeat sequences [55], homologous replication [76] and topoisomerase II cleavage [8] have been suggested to be the possible mechanisms for mtDNA deletions. Presumably due to their stem loop structures, the mitochondrial tRNA genes are thought to be hot spots for point mutations [66]. Indeed, more than 10 different point mutations have been found in the $tRNA^{Leu(UUR)}$ gene [53]. 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 [35, 69]. Moreover, certain regions of mtDNA have been demonstrated to be particularly sensitive to oxidative insult of ROS and are prone to mutation [26]. 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 [26]. These observations suggest that the unusual structure and/or nucleotide sequence of human mtDNA are the important factors involved in aging-associated mtDNA mutations. In addition, it was hypothesized that genotoxic intermediates of lipid peroxidation may play a role in eliciting age-associated DNA mutations [28]. The region of mtDNA that is attached to the ROS-generating sites in the mitochondrial inner membrane should be more susceptible to oxidative damage, strand breakage and mutation [28]. Furthermore, ROS-induced mutagenesis has been observed to be DNA polymerase specific [16]. 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.

In addition, several mtDNA mutations have been reported to occur more frequently in sun-exposed skin at relatively high levels [47, 72]. This observation suggests that free radicals generated by environmental insult (e.g. sunshine, air pollutants and cigarette smoke) may also play an important role in the induction of mtDNA mutations during the aging process.

Mitochondrial Respiratory Function Declines with Age

It was first demonstrated in 1989 that the respiratory function of mitochondria gradually declines with age in the human liver [73] and skeletal muscle [65], respectively. This phenomenon has been confirmed by several investigators [48]. The respiratory control, oxidative phosphorylation efficiency, the rates of resting (state 4) and adenosine-diphosphate-stimulated (state 3) respiration and the activities of the respiratory enzyme complexes all decline with age in various human tissues [48, 65, 73]. In addition, the number of skeletal and heart muscle fibers deficient in cytochrome *c* oxidase was found to increase with age [40, 41]. Since the age-dependent decline of the glutamate-malate-supported respiration was found to be more dramatic than that of the succinate-supported respiration, we conjectured that mutation(s) in the 7 genes of NADH dehydrogenase encoded by mtDNA may be involved in this aging-associated respiratory function decline. We quickly confirmed this idea by showing that both the frequency of occurrence and abundance of the 4,977-bp deleted mtDNA increases with age in the liver [34, 74] and many other tissues [15, 34, 69, 72]. It was recently observed that the extent of mtDNA mutation strongly correlates with the progressive decrease in cyto-

chrome *c* oxidase activity in aging human muscle [48]. Because mtDNA has very little redundancy and high information density, the large-scale deletions often cause the removal or truncation of multiple structural genes and tRNA genes and thereby lead to multiple respiratory-chain deficiencies. In addition, we recently found several tandem duplications in the D loop of human mtDNA [35, 69], which contains the replication origin O_H and two transcriptional promoters for each strand of mtDNA [5, 11]. The D loop region is the only and most important control region in human mtDNA. Therefore, any type of mutation in the regulatory elements in the D loop of mtDNA can cause alterations of mtDNA replication and transcription. In addition, oxidative modification to the nucleobases could also elicit the errors of mtDNA replication and gene expression. Therefore, accumulation of oxidatively damaged and mutated mtDNA may contribute to the age-dependent progressive decline of respiratory function especially in postmitotic cells [4, 39, 48, 67, 68]. On the other hand, it has been reported that the steady-state levels of mitochondrial transcripts are significantly reduced during aging of *D. melanogaster*; these changes correlate very well with the life span of the insect [9]. This decline in the expression of mitochondrial genes might be in part caused by damage to mtDNA. Interestingly, it was recently found that not only cytochrome *c* oxidase, which contains subunits encoded by the mitochondrial genome, but also glutamate dehydrogenase, a nuclear DNA-encoded enzyme present in the mitochondrial matrix, gradually lose enzymatic activity during the aging of *D. melanogaster* [9]. These observations suggest that although mtDNA is more vulnerable to oxidative damage, some age-related defects in the nuclear genome may also be involved in aging.

However, it is important to note that the proportions of the age-related mtDNA mutations in various human tissues are not so high as those seen in the target tissues of patients with mitochondrial myopathies [67]. It thus appears difficult for us to comprehend any significant deleterious effect on mitochondrial functions exerted by such low proportions of the mutant mtDNA in human tissues. The age-dependent decline of mitochondrial respiratory function may also be due to the direct ROS damage to proteins, aside from the deleterious effects of mutation or oxidative damage to mtDNA. Moreover, it is possible that all the mutations and oxidative damages to mtDNA impair, in a synergistic manner, the function of the electron transport chain and elicit a profound increase in the rate of ROS generation. A broad spectrum of oxidative damage and mutation of mtDNA may be effected through a recently proposed vicious cycle in the aging process [68]. However, it is worth noting that a clear causal relationship between oxidative modification and mutation of mtDNA, mitochondrial dysfunction and aging remains to be established.

Concluding Remarks

Mitochondria are responsible for the supply of metabolic energy and are also the main intracellular source and target of ROS and free radicals, which are generated as by-products in the respiratory chain. In the mammalian cells, the proper assembly and function of mitochondria are effected through the coordination between gene products encoded by mitochondrial and nuclear genomes [50]. Communication between the nucleus and the mitochondrion is essential for delicate regulation of the synthesis of proteins in the cytosol and their 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 the signals for communication between the mitochondrion and the nucleus [50, 76]. Besides the effects of the nuclear genome on the expression of mitochondrial genes, the mitochondrial genome can also affect the expression of nuclar gene-encoded mitochondrial proteins [50]. Oxygen concentration, exercise and hormone levels have been demonstrated to be able to regulate the mRNA level of cytochrome *c* oxidase in the mammal [50, 51]. Therefore, mitochondria may act as a sensor in regulating energy metabolism and the release of ROS in response to extracellular stimuli. Moreover, within a certain concentration range, ROS and free radicals may act as a secondary messenger in some signal transduction pathways [32, 54]. Normally, the overproduced ROS can be scavenged by enzymatic and nonenzymatic antioxidant systems to prevent deleterious oxidative damage. However, as a result of an aging-associated increase in ROS generation in the respiratory chain and a decrease in the intracellular concentrations of antioxidants and activities of free-radical-scavenging enzymes, the age-related elevation of ROS and oxidative stress is harmful to the cell [51]. As the major intracellular source of ROS, mitochondria are particularly vulnerable to oxidative damage. Experimental data from our and other laboratories have provided ample evidence to support the notion that mutation and oxidative damage to mtDNA and mitochondrial respiratory function decline are important contributors to human aging [68]. Although a causal relationship between oxidative modifi-

cation and mutation of mtDNA, mitochondrial dysfunction and aging has emerged, the detailed mechanism by which these molecular and biochemical events cause aging remains to be established. Understanding of the age-dependent changes of the structure and function of mitochondria in the aging process should be of prime importance in unraveling the molecular basis of aging in the coming years.

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