Respiratory Function Decline and DNA Mutation in Mitochondria, Oxidative Stress and Altered Gene Expression during Aging

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Aging is a biological process that is characterized by the gradual loss of physiological function and increases in the susceptibility to disease of an individual. During the aging process, a wide spectrum of alterations in mitochondria and mitochondrial DNA (mtDNA) has been observed in somatic tissues of humans and animals. This is associated with the decline in mitochondrial respiratory function; excess production of the reactive oxygen species (ROS); increase in the oxidative damage to mtDNA, lipids and proteins in mitochondria; accumulation of point mutations and large-scale deletions of mtDNA; and altered expression of genes involved in intermediary metabolism. It has been demonstrated that the ROS may cause oxidative damage and mutations of mtDNA and alterations of the expression of several clusters of genes in aging tissues and senescent cells. We found that intracellular levels of hydrogen peroxide (H_2O_2) and oxidative damage to DNA in the tissue cells and skin fibroblasts of old donors were higher than those of young donors. In H_2O_2-induced senescent skin fibroblasts, we observed an increase in the protein expression and activity levels of manganese-dependent superoxide dismutase and a concurrent decrease in the activity of cytochrome *c* oxidase and the rate of oxygen consumption. Moreover, the mRNA and protein expression levels of pyruvate dehydrogenase (PDH) were decreased but those of PDH kinase and lactate dehydrogenase were increased in senescent skin fibroblasts. The changes in the expression of these enzymes suggest a metabolic shift from mitochondrial respiration to glycolysis as a major supply of ATP in aging human cells. On the other hand, recent studies on mitochondrial mutant mice, which carry a proofreading-deficient subunit of DNA polymerase γ, revealed that mtDNA mutations accumulated in somatic tissues in the mice that displayed prominent features of aging. Taken together, we suggest that the respiratory function decline and increase in the production of the ROS in mitochondria, accumulation of mtDNA mutation and oxidative damage, and altered expression of a few clusters of genes that culminated in the metabolic shift from mitochondrial respiration to glycolysis for major supply of ATP were key contributory factors in the aging process in the human and animals. *(Chang Gung Med J 2009;32:113-32)*

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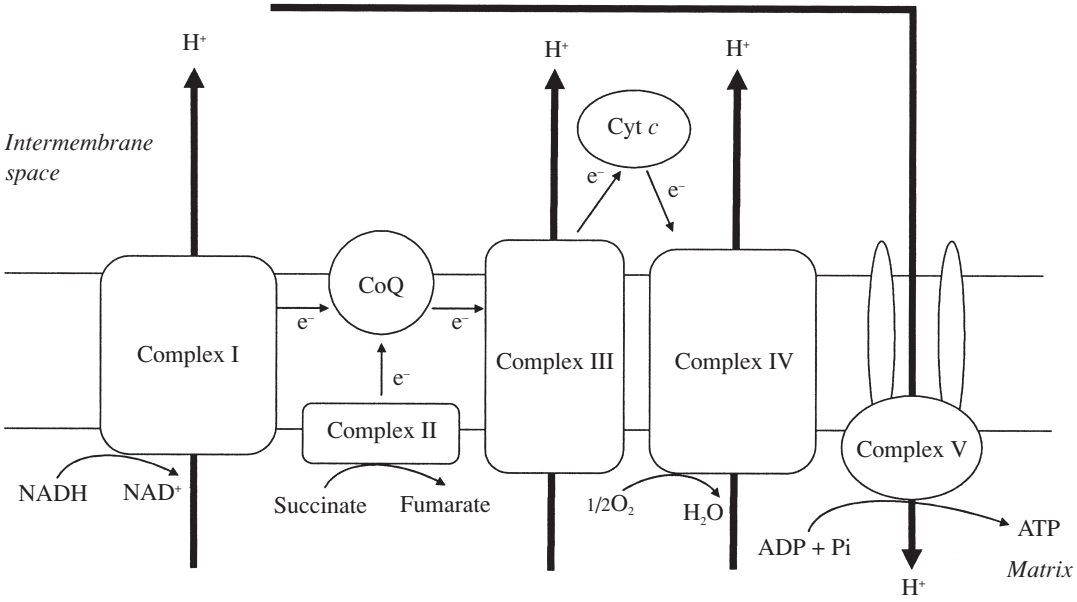
In mammalian cells, mitochondria are the organelles that generate the majority of energy in the form of ATP via the respiratory chain and the oxidative phosphorylation system (Fig. 1). This is a more efficient and sophisticated way of generating ATP from the nutrient molecules that humans and animals obtain from the food. It has been documented that use approximately 90% of tissue oxygen and 1-5% of the mitochondria are metabolized to form the reactive oxygen species (ROS) under normal physiological conditions.[1] Therefore, mitochondria are not only the major supplier of ATP but also the major organelles producing the ROS. Conceivably, mitochondria are the immediate targets of the ROS generated in the organelles of tissue cells. It was Miquel and coworkers who first suggested that respiratory function decline and increase in the production of the ROS in mitochondria played a role in cell aging.[2] These changes and subsequent oxidative stress-induced oxidative damage to various biological molecules have been proposed as important factors in the aging of humans and animals.[3]

In the early 1950's, Dr. Harman first proposed the "free radical theory of aging" to provide a theoretical framework to explain the fundamental aspects of human aging.[4] In the original hypothesis, he contended that oxygen free radicals were generated as by-products from aerobic metabolism and caused cumulative oxidative damage to tissue cells, which eventually resulted in aging and age-related diseases in humans. After realizing that mitochondria were the major source and target of the ROS, Dr. Harman refined the hypothesis and proposed that mitochondria played a key role in the aging process.[5,6]

Every cell in the somatic tissues of the human body contains hundreds of mitochondria, and each



Subunits	Complex I	Complex II	Complex III	Complex IV	Complex V
mtDNA-encoded	7	0	1	3	2
nDNA-encoded	36	4	10	10	14

Fig. 1 Energy production through the coupling of electron transport chain with oxidative phosphorylation in the mitochondria. The reducing equivalents in NADH or $FADH_2$ enter the electron transport chain through Complex I and Complex II, respectively. During the transfer of electrons from NADH to coenzyme Q (CoQ), from CoQ to Complex III, and then from cytochrome *c* to Complex IV, protons are translocated from matrix to the intermembrane space. A proton gradient is thus established across the mitochondrial membranes, which is the driving force of ATP synthesis catalyzed by the membrane-located ATP synthase (Complex V). Among the ~80 polypeptides constituting the electron transport chain, 13 are encoded by mtDNA and the rest are all synthesized in the cytosol and are translocated, with the help of the respiration-generated transmembrane proton gradient, to the mitochondria in a post-translational manner. (nDNA: nuclear DNA)

mitochondrion harbors 2-10 copies of mitochondrial DNA (mtDNA). Because the mitochondrial genome encodes 2 rRNAs, 22 tRNAs, and 13 polypeptides that constitute four respiratory enzyme complexes essential for respiration and oxidative phosphorylation system, it is conceivable that somatic mutations in mtDNA directly affect the bioenergetic function of mitochondria. In the late 1980's, several investigators involved in mitochondrial research thought that respiratory chain dysfunction may be caused by mtDNA mutation and that it is involved in the aging process.[3] Consistent with this line of arguments, Linnane and coworkers proposed in 1989 that the accumulation of mutations in mtDNA in somatic tissues was a major contributor to human aging and degenerative diseases.[7] As a result of this new paradigm of mitochondrial research, the free radical theory of aging quickly evolved to the so-called "Mitochondrial theory of aging".[8]

The mitochondrial theory of aging proposes that progressive accumulation of somatic mutations in mtDNA during the lifetime of an individual leads to a decline in mitochondrial function and is a key contributory factor to human aging. ROS are generated at very low levels during normal respiration of mitochondria and most of them can be removed efficiently by antioxidants and free radical scavenging enzymes. However, mitochondrial production of ROS is increased but the removal efficiency is decreased in aging tissue cells.[1] Oxidative damage to mtDNA by ROS may produce various modified nucleotides and contribute to the occurrence of somatic mtDNA mutations. Accumulation of mutations and oxidative damage to mtDNA may result in respiratory chain dysfunction, leading to increased production of ROS in mitochondria and induction of further mtDNA mutations.[8,9] This vicious cycle will gradually diminish the functional capacity of mitochondria and has been proposed to account for an increase in oxidative damage during aging, which leads to the progressive decline of cellular functions as a result of both insufficient supply of energy and over-production of ROS in somatic tissues.[2,8]

In the past few decades, free radical theory of aging has been extensively examined from many approaches and substantial support has been gained from the results of molecular and cellular biological studies. A large number of studies on various human and animal tissues have shown increases in the production of ROS, decline in mitochondrial function, and accumulation of mtDNA mutations in somatic tissues during aging (Table 1). In this review, we discuss recent advances in the studies of the roles of mitochondrial function decline, oxidative stress, and mtDNA mutations during aging. In addition, changes in the expression of several clusters of genes involved in metabolism that lead to a metabolic shift from oxidative phosphorylation to glycolysis as the major supply of ATP in senescent human cells are discussed.

Table 1. Aging-associated Mitochondrial DNA Mutations in Human Tissues and Cells

Type of mtDNA mutation	Nucleotide position	Human tissues where the mtDNA mutations have been detected
Deletions		
4977 bp	8483 to 13459	Heart, skeletal muscle, liver, lung, spleen, diaphragm muscle, skin, spermatozoa, testis, adrenal gland, kidney, oocytes, brain, neurons, skin fibroblasts
7436 bp	8649 to 16084	Heart, skeletal muscle, liver, skin, spermatozoa
6063 bp	7842 to 13904	Skeletal muscle, liver
3610 bp	1837 to 5446	Skeletal muscle
5827 bp	7993 to 13786	Skeletal muscle
6335 bp	8477 to 14811	Skeletal muscle
7635 bp	8440 to 16074	Skeletal muscle
8041 bp	8035 to 16075	Brain
Point mutations		
A3243G	3243	Skeletal muscle
A8344G	8344	Extra-ocular muscle, skeletal muscle
T414G	414	Skeletal muscle, skin fibroblasts
T408A	408	Skeletal muscle, skin fibroblasts
C150T	150	Leukocytes, skin fibroblasts
Tandem duplications		
200 bp	–493/301	Skeletal muscle, liver, breast, skin
260 bp	–567/301	Skeletal muscle, liver, breast, skin

The mtDNA mutations listed in this table are compiled from the published data obtained from our laboratory[8,30,74,76,84,85] and from those reported by other investigators.[18,75,77,80-83,87-90,92-96]

ROS production and oxidative damage increase with age

Mitochondria utilize most of the oxygen in human tissue cells to generate ATP through aerobic metabolism and at the same time produce ROS including superoxide anions, hydrogen peroxide, and hydroxyl radicals.[10,11] It has been demonstrated that the average lifespan of the dipteran fly is inversely correlated with the rate of production of superoxide anions and H_2O_2 from mitochondria, which is correlated with the level of protein carbonyls in tissue cells.[12] Moreover, an *in vitro* study of human skin fibroblasts revealed that the rate of ROS production is increased in cells at the later proliferation stages as compared with cells during the early proliferation stages.[13] Previously, researcher have demonstrated that ROS and organic free radicals (e.g., ubisemiquinone and flavosemiquinone) are generated and maintained at certain steady- state levels in the mitochondria of tissue cells under normal physiological conditions.[8,14] It has been established that Complexes I and III of the respiratory chain are the major sites that generate most ROS in the mitochondria.[1,11] It has been shown that the rates of production of superoxide anions and H_2O_2 in the mitochondria of animal tissues increased with age.[15] In fact, it was demonstrated by several investigators that the rate of the ROS production by mitochondria is an important determinant of lifespan of the mammals in the studied.[16]

Increased ROS production may cause oxidative damage to cellular constituents, including DNA, RNA, proteins, and lipids. Being the major intracellular producer of ROS, mitochondria are subject to direct attack by ROS in human and animal cells. Among the constituents of mitochondria, mtDNA has been shown to be extremely sensitive to ROS and thus prone to mutation. Mammalian mtDNA is highly susceptible to oxidative damage due to its close proximity to the sites of ROS production from the respiratory chain, lack of protection by histones, and limited capacity of mitochondria to repair DNA damage. This was confirmed by the observation that oxidative modification to mtDNA, indicated by the formation of 8-hydroxy 2'-deoxyguanosine (8-OHdG), is much more extensive than that of nuclear DNA.[17] Damage to mtDNA may appear in the form of base modification, abasic sites, DNA strand breaks, or other types of DNA lesions. HPLC analysis of the modified nucleosides revealed that the 8-OHdG content in mtDNA of human diaphragm or heart muscle increased with the age of the subjects.[18,19] A study using a gene-specific DNA damage assay based on quantitative polymerase chain reaction (PCR) revealed that damaged nucleotides blocked the progression of the DNA polymerase resulting in decreased amplification of the target sequence, which suggests that mtDNA is more susceptible to oxidative damage than is nuclear DNA.[20] A large number of studies have demonstrated that damage and mutation of mtDNA are accumulated in post-mitotic tissues in the process of human aging.[8,21] Moreover, it has been shown that the amounts of lipid peroxides and proteins with oxidative modification in mitochondria of somatic tissues increased with age.[15,22,23] These observations are consistent with the findings that glutathione is markedly oxidized in mitochondria in aging tissues of rats and mice.[24] The ratio between the oxidized and reduced glutathione (GSSG/GSH) and the 8-OHdG content of mtDNA were found to increase concurrently with age in the livers, kidneys, and brains of these animals. These data have provided staunch support for the notion that oxidative stress level is increased in somatic tissues in aging animals.

In recent studies we found that mild stress increased mitochondrial biogenesis of human cells.[13] However, age-associated increases in the number of mitochondria may result in further increases of ROS production and elevation of oxidative stress in tissue cells. Mitochondrial mass can be increased in cultured cells undergoing *in vitro* replicative senescence.[13,25] This phenomenon may be due to the increase in the expression of transcription factors mtTFA, NRF-1, and NRF-2 that are involved in the up-regulation of genes encoding polypeptides constituting respiratory enzymes.[13,26,27] Moreover, intracellular production of ROS was increased in human cells with higher density of mitochondria.[13,28] These observations support the idea that mitochondrial production of ROS and oxidative stress in tissue cells is increased during aging.

Disturbance of antioxidant system and increase of ROS and oxidative damage during aging

During the long process of evolution, humans and animals have developed efficient antioxidant defense systems to cope with the ROS generated by

mitochondria in aerobic metabolism under normal physiological conditions. This system is composed of antioxidant enzymes, sulfhydryl group-containing proteins, and small-molecular-weight antioxidants. The enzymes including manganese superoxide dismutase (MnSOD), copper/zinc superoxide dismutase (Cu/ZnSOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR). MnSOD and Cu/ZnSOD convert superoxide anions to H_2O_2 which is then transformed to water by glutathione peroxidase or catalase.[16] Reduced glutathione (GSH) is the primary physiological antioxidant, which is usually used to protect the sulfhydryl groups of proteins and maintain a suitable redox state of the cells. Oxidized glutathione (GSSG) generated by the action of GPx is then reduced to GSH by GR or by other small-molecular-weight sulfhydryl group-containing proteins such as thioredoxins and glutaredoxin. These antioxidant enzymes and redox-active proteins together with small-molecular-weight antioxidants (e.g., GSH, α-tocopherol, and lipoic acid) can effectively dispose of ROS and free radicals under normal physiological conditions.[10] However, an excess production of ROS may overwhelm the antioxidant defense system and cause oxidative damage to various cellular constituents including DNA, RNA, proteins, and lipids. In addition to the increased production of mitochondrial ROS, oxidative stress can also be elicited by a decline in the capacity of intracellular antioxidant defense systems. It was demonstrated in human skin fibroblasts that the activities of Cu/ZnSOD, CAT, and GPx decreased with age, but that of MnSOD increased with age up to 65 years and decreased thereafter.[29] Based on these findings and the work from other investigators, we have proposed that a decrease in the antioxidant capacity and an imbalance in the expression of free radical scavenging enzymes are contributors to the increase in oxidative stress and damage to tissue cells during the aging process.[30-32]

Studies of fruit flies and animals deficient in some free radical scavenging enzymes have provided evidence to substantiate the important role of ROS in mitochondrial function decline during aging. The fruit flies with homozygous mutations in either the Cu/ZnSOD or CAT gene were found to exhibit increased sensitivity to oxidative stress and had reduced viability and shorter lifespans.[33,34] It was found that mice deficient in the MnSOD exhibited neonatal lethality in association with dilated cardiomyopathy and lipid accumulation in the liver.[35] These mice also displayed severe defects in the respiratory chain and enormous oxidative damage to mitochondria in affected tissues.[35,36] Because inactivation of Cu/ZnSOD had little effect on animal viability, the pathologies observed in MnSOD-deficient mice have thus provided evidence to substantiate the deleterious effect of mitochondrial superoxide anions in cellular toxicity of the ROS.[37] In addition, studies of mice with partial deficiency in MnSOD revealed that animals with reduced MnSOD activity exhibited increased oxidative damage to mtDNA and mitochondrial proteins,[38] a significant decrease in the respiratory control and increase in uncoupled respiration, enhanced sensitization of mitochondrial permeability transition pores, and increased apoptosis in somatic tissues in aging.[39,40] It is possible that increased expression of MnSOD but no changes in the expression of the other antioxidant enzymes produced more H_2O_2, which is not beneficial to the animals due to the deleterious effects of its chemically reactive product in the presence of Fe^{2+} or Cu^{+}. Taken together, these observations suggest that an increase in the production of ROS by mitochondria is one of the major factors contributing to the aging process of animals.

On the other hand, it has been shown that overexpression of SOD and/or CAT enhances the lifespan of the *Drosophila melanogaster*.[41-43] Furthermore, the flies over-expressing only Cu/ZnSOD or together with the over-expression of CAT exhibited higher resistance to oxidative stress and had significantly less oxidative damage to proteins and lived longer.[42,43] A recent study on transgenic mice that over-expressed human CAT revealed that the enzyme could be localized to the peroxisomes, nucleus, or mitochondria, and that overexpression of mitochondria-targeted CAT could extend the median and maximum lifespan of the mouse.[44] The extension of lifespan was associated with attenuation of mitochondrial ROS toxicity and oxidative mtDNA damage and a reduction in the amount of deletions of mtDNA. These results have provided strong support of the "Mitochondrial theory of aging" and reinforce the long-held notion that mitochondrial ROS are an important factor in the determination of the lifespan of mammals.[16]

Mitochondrial respiratory function decline during aging

In the past two decades, abundant evidence obtained from studies of human and animal tissues have substantiated that the efficiency and capacity of mitochondrial respiration and oxidative phosphorylation decline with age.[8,30-32] By using immuno-histochemical staining techniques, Müller-Höcker first observed cytochrome *c* oxidase (COX) deficiency in the heart, limb, diaphragm, and extraocular muscle of healthy elderly subjects, and that the number of COX-negative muscle fibers was increased with age in humans and animals.[45-47] Several investigators have demonstrated that bioenergetic functions of isolated mitochondria and electron transport activities of respiratory enzyme complexes gradually decline with age in the liver[48] and skeletal muscle[49-51] of healthy human subjects, as well as in the skeletal muscle, heart, and liver tissues of dogs[52] and rats.[52-54] Most important, we demonstrated that the age-dependent decline of glutamate-malate-supported respiration was more dramatic than that of the succinate-supported respiration in human livers.[48] This finding implies that the function of Complex I might be more severely affected when compared with the other respiratory enzyme complexes in aging mitochondria. The respiratory control and ADP/O ratios (index of oxidative phosphorylation efficiency), the rates of resting (State 4) and ADP-stimulated (State 3) respiration, and the activities of the respiratory enzyme complexes were found to decline, although at different degrees, with age in various human tissues.[48,51] These bioenergetic changes, which were originally observed in the late 1980's in our laboratory and later confirmed by study results from other laboratories, have led to the conclusion that the respiratory function of mitochondria in animal and human tissues declines with age.[30-32]

Because the ROS production of mitochondria increased with age, an increase in the oxidative damage or modification of proteins can cause a decline in the activity of the targeted enzyme. It has been reported that the treatment of human skin fibroblasts with 200 μM H_2O_2 resulted in a significant decline of bioenergetic function of the mitochondria.[55] In addition to exogenous oxidative insults, endogenous oxidative stress elicited by electron leakage from the respiratory chain can result in a decline of mitochondrial function.[56]

Aconitase exists as an isoform localized to the cytosol and mitochondria, and the latter has been demonstrated to be the preferred target of oxidative damage during the aging of animals.[57] It has been established that aconitase plays a very important role in the regulation and maintenance of iron homeostasis in healthy human cells.[58] In addition, among the proteins containing iron-sulfur-clusters in the mitochondria, aconitase has been shown to be extremely susceptible to oxidative damage in aging tissue cells.[59-61] The mitochondrial aconitase is very sensitive to superoxide anions, which results in the release of one iron atom from the iron-sulfur cluster in its active site.[61] This not only inactivates the TCA cycle in aerobic metabolism but also blocks normal electron flow to oxygen, which results in an increase in ROS production and oxidative damage to biological molecules in the mitochondria. The most deleterious effect is that the release of iron from damaged aconitase molecules may catalyze the Fenton reaction to generate more reactive hydroxyl radicals in the mitochondria. In aging tissues, the above-mentioned changes may disturb iron homeostasis and result in damage to other biological molecules in the affected cells.[58]

In a study of a mitochondrial disease animal model, Esposito and coworkers demonstrated that mitochondrial oxidative phosphorylation was impaired in the mice carrying homozygous mutations of the gene encoding adenine nucleotide translocase (ANT), which catalyzes the exchange of ADP and ATP across the mitochondrial inner membrane.[62] Interestingly, they found that the mitochondria isolated from the skeletal muscle, heart, and brain of the ANT-deficient mice produced more ROS and accumulated more mtDNA rearrangements. It was thus concluded that the oxidative damage and inactivation of ANT impaired respiratory function of the mitochondria and resulted in increased oxidative stress and mtDNA mutations. The increased ROS production by defective mitochondria further resulted in impairment of mitochondrial function and elicited oxidative damage, and ultimately lead the tissue cells to enter a vicious cycle of aging.[30-32,63]

In addition, it was observed that the steady-state levels of mitochondrial RNA transcripts decreased with age in somatic tissues of *D. melanogaster*[64] and rats,[65] as well as in elderly humans and animals.[66-68] Researchers have suggested that gene expression of

the mammalian mitochondrial genome was altered during aging. Laderman and colleagues demonstrated that the mtDNA/nuclear DNA ratio of the cybrids established from skin fibroblasts of elderly subjects was significantly lower than those of young donors.[69] These changes in mtDNA may affect, in a synergistic manner, the bioenergetic function of mitochondria in the aging process of the human and animals.[8,30-32] In a following study, they demonstrated that the biogenesis and bioenergetic function of mitochondria in skin fibroblasts from donors older than 40 years were significantly decreased as compared with those of donors younger than 40 years.[70] On the other hand, an age-dependent decline in the rate of protein synthesis was observed in *D. melanogaster*, mouse livers and kidneys,[71,72] and human skeletal muscles.[73] Further study on 146 healthy subjects with ages of 18-89 years showed that in skeletal muscle the content of mtDNA, the levels of mitochondrial gene transcripts and proteins and ATP production all decreased with age, whereas the amount of oxidative DNA lesions increased.[74] Recently, it was reported that overexpression of mitochondrial transcription factor A (mtTFA) in mice not only attenuated age-related accumulation of oxidative DNA damage and lipid peroxidation products but also improved the memory function of the brains in mice.[75] These findings suggest that the decrease in the rates of mitochondrial transcription and protein synthesis may contribute to the age-related decline in the capacity of aerobic metabolism, particularly the respiratory function of the mitochondria. It is conceivable that as the somatic mutations of mtDNA accumulate, they can exacerbate the pre-existing functional defects in the mitochondria until the combined defects reach a threshold and result in bioenergetic failure of the affected tissues of the elderly subjects.[8,48,52]

Aging-associated mtDNA mutations

It has been well documented that mutations in mtDNA are progressively accumulated in a variety of tissues during aging of humans[8,30,32,76-79] and animals such as monkeys[80] and mice.[81] Most of these mtDNA mutations occur and accumulate with age in post-mitotic tissues after the mid-thirties in the human subjects.[76,78] A wide spectrum of mtDNA mutations, including point mutations,[79,81-84] large-scale deletions,[76,78,85] and tandem duplications[86,87] have been observed in the tissues of elderly subjects. The most prevalent aging-associated mutations of mtDNA are A3243G and A8344G transitions and large-scale deletions including the common 4,977 bp deletion (Fig. 2). These mtDNA mutations may occur alone or in various combinations in somatic tissues of humans during the aging process.

A number of investigators screened for mtDNA mutations in the skeletal muscle, heart, brain, and other tissues of humans and mice[88-92] and found that a broad spectrum of mtDNA deletions and rearrangements accumulated with age in most of the tissues examined (Table 1). Moreover, by using PCR with the back-to-back primers we detected 10 types of tandem duplication in the D-loop region of mtDNA from the skeletal muscle, skin, and testis tissues of elderly subjects, and the incidence and abundance of some of the tandem duplications increased with age.[86,87] High levels of point mutations in the D-loop region of mtDNA have also been found to accumulate with age in human tissues and cultured human cells.[89,93]

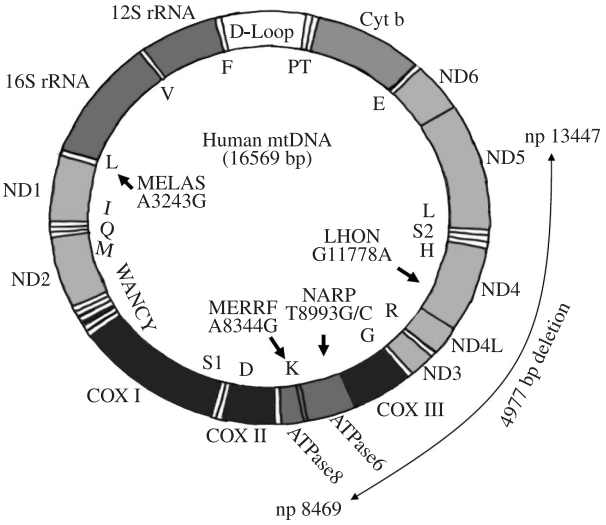


Fig. 2 Aging-associated point mutations and the common deletion of mtDNA found in human tissues. The most prevalent aging-associated point mutations of mtDNA are A3243G and A8344G transitions and the most common mtDNA deletion in aging human tissues is the 4,977 bp deletion, which was first detected in muscle biopsies of some patients with Kearns-Sayre syndrome or CPEO syndrome. They may occur alone or in different combinations with other mtDNA mutations in somatic tissues of aged individuals.

Due to the multiple copy nature of mtDNA within a cell, mutated mtDNA molecules may coexist with the wild-type mtDNA, a condition termed "heteroplasmy".[93] It has been generally accepted that mtDNA mutations cannot cause mitochondrial dysfunction until they reach a threshold. Although most researchers have shown that the overall proportion of mutated mtDNA is low,[32] we have argued that the observed mutations may be just the tip of iceberg of the aging-associated alterations of mtDNA.[8,30-32] In fact, most investigators used the whole aging tissues rather than individual cells to screen for mtDNA mutations. Evidence has been accumulated to support the notion that mutated mtDNA molecules are unevenly distributed and can accumulate clonally in certain cells, causing a mosaic pattern of respiratory chain deficiency in tissues during aging.[91,94-96] It was shown that the respiratory function of mitochondria was severely impaired in the fiber segments of skeletal muscle harboring high levels of mutated mtDNA. Many types of rearrangements of mtDNA have been shown to be abundant in COX-negative fibers in the skeletal muscle of elderly subjects and the proportion of mutated mtDNA is correlated with the decrease of cytochrome *c* oxidase activity.[91,94-96] Recent studies on the finely dissected substantia nigra of postmortem human brains revealed that very high levels ($52.3 \pm 9.3\%$) of mtDNA deletions accumulated in individual dopaminergic neurons, and slightly lower levels ($43.3 \pm 9.3\%$) of mtDNA deletions were present in neurons from the hippocampus of healthy elderly subjects.[97] It is noteworthy that the amount of mtDNA with large-scale deletions increased significantly with age and was related to the impairment of respiratory chain functions such as cytochrome *c* oxidase.[97,98] The authors suggested that the mtDNA deletions were somatic, clonally expanded, and may account for half of the mtDNA population in individual neurons in the elderly subjects. Moreover, it was found that the proportion of the COX-deficient substantia nigra neurons in patients with Parkinson's disease was higher than in the neurons with COX-positive staining in age-matched control subjects. This implies that mtDNA deletions play a causal role in the selective neuronal loss, which is well documented in patients with Parkinson's disease.[97,98] These findings suggest that accumulation of mtDNA mutations is related to, and possibly responsible for, the decrease of mitochondrial oxidative phosphorylation function observed in brain tissues of elderly subjects and patients with neurodegenerative diseases.

Although many mechanisms have been proposed to explain the deletions of mtDNA, many controversies still exist. It is most probable that oxidative damage-associated single- or double-stranded DNA breaks are involved in the formation of deleted mtDNA.[30,32] Ozawa and coworkers showed that the proportion of mtDNA with large-scale deletions correlated well with the 8-OHdG content of mtDNA in aging human tissues.[3,18] It was demonstrated that treatment of human skin fibroblasts with sublethal dose of oxidative stress could result in the formation and accumulation of the common 4977 bp deletion of mtDNA.[99] Moreover, the proportion of the mtDNA with deletion can be increased by environmental insults, such as UV irradiation,[100-102] cigarette smoking,[103,104] and betel nut chewing.[105] It was recently demonstrated that H_2O_2 induced large-scale deletions of mtDNA through formation of double-strand DNA breaks.[106] Most importantly, the frequency of mtDNA deletions was significantly decreased in mCAT mice, which expressed the human catalase gene that was targeted at mitochondria.[44] It was recently shown that the clonal mutations and deletions of mtDNA, which were caused by deficient proofreading DNA polymerase γ, resulted in premature aging.[107] The studies provided direct evidence to support the notion that ROS and free radicals are involved in the mechanisms underlying the somatic mutations of mtDNA, which indeed can drive premature aging in animals. In summary, ROS and free radicals generated by defective mitochondria or environmental insults can cause the formation and accumulation of mtDNA mutations in somatic tissues during the aging process.

Consequences of somatic mutations of mtDNA during aging

Because of the crucial role of mitochondria in energy metabolism, mitochondria with a pathogenic mtDNA mutation can result in respiratory chain dysfunction, ROS overproduction, and further oxidative damage or mutation to mtDNA. This "vicious cycle" operates in different tissues at different rates and ultimately leads to cell dysfunction, degenerative cell loss or cell death, and tissue dysfunction in the aging process (Fig. 3). Recently, several researchers demonstrated that human cells harboring mutated

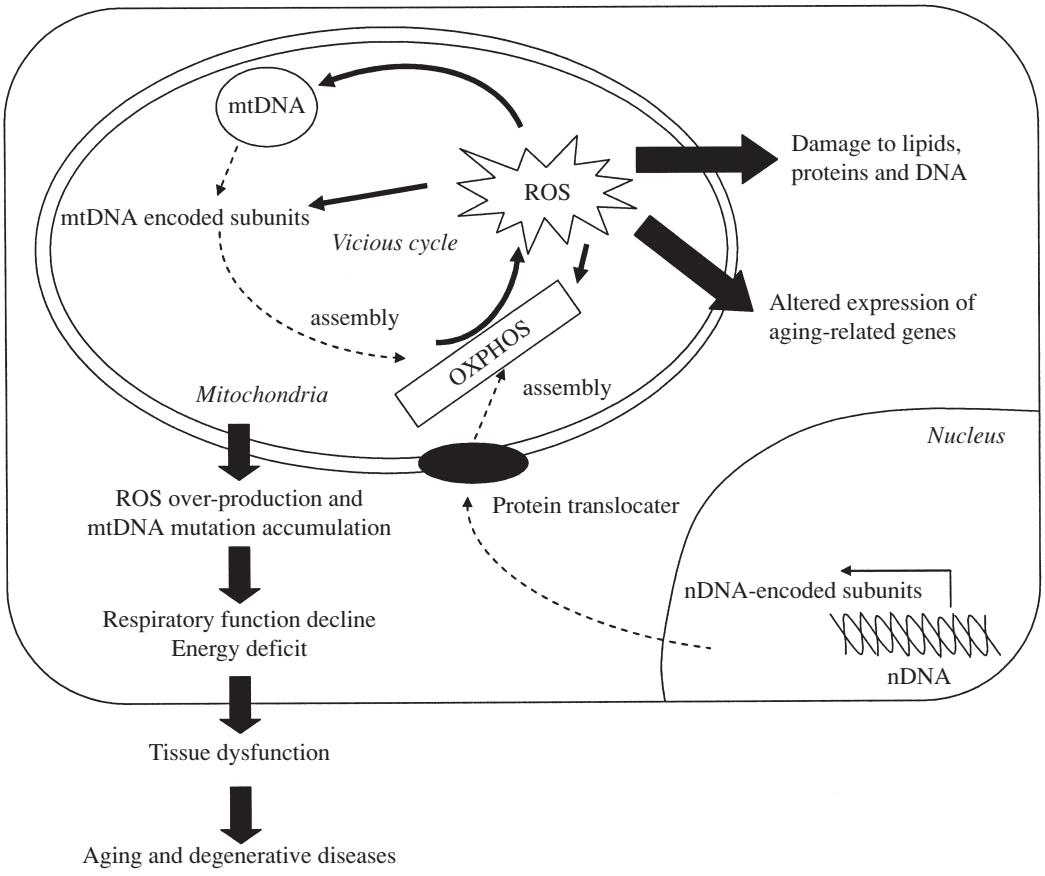


Fig. 3 Mitochondrial role in human aging and age-related degenerative diseases. The oxidative phosphorylation (OXPHOS) system in the mitochondrial inner membrane, which is composed of protein subunits encoded by mtDNA and nDNA and drives ATP synthesis through coupling with the trans-membrane proton gradient generated by respiration, consumes about 90% of cellular oxygen uptake. However, a fraction of the oxygen is incompletely reduced due to the electron leakage from respiratory chain and results in the generation of ROS. If escaped, ROS may cause oxidative damage and mutation of the nearby mtDNA molecules that are attached to the inner membrane. The mtDNA molecules with oxidative damage or mutation are transcribed and translated to produce defective protein subunits that are assembled to form defective OXPHOS system. The mitochondria with defective OXPHOS not only work less efficiently in ATP synthesis, but also generate more ROS, which will further increase oxidative stress and oxidative damage to various biomolecules and alter the expression of aging-related genes. This "vicious cycle" is operated 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 of physiological functions of tissue cells in the aging process of the human.

mtDNA were defective in respiratory function, had higher rate of ROS production, and were more susceptible to apoptosis elicited by various external stimuli.[108,109]

The causal role of mtDNA mutations in the aging of animals has been supported by studies using mice with homozygous knock-in mutant mitochondrial DNA polymerase γ gene (PolgA) that expressed a proof-reading-deficient version of PolgA, the nuclear DNA-encoded catalytic subunit of mtDNA polymerase.[110,111] The knock-in homozygous mutant mice developed an mtDNA mutator phenotype with a 7-11 fold increase in the proportions of point mutation and large-scale deletion of mtDNA.[107] The large increase in somatic mtDNA mutations and defects in mitochondrial respiratory function have been associated with reduced lifespan and premature onset of age-related phenotypes such as weakness, weight loss,

reduced subcutaneous fat, alopecia (hair loss), kyphosis (curvature of the spine), osteoporosis, anemia, reduced fertility and heart enlargement.[110, 112] In further study of the heterozygous PolgA$^{+/-}$ mutant mice, Vermulst et al.[107] found that the rate at which mtDNA mutations reached aging-related phenotypic changes was markedly different among the tissues examined. The brain and heart tissues accumulated mtDNA mutations much more quickly than did the other tissues. This may explain, at least partly, why age-related declines in the biochemical and physiological functions are usually much more pronounced in the tissues with higher energy demand.

Similar mice expressing a proofreading-deficient version of the PolgA were used by other investigators to study other aspects of the molecular and cellular biology changes by which mtDNA mutations contribute to aging.[111] It was further shown that the PolgA mutant mice accumulated mtDNA mutations and displayed features similar to that of accelerated aging. However, it was found that the mouse embryonic fibroblasts from the mutant mice displayed normal ROS production and none increased sensitivity to oxidative stress-induced cell death, but they were more susceptible to apoptosis, particularly in the tissues characterized by rapid cellular turnover.[112] It has been generally proved that the levels of apoptotic biomarkers, such as caspase 3 activation and release of pro-apoptotic proteins from the mitochondria, increased during aging in animals. These results further substantiate the notion that accumulation of mtDNA mutations, which also promote cell apoptosis, plays an important role during mammalian aging.

However, it should be noted that the oxygen consumption rate was dramatically reduced by 95% in mouse embryonic fibroblasts established from mtDNA mutant mice.[112] The error-prone DNA polymerase γ might have caused extensive mutations in the mtDNA, respiratory function defects, and increased apoptosis. These findings further support the causal role of the loss of mtDNA integrity and mitochondrial function during aging. However, they can not rule out the role of mitochondrial ROS production during the normal aging process.[113] One may argue that mitochondria may play different roles during the aging process of the normal animals, in which somatic mtDNA mutations accumulate much more slowly than what occur in the PolgA mutant mice.

Alteration of gene expression during aging

To fully identify the molecular events associated with aging, a number of investigators have examined the age-related genome-wide changes in the gene expression profile in *D. melanogaster*,[114] in the skeletal muscle,[115] brain,[116-118] heart,[119-121] and liver[122] of mice, in the liver[123] and skeletal muscle[124] of rats, in the skeletal muscle of rhesus monkeys,[125] and in the colon epithelial tissue[126] and skeletal muscle[127] of humans. It was found that the expression levels of several clusters of genes were consistently altered in aging animal tissues. In addition, the majority of the aging-related changes in the gene expression profiles in the tissues of animals could be reversed, although to different extents, by caloric restriction.[115,116,119,122,125]

Lee at al.[115,116] used cDNA microarrays to analyze the transcriptional alterations during the aging process in gastrocnemius muscle and neocortex and cerebellum of the mouse, and found that aging resulted in a differential gene expression pattern indicative of a marked increase of stress response and lower expression of metabolic and biosynthetic genes in skeletal muscle of the mouse. They contended that an induction of stress response genes was a result of an increase of tissue damage to proteins and other macromolecules during aging. A decline in the enzyme systems required for the turnover of damaged molecules may result from an energetic deficit in aged tissue cells. The observed decrease in the transcription of genes associated with energy metabolism and mitochondrial function clearly indicates a decrease in mitochondrial biogenesis or turnover secondary to cumulative ROS-induced mitochondrial damage. Table 2 is a summary of alterations in the expression of several clusters of genes observed in various tissues of aging animals.

Researchers studying the gene expression profile of aging brain tissues also found an increase of inflammatory response, oxidative stress and reduced neurotrophic support in the neocortex and cerebellum of mice.[116] Induction of the genes involved in stress response is consistent with a state of higher oxidative stress and accumulation of damaged protein present in the neocortex and cerebellum of aging animals. It is noteworthy that a stress response characterized by the induction of heat-shock proteins and other oxidative stress-related enzymes also occurred in aging brains and skeletal muscles of old mice.

Most age-related alterations of gene expression

Table 2. Aging-associated Alterations in Gene Expression of the Human or Animal Tissues

Genes	Affected tissue	Alteration	References
Stress response			
Heat shock response genes	Skeletal muscle (mouse, monkey), Neocortex (mouse), Liver (mouse and rat)	Increase	(113,114,120,122,123)
Heat shock response genes	Heart (mouse), Cortex (mouse), Colon epithelial tissue (human)	Decrease	(115,119,124)
DNA damage-inducible genes	Skeletal muscle (mouse), Neocortex (mouse)	Increase	(113,114)
Oxidative stress-inducible genes	Skeletal muscle (mouse, monkey), Neocortex (mouse)	Increase	(113,114,123)
Lysosomal proteases	Neocortex and cerebellum (mouse), Liver (mouse)	Increase	(114,120)
GPx, Catalase	Colon epithelial tissue (human)		(124)
Inflammatory response			
Complement cascade	Neocortex and cerebellum (mouse), Liver (mouse), Skeletal muscle (mouse, monkey)	Increase	(113,114,120,123)
MHC molecule	Neocortex and cerebellum (mouse), Skeletal muscle (monkey)	Increase	(114,123)
Microglia activation factors	Neocortex (mouse), Skeletal muscle (monkey)	Increase	(114,123)
Inflammatory peptides	Neocortex and cerebellum (mouse), Skeletal muscle (monkey)	Increase	(114,123)
Energy metabolism			
Glycolysis	Skeletal muscle (mouse and rat), Skeletal muscle (human)	Decrease	(113,122,125)
Glycolysis	Heart (mouse), Neocortex (mouse), Liver (rat), Skeletal muscle (rat)	Increase	(114,117,121,122)
Oxidative phosphorylation	Skeletal muscle (mouse, monkey), Heart (mouse), Liver (rat), Colon epithelial tissue (human)	Decrease	(113,114,123,124)
Oxidative phosphorylation	Hypothalamus (mouse), Skeletal muscle (human)	Increase	(115,125)
Fatty acid transport	Heart (mouse)	Decrease	(117)
Mitochondrial β-oxidation	Heart (mouse), Skeletal muscle (monkey),	Decrease	(117,123)
Mitochondrial β-oxidation	Liver (rat), Skeletal muscle (human)	Increase	(121,125)
Creatine kinase	Skeletal muscle (mouse), Neocortex (mouse), Brain (mouse), Heart (mouse), Colon epithelial tissue (human)	Increase	(113,114,116,117,124)
Creatine kinase	Skeletal muscle (rat), Skeletal muscle (human)	Decrease	(122,125)
Protein turnover			
Protein degradation	Skeletal muscle (mouse), Neocortex and cerebellum (mouse), Brain (mouse)	Decrease	(113,114,116)
Protein degradation	Heart (mouse), Hypothalamus and cortex (mouse)	Increase	(115,117)
Protein degradation	Liver (rat)	No effect	(121)
Protein synthesis	Heart (mouse), Cerebellum (mouse)	Decrease	(114,117)
Protein transport	Skeletal muscle (human)	Decrease	(125)

This table was made by compiling data obtained from cDNA microarray and proteomics studies on aging-associated changes in the gene expression profiles of the human and/or animal tissues.

in the skeletal muscles and brains of mice were completely or partially prevented or alleviated by caloric restriction.[115,116] Caloric restriction has been shown to slow down the intrinsic rate of aging in mammals, retard age-related decline in psychomotor function and ability to fulfill spatial memory tasks, and decrease the age-associated loss of dendritic spines and reduce neuronal degeneration in animal models of Parkinson's disease.[119] Therefore, the findings that calorie restriction could attenuate the changes of gene expression in aging animals have validated the use of cDNA microarray for the analysis of genome-wide aging-associated alterations in gene expression profiles. These results further support the long-held belief that oxidative stress and oxidative damage of post-mitotic tissues is an important cause of aging and diet restriction is an effective strategy to prolong the lifespan of animals.

Most of the aging-associated changes in gene expression profiles were tissue-specific as revealed by comparative analysis of the gene expression profiles for various tissues of mice[115-117,119,121-125] and rats.[128] It was noted that aging induced the expression of a number of stress response genes in the skeletal muscles, neocortex, cerebellum, and liver of laboratory animals.[115,116,122,124] However, the expression levels of some other stress response genes in the cortex and hypothalamus were reduced in aging tissues.[117] Likewise, it was found that aging is associated with increases in the expression of several inflammatory genes in the neocortex, cerebellum, and liver, but not in the skeletal muscle, cortex or hypothalamus.[117] These results indicate that tissues are subject to different stresses during aging, and that oxidative stress plays an important role in the aging process of animals. Additionally, intensive studies of aging-associated alterations in gene expression profile in mice and fruit flies have led to the conclusion that the expression levels of the genes involved in energy metabolism and mitochondrial respiratory function are reduced during aging.[114-117,119-123,125] This may be caused by the accumulation of oxidative damage and mutation of mtDNA in tissue cells during the aging process, which in turn leads to decreases in the function or biogenesis of mitochondria. Aging-associated reduction in the efficiency of oxidative phosphorylation may be caused by alterations in mitochondrial gene expression. This was supported by an observation of the induction of free radical scavenging enzymes and inflammatory response proteins in tissue cells or cultured cells in elderly humans and animals. Alterations in the expression profiles of genes in aging human tissues have been investigated using both cDNA microarray and proteomic analysis. In one such study, Welle and coworkers demonstrated differences in the skeletal muscle gene expression profiles between 20-29 year-old women and 65-71-year-old women.[129] In addition, Hong et al examined the mRNA expression profile of brain tissues in 191 individuals with age-at-death of 65-100 years and compared them with the mRNA expression profile of lymphocytes in 1240 living individuals with ages that ranged from 15 to 94 years.[130] The results showed that the genes involved in the regulation of oxidative phosphorylation emerged as a highly significant term associated with aging in the brain sample, but not in the lymphocytes. This finding indicates that there are likely to be tissue specific differences in the component genes related to mitochondrial function.[130] However, these age-related alterations in mRNA expression levels may reflect changes in gene expression, in mRNA stability or both. At present, it cannot be determined whether the aging-related changes in mRNA levels of these genes are causal factors or consequences of aging. A recent phosphoproteomic analysis of the skeletal muscle of old rats revealed that aging may induce alterations in the levels of phosphoproteins, which are involved in complex cytosolic and mitochondrial metabolism.[131] Further functional studies of the proteins encoded by aging-related genes and their roles in the biology of aging are warranted.

Another important aspect of age-related alteration of gene expression is the change in the flux through major metabolic pathways. Several researchers have demonstrated that the protein and activity levels of certain enzymes participating in glycolysis were up-regulated in senescent human skin fibroblasts.[132,133] It was observed that senescent skin fibroblasts displayed higher rates of utilization of glucose and amino acids and produced more pyruvate and lactate as compared with young skin fibroblasts.[133] Recently, we discovered that the protein expression level of pyruvate dehydrogenase (PDH) decreased while those of PDH kinase (PDK) and lactate dehydrogenase (LDH) increased in senescent human skin (CCD) and lung (MRC-5) fibroblasts induced after 90 min of treatment with 250 μM H_2O_2

(Fig. 4). The increase in the expression of PDK may enhance the phosphorylation of PDH to further decrease the activity of the enzyme and decrease the flux of pyruvate into the TCA cycle. This is in line with the well-documented observation that the lactate production increased in senescent human skin fibroblasts.[133] Furthermore, the activities of mitochondrial respiratory enzymes decreased in senescent cells induced by exogenous H_2O_2. In senescent human skin fibroblasts induced after treatment with 250 μM H_2O_2 for 90 min, we found that cytochrome *c* oxidase activity decreased to 65% and the oxygen consumption rate decreased to about 70% of the control subjects, respectively. Moreover, the lactate production rate of H_2O_2-induced senescent human skin fibroblasts was 1.8 times higher than that of the control samples, which indicates that anaerobic glycolysis was dramatically increased in oxidative stress-induced senescent cells. Most importantly, we found that the mRNA level of HIF-1 in the senescent skin fibroblasts was about 3-fold higher than that of the skin fibroblasts without H_2O_2 treatment. It has been reported that HIF-α up-regulated the expression of glycolytic enzymes in cancer cell lines through an increase in the expression of PDK and decrease in the activity of PDH. This molecular mechanism might be also involved in the aging-related down-regulation of mitochondrial respiration and up-regulation of glycolysis because of the decrease in the supply of metabolites (e.g., acetyl CoA) to the TCA cycle caused by the decreased activity of PDH. These observations have led us to suggest that a shift of the major energy supply from mitochondrial respiration to glycolysis is a metabolic signature of the senescence of human cells.

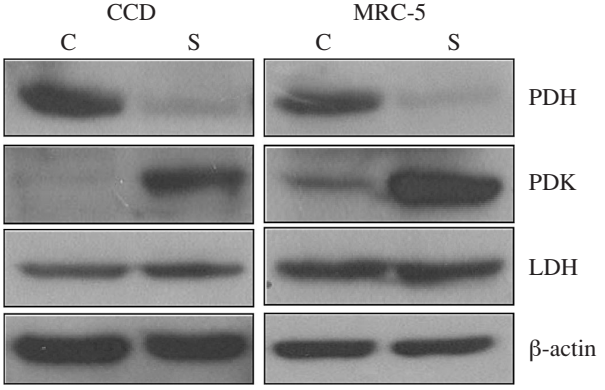


Fig 4. Change in the expression of glycolytic enzymes during H_2O_2-induced cellular senescence in human skin fibroblasts. The protein expression levels of pyruvate dehydrogenase (PDH), PDH kinase (PDK) and lactate dehydrogenase (LDH) in 250 μM H_2O_2-induced senescent skin fibroblasts were analyzed by Western blots. We found that the protein expression level of PDH was decreased while those of PDK and LDH were increased in H_2O_2-induced senescent skin fibroblasts. The changes of PDH, PDK, and LDH expression between control cells (C) and senescent cells (S) suggest that aerobic metabolism is inhibited and glycolytic pathway is up-regulated in H_2O_2-induced cellular senescence of human skin fibroblasts.

Concluding remarks

During the past two decades, we have witnessed the maturation of "Mitochondrial theory of aging". Many of the studies conducted on cultured human cells and animals have revealed that aging is associated with the impairment of bioenergetic function, increased oxidative stress, attenuated ability to respond to stresses, and alterations in gene expression. Most of these events gradually occur during aging of organs and tissue cells. Indeed, most aging-related changes have been correlated with mitochondrial function decline and overproduction of ROS, oxidative damage, and accumulation of mtDNA mutations in somatic tissues.

It is noteworthy that aging is not only associated with or caused by qualitative and quantitative changes in mtDNA, but also in mammalian cells, the biogenesis of mitochondria may be disturbed by defects in the coordination between a wide array of proteins encoded by genes in the nuclear DNA and mtDNA.[134,135] Efficient communication between the two genomes is essential for the regulation of the gene expression to meet the needs in response to intracellular stress and/or environmental chances the individual cells. Oxygen tension and oxidative stress in tissue cells, as well as exercise and hormone levels have been shown to be able to regulate the mRNA levels of mitochondrial genes in the mammals.[136-138] Mitochondria of the human and animal cells may act as sensors in regulating energy metabolism through modulation of the expression of respiratory genes in response to extracellular stimuli. On the other hand, a loss of mtDNA integrity or a mitochondrial defect might trigger a signal from mitochondria to the nucleus, the so called "retrograde signaling" during aging.[135,136] This can best be illustrated by the recent

observation that human fibroblasts shift the majority of their energy supply from aerobic metabolism to glycolysis upon H_2O_2-induced senescence or replicative senescence (Fig. 4). However, it remains to be established as to how the aging-related alterations in gene expression lead to functional changes of mammalian cells during the aging process.

Although experimental data from this and other laboratory studies have provided abundant evidence to support the "Mitochondrial theory of aging", the detailed mechanisms by which these molecular and biochemical events cause human aging remain to be established.[32,139] Functional genomics and proteomics approaches used to study aging at the genome-wide scale have provided novel information for us to better understand the aging-related alterations in the structure and function of mitochondria in the aging process. A number of biomarkers for aging, which include the 4,977 bp deletion and 8-OHdG of mtDNA, increased glucose utilization and lactate production, and decreased cytochrome *c* oxidase and O_2 consumption, have been compiled from studies on aging human tissues and senescent cell cultures *in vitro*. We believe that these markers will be useful in further studies to elucidate the molecular mechanisms of aging and to evaluate the methods to slow aging or prevent age-related diseases in humans and animals.

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老化過程中粒線體呼吸功能衰退與粒線體 DNA 突變，氧化壓力及基因表現的變化

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老化是一個伴隨著生理功能的逐漸喪失和容易發生疾病的生物現象，同時體細胞內的生化功能下降及各種生化分子的損傷增加。在人類和動物的老化過程中，粒線體和粒線體 DNA (mtDNA) 發生許多的變化。這包括粒線體呼吸功能衰退，粒線體所產生的活性氧分子 (ROS) 增加，粒線體內的 mtDNA、脂質和蛋白質的氧化損傷增加，mtDNA 點突變和大段刪除突變的累積以及中間代謝相關基因表現的變化。這些與老化相關的變化，多數與體組織中粒線體之生物能量功能衰退及 ROS 的增加同時發生。最近的研究證實 ROS 在老化的人體組織或人類細胞中累積 mtDNA 的氧化損傷與各種突變及基因表現的變化扮演重要的角色。我們發現老年人的皮膚纖維母細胞所含之過氧化氫 (H_2O_2) 與 DNA 之氧化損傷較年輕人的皮膚纖維母細胞所含者要來得高。除此之外，人類皮膚纖維母細胞在 H_2O_2 誘導老化後，錳型超氧化物歧化酶之蛋白質量及酵素活性增加，但是細胞色素氧化酶活性及粒線體耗氧速率有降低的現象；再者，丙酮酸去氫酶的蛋白質量及酵素活性降低，而丙酮酸去氫酶激酶與乳酸去氫酶的蛋白質量及酵素活性反而增加。這些糖解代謝酵素表現的變化顯示老化的人類組織細胞由粒線體呼吸轉到糖解代謝作爲主要的 ATP 供應途徑。另一方面，最近有關缺乏校讀功能之粒線體 DNA polymerase γ 突變鼠的研究，顯示體組織中，mtDNA 的各種點突變及大段刪除突變的累積與加速老化的表現型有密切關係。將以上這些我們及其他研究室的發現納入考慮，我們認爲粒線體呼吸功能衰退、ROS 增加、mtDNA 的氧化損傷與突變之累積以及基因表現變化造成細胞由粒線體呼吸轉而仰賴糖解代謝作爲主要的 ATP 供應途徑是人類和動物老化過程的重要因素。(長庚醫誌 2009;32:113-32)

關鍵詞：老化，粒線體，粒線體 DNA 突變，氧化壓力，基因表現變化，代謝轉移

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