MINIREVIEW

Oxidative Stress, Mitochondrial DNA Mutation, and Apoptosis in Aging

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A wide spectrum of alterations in mitochondria and mitochondrial DNA (mtDNA) with aging has been observed in animals and humans. These include (i) decline in mitochondrial respiratory function; (ii) increase in mitochondrial production of reactive oxygen species (ROS) and the extent of oxidative damage to DNA, proteins, and lipids; (iii) accumulation of point mutations and large-scale deletions of mtDNA; and (iv) enhanced apoptosis. Recent studies have provided abundant evidence to substantiate the importance of mitochondrial production of ROS in aging. On the other hand, somatic mtDNA mutations can cause premature aging without increasing ROS production. In this review, we focus on the roles that ROS play in the aging-associated decline of mitochondrial respiratory function, accumulation of mtDNA mutations, apoptosis, and alteration of gene expression profiles. Taking these findings together, we suggest that mitochondrial dysfunction, enhanced oxidative stress, subsequent accumulation of mtDNA mutations, altered expression of a few clusters of genes, and apoptosis are important contributors to human aging. Exp Biol Med 232:592–606, 2007

Key words: aging; mtDNA; mitochondria; oxidative stress; oxidative damage; apoptosis

Introduction

Mitochondria are the major energy suppliers that generate ATP through oxidative phosphorylation (OXPHOS) in mammalian cells. The mitochondrial respiratory chain is also the major intracellular source of reactive oxygen species (ROS) and free radicals under normal physiologic and pathologic conditions. It has been thought that loss of mitochondrial function and increased mitochondrial ROS production are important causal factors in aging.

As early as half a century ago Harman (1) first proposed the free radical theory of aging. He contended that oxygen free radicals, by-products from aerobic metabolism, cause cumulative oxidative damage, which eventually results in aging and age-related diseases in humans and animals. Subsequently he refined his theory and suggested that mitochondria play a key role in the aging process because these organelles are the major source as well as the major target of free radicals (2, 3).

Every human cell contains hundreds of mitochondria, and each mitochondrion has multiple copies of mitochondrial DNA (mtDNA). Because the mitochondrial genome codes for 13 polypeptides constituting the respiratory enzyme complexes required for normal functioning of the OXPHOS system, somatic mutations in mtDNA may be directly involved in the mechanism by which ROS initiate a vicious cycle and cause aging. Linnane *et al.* (4) further proposed in 1989 that accumulation of somatic mutations in mtDNA is a major contributor to human aging and degenerative diseases. As a result of this new wave of mitochondrial research, the free radical theory of aging has thus been extended to the "mitochondrial theory of aging."

The mitochondrial theory of aging proposes that progressive accumulation of somatic mutations in mtDNA during an individual's lifetime leads to a decline in the bioenergetic function of mitochondria and is a contributory factor to human aging. ROS are generated at very low levels during mitochondrial respiration under normal physiologic conditions. Oxidative damage to mtDNA by ROS may lead to DNA strand breaks and the occurrence of somatic

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mtDNA mutations. Accumulation of these mtDNA mutations may result in dysfunction of the respiratory chain, leading to increased ROS production in mitochondria and subsequent accumulation of more mtDNA mutations. This vicious cycle has been proposed to account for an increase in oxidative damage during aging, which leads to the progressive decline of cellular and tissue functions as a result of an insufficient supply of energy and/or increased susceptibility to apoptosis (4, 5).

In the past few decades these aging theories have been extensively tested by many approaches, and substantial supportive evidence has been gained from molecular and cellular biologic studies. Studies from aging humans and animals have shown good correlations between aging and increased mitochondrial production of ROS and between mitochondrial function decline and accumulation of mtDNA mutations. Moreover, in the past decade it has been established that mitochondria play a key role in the initiation, execution, and regulation of apoptosis of mammalian cells. In this review, we discuss recent advances in the understanding of the roles that alterations of mitochondria and mtDNA may play in aging. In addition, the key roles of ROS in aging-associated mitochondrial functional decline, somatic mtDNA mutations, apoptosis, and gene expression changes are discussed.

Mitochondrial OXPHOS Function Declines with Age

Bioenergetic studies of humans and animals indicate that the respiratory function of mitochondria declines in aging postmitotic tissues. By using immunohistochemical staining, it has been observed that cytochrome *c* oxidase (COX)–negative cardiomyocytes and muscle fibers are present in the heart, limb, diaphragm, and extraocular muscles of normal elderly subjects and that their number increases with age of the human subjects (6–8). The electron transport activities of respiratory enzyme complexes gradually decline with age in the brain, skeletal muscle, and liver of normal human subjects (9–14). Similar changes have been reported in various tissues of experimental animals (14–17).

In addition to the age-related decline in the activities of respiratory enzyme complexes, the respiratory control ratio, OXPHOS efficiency, the rates of resting (State 4) and ADP-stimulated (State 3) respiration, and ATP synthesis of mitochondria all decline to varying degrees with age in various human tissues (10, 12) and skin fibroblasts (18). The age-associated decrease in mitochondrial membrane potential, the driving force for OXPHOS, and the increase in proton leakage of the respiratory chain were found to correlate with reduced ATP synthesis in tissues of old animals (19–21) and in skin fibroblasts from elderly human subjects (22). A recent study of 146 healthy human subjects further confirmed that the activities of mitochondrial respiratory complexes and ATP production in skeletal muscle declined with age (23). These observations led to the conclusion that bioenergetic function of mitochondria in animal and human tissues declines with age. The decline in mitochondrial respiratory function can lead to lower ATP production and more ROS formation in aged tissues (Fig. 1).

Mitochondrial ROS Production and Oxidative Damage Increase with Age

Approximately 1%–5% of the oxygen consumed by mitochondria in human cells is converted to ROS, including superoxide anions, H_2O_2 , and hydroxyl radicals (24, 25). A majority of intracellular ROS are generated as by-products of oxidation-reduction reactions in the respiratory chain of mitochondria (24). Under normal physiologic conditions, ROS and organic free radicals (e.g., ubisemiquinone and flavosemiquinone) are generated and maintained at a relatively high steady-state level in mitochondria of animal tissues (25, 26). Respiratory enzyme complex I and the protonmotive Q cycle operating in complex III are the major sites that generate ROS in the mitochondrial respiratory chain (25). It has been observed that the average life span of dipteran flies is inversely correlated with the rate of production of superoxide anions and H_2O_2 from mitochondria in tissue cells (27). Moreover, the rates of ROS production from mitochondria increase with age in mammalian tissues (28) and are elevated in later passages of cultured human lung fibroblasts (29).

Being the major intracellular source of ROS, mitochondria are subjected to direct attack by ROS in animal and human cells. It has been reported that treatment of human skin fibroblasts with 200 μM H_2O_2 for 1 hr can result in a sharp decline of bioenergetic function of mitochondria (30). Besides exogenous oxidative insults, endogenous oxidative stress elicited by electron leakage of the mitochondrial electron transport chain can result in the loss of mitochondrial function (31). Increased production of ROS in mitochondria may cause oxidative damage to cellular constituents, including nucleic acids, lipids, and proteins (Fig. 1).

Oxidative damage to DNA causes modification of the purine and pyrimidine bases, single and double-strand breaks, and cross-links to other molecules. Many of these modifications in nuclear DNA and mtDNA of tissue cells are increased with age in mammals (24). Several characteristics of mammalian mtDNA render it to be highly susceptible to oxidative damage. These include its close proximity to the sites of ROS production from the respiratory chain, lack of protection by histones, and limited capacity for repair of DNA damage. It has been documented that oxidative modification to mtDNA is much more extensive than that of nuclear DNA (32). The oxidative modifications in mtDNA of the diaphragm and heart muscle were found to increase with the age of human subjects (33, 34). Studies using a gene-specific DNA damage assay based on quantitative polymerase chain reaction revealed that

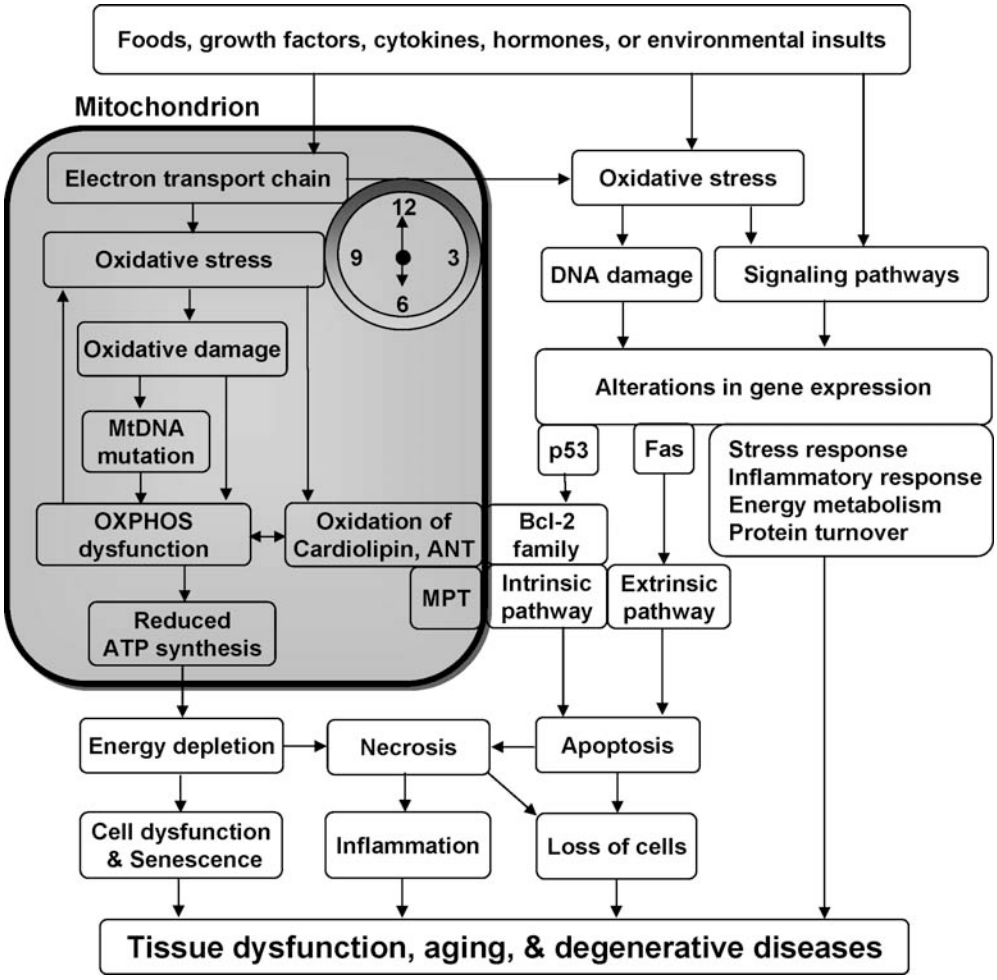

Figure 1. Mitochondrial role in human aging. The electron transport chain (ETC) in the mitochondrial inner membrane is actively involved in ATP synthesis through coupling with respiration, which consumes approximately 90% of the oxygen uptake of the tissue cells. A fraction of the oxygen is incompletely reduced to generate ROS and organic free radicals, which are usually disposed of by the coordinated function of antioxidant enzymes. If ROS escape, they may cause oxidative damage and mutation of 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 ETC. The impaired ETC works less efficiently in ATP synthesis and generates more ROS, which will cause further oxidative damage to various biomolecules in mitochondria. In the aging process, oxidative damage and mutations of mtDNA are accumulated, which ultimately leads to a progressive decline in bioenergetic function and enhanced mitochondrial oxidative stress. The energy depletion and enhanced oxidative stress can lead to functional decline and apoptosis or necrosis of tissue cells in the aging process. In addition, uptake foods; changed levels of growth factors, hormones, and cytokines; and exposure to environmental insults may modulate mitochondrial metabolism rate, oxidative stress, and gene expression. The enhanced oxidative stress can induce apoptosis and/or necrosis by activation of p53, up-regulated Fas expression, or alterations in the expression of other genes. These aging-related changes can facilitate lipid peroxidation and the opening of permeability transition pores, leading to the subsequent release of cytochrome *c* and the ultimate activation of apoptosis. Therefore, the accumulation of mtDNA with oxidative damage and/or mutations, defective mitochondria, enhanced apoptosis, and altered gene expression act synergistically to cause the general decline of biochemical and physiologic functions of tissue cells in elderly humans.

damaged nucleotides block the progression of DNA polymerase, resulting in decreased amplification of the target sequence (30). This suggests that mtDNA is more susceptible to oxidative damage than is nuclear DNA (30) and that mtDNA damage accumulates with age in postmitotic tissues (35). It has been proposed that oxidative damage to mtDNA is a major cause of instability and mutations (point mutations and deletions) of the mitochondrial genome, leading to respiratory dysfunction (4, 24).

Polyunsaturated fatty acids in the phospholipids of mitochondrial membranes are extremely sensitive to oxidation. The hydroxyl radical is one of the most potent inducers of lipid peroxidation. It has been shown that the amount of lipid peroxides in mitochondria increases with age (36). Iron-induced lipid peroxidation has been shown to alter mitochondrial respiration and OXPHOS, inner membrane barrier properties, maintenance of mitochondrial membrane potential, and mitochondrial calcium-buffering capacity (37–39). Among the phospholipids, cardiolipin is present only in mitochondria and resides primarily in the inner membrane of mitochondria. The highly unsaturated nature of the fatty acyl chains in cardiolipin appears to be

required for optimal function of many of the proteins involved in the mitochondrial respiratory chain. It has been shown that increased ROS production from mitochondria may result in oxidation and depletion of cardiolipin, as well as inhibition of cytochrome *c* oxidase activity (40). Peroxidation of cardiolipin has been suggested to impair the barrier function of the inner membrane and facilitate the detachment of cytochrome *c* from the electron transport chain of mitochondria (40, 41) (Fig. 1).

The direct oxidation of amino acid residues promotes oxidation of the sulfhydryl groups of proteins or the formation of protein carbonyls, which can dramatically alter protein structure and lead to loss of its normal function (42). It has been shown that the amount of proteins with oxidative modification in mitochondria increases with age (28, 43). A recent study on human skin fibroblasts from individuals of various ages confirmed that fibroblasts from older subjects (60–80 years) contained significantly higher levels of oxidized proteins than skin fibroblasts from younger donors (<60 years) (44). The rate of protein carbonyl accumulation was significantly higher in the mitochondrial fraction than in the whole-cell lysate of these skin fibroblasts (44). These observations are consistent with the report that mitochondrial glutathione is markedly oxidized in aging tissues of the rat and mouse (45). The ratio between the oxidized glutathione and reduced glutathione (GSH) and the content of mtDNA with oxidative modification was found to increase concurrently with age in the liver, kidney, and brain of these animals.

Among the proteins in mitochondria, aconitase and adenine nucleotide translocase (ANT) have been found to be the preferred targets of oxidative damage during aging of animals (46, 47). Mitochondrial aconitase is highly sensitive to superoxide anions, which inactivate the iron-sulfur cluster in its active site (48). In another study of a mouse model with disruption of the ANT gene, Esposito *et al.* (49) demonstrated that the absence of ANT blocks the exchange of ADP and ATP across the mitochondrial inner membrane, thus inhibiting OXPHOS. Mitochondria isolated from skeletal muscle, heart, and brain of the ANT-deficient mice produced greater amounts of ROS and accumulated more mtDNA rearrangements (49). Thus, functional inactivation of ANT impairs mitochondrial OXPHOS and results in increased oxidative stress and mtDNA mutations.

The age-related increase in mitochondrial mass may result in further increase of oxidants in the tissue cells. It has been observed that mitochondrial mass increases in tissue cells and in human cells upon *in vitro* replicative senescence (29, 50). We found that mild oxidative stress, elicited by a sublethal dose of H_2O_2, caused an increase in the mitochondrial mass of human cells (29, 51) and that ROS production was elevated in human cells with a higher density of mitochondria (29). These observations support the notion that mitochondrial ROS production and oxidative damage in tissue cells are increased during aging.

Role of Mitochondrial ROS in Aging

During the process of evolution mammalian cells have developed an array of antioxidant enzymes to cope with and dispose of ROS generated by aerobic metabolism under normal physiologic conditions. These enzymes include 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 GPx or CAT (24). GSH is used to protect the sulfhydryl groups of proteins and to maintain a suitable redox status of the cells. Oxidized glutathione generated by the action of GPx is then reduced to GSH by GR or other small–molecular-weight thiol-containing proteins such as thioredoxin. These antioxidant enzymes and proteins, together with other small–molecular-weight antioxidants, can dispose of ROS and free radicals under normal physiologic conditions (24). However, an excess production of ROS may overwhelm the antioxidant defense system and cause oxidative damage. In addition to the aging-associated increase in mitochondrial ROS production, oxidative stress can be elicited by a decline in the capacity of intracellular antioxidant systems during the aging process. It has been demonstrated in human skin fibroblasts that the activities of Cu/ZnSOD, CAT, and GPx decrease with age but that of MnSOD increases with age up to 65 years and decreases thereafter (52). We have suggested that the decrease in antioxidant capacity and the imbalance in the expression of free radical scavenging enzymes contribute to the increased oxidative stress and oxidative damage to tissue cells in the aging process (53–55).

Studies of flies and animals deficient in some free radical scavenging enzymes have provided evidence to substantiate the importance of ROS in mitochondrial function and aging. It was observed that fruit flies with homozygous mutations in either the Cu/ZnSOD or CAT gene exhibit increased sensitivity to oxidative stress and have reduced viability and shorter life spans (56, 57). Because the primary source of oxidative stress in the cell is mitochondrial production of the superoxide anion, MnSOD, the only known scavenger of superoxide anion in the mitochondrial matrix, plays a critical role as the first line of defense in protecting the mitochondria. Mice deficient in MnSOD exhibited neonatal lethality in association with dilated cardiomyopathy and lipid accumulation in the liver (58). These mice also displayed severe mitochondrial dysfunction and enhanced oxidative damage to mitochondria (58, 59). Because inactivation of Cu/ZnSOD (60) or extracellular SOD (61) had little effect on animal viability, the pathologies observed in MnSOD-deficient mice provide critical evidence to demonstrate the importance of the mitochondrial superoxide anion in cellular ROS toxicity.

The $Sod2^{+/-}$ mouse, which has a partial deficiency in mitochondrial MnSOD, provides a good model to examine

the roles that increased endogenous mitochondrial oxidative stress play in the alterations of mitochondria during aging (e.g., mtDNA mutation, mitochondrial dysfunction, and apoptosis), as well as on the control of life span (62–65) (Table 1). These mice with reduced (30%–80%) mitochondrial MnSOD activity in all tissues throughout life had lower levels of total glutathione in isolated mitochondria (62) but showed no significant alteration in the activities of the other major antioxidant enzymes (e.g., Cu/ZnSOD, GPx, and CAT) in any of the tissues from either young or old mice (62, 64). Although the H_2O_2 release from mitochondria was not greater in either young or old $Sod2^{+/-}$ mice compared with age-matched wild-type mice (65), a significant increase in oxidative damage to mitochondria was demonstrated in $Sod2^{+/-}$ mice (62, 64, 65). Young $Sod2^{+/-}$ mutant mice (3–5 months) exhibited increased oxidative damage to DNA (62, 64, 65) and increased carbonyl groups in proteins (62) but had the same levels of lipid peroxidation (63) compared with $Sod2^{+/+}$ mice. Activities of the iron-sulfur proteins (aconitase and NADH:coenzyme Q oxidoreductase), sensitive to oxidative inactivation, were decreased in the $Sod2^{+/-}$ mice (62). These results suggest that the reduced activity of mitochondrial MnSOD enhances endogenous mitochondrial oxidative stress.

The reduced activity of mitochondrial MnSOD was correlated to a decline in mitochondrial function (Table 1). Young $Sod2^{+/-}$ mice exhibited decreased electron transport activity of complex I (62, 65); increased proton leak (63); reduced respiration rate (63); decreased respiratory control ratio for substrates metabolized by complexes I, II, and III (62); lower mitochondrial membrane potential (63); and reduced ATP synthesis (65). These findings suggest that enhanced endogenous mitochondrial oxidative stress leads to mitochondrial respiratory enzyme deficiency.

In addition, the mice with reduced mitochondrial MnSOD activity showed an increased sensitization of the mitochondrial permeability transition pores as well as susceptibility to oxidative stress–induced apoptosis (62, 63). The increased endogenous oxidative stress in the mitochondria of the young animals can result in oxidative damage to mtDNA, mitochondrial respiratory enzyme deficiency, and apoptosis, which have been observed previously in mitochondria during aging.

Although the mitochondrial oxidative stress generated by reduced activity of MnSOD increased with age (63–65), it was not higher in $Sod2^{+/-}$ mice (65). Activities of mitochondrial electron transport complex I and ATP synthase were not significantly reduced in old $Sod2^{+/-}$ mice compared with age-matched wild-type mice (65)—even the activities of OXPHOS enzymes were up-regulated in old $Sod2^{+/-}$ mice (63). In addition, biomarkers of aging, such as cataract formation, defective immune response, and formation of glycoxidation products such as carboxymethyl lysine and pentosidine in skin collagen changed with age to a similar extent in both wild-type mice and the mice with a partial deficiency in MnSOD (64). Importantly, the life spans (mean and maximum survival) of the mice with a partial deficiency in mitochondrial MnSOD were not shorter than those of wild-type mice, suggesting that increased mitochondrial oxidative damage is not sufficient for accelerated aging (64). However, the increased levels of oxidative damage to DNA in the mice with a partial deficiency in MnSOD were associated with an increased incidence of cancer (64). These results suggest that mitochondrial oxidative stress plays an important role in aging-related oxidative damage to mtDNA, decline of mitochondrial respiratory function, and apoptosis, but mitochondrial oxidative stress seems not to cause a vicious cycle of amplified damage and dysfunction in mitochondria with age.

On the other hand, it was shown that overexpression of SOD and/or CAT enhances the life span of the fruit fly *Drosophila melanogaster* (66–68). Moreover, flies overexpressing Cu/ZnSOD alone or in combination with overexpression of CAT exhibited higher resistance to oxidative stress and had significantly less oxidative damage to proteins and lived longer (67, 68). Recent studies in transgenic mice that overexpressed human CAT localized to the peroxisome, nucleus, or mitochondria revealed that overexpression of CAT targeted to mitochondria extends both median and maximum life span (69). The extension of life span was in association with attenuated mitochondrial ROS toxicity and oxidative damage to mtDNA, as well as reduced accumulation of mtDNA deletions (69). These results support the free radical theory of aging and reinforce the notion that mitochondrial ROS and mitochondrial accumulation of oxidative damage can be important limiting factors in the determination of life spans of animals.

Accumulation of mtDNA Mutations in Aging

A number of somatic mutations in mtDNA have been found to progressively accumulate in a variety of tissues during aging in humans, monkeys, and rodents (4, 70–75). Many of these mtDNA mutations start to occur after the mid-thirties and accumulate with age in postmitotic tissues of humans (70, 72). There are different types of age-related mtDNA mutations, including point mutations (73, 75–78), large-scale deletions (70, 72, 79), and tandem duplications (80, 81).

A number of investigators have screened large-scale deletions of mtDNA in the skeletal muscle, heart, and brain of humans and mice (82–86) and confirmed that a broad spectrum of different mtDNA rearrangements are accumulated with age in all the tissues examined. Moreover, many types of tandem duplication were detected in the D-loop region of mtDNA from skeletal muscle, skin, and testis tissues of elderly subjects, and the incidence and abundance of some of the mtDNA with tandem duplication increased with age (80, 81). High levels of point mutations in the D-loop region of mtDNA were also found to accumulate with

Table 1. Effect of Reduced MnSOD Activity on Oxidative Damage, Mitochondrial Function, Apoptosis, Aging Biomarkers, and Tumor Formation During Aging Compared with Age-Matched Wild-Type Mice

	Young MnSOD$^{+/-}$ mice			Old MnSOD$^{+/-}$ mice		
	Tissue	Alteration	Reference(s)	Tissue	Alteration	Reference(s)
Mitochondrial H_2O_2 release						
State 1	Heart	NS[a]	65	Heart	NS	65
State 1	Skeletal muscle	NS	65	Skeletal muscle	NS	65
Pyruvate/malate	Heart	NS	65	Heart	NS	65
Glutamate/malate	Skeletal muscle	NS	65	Skeletal muscle	NS	65
Oxidative damage						
mtDNA	Liver	Increase	62, 64	Liver	Increase	64
	Brain	Increase	64	Brain	Increase	64
Nuclear DNA	Liver	NS	62			
	Liver	Increase	64	Liver	Increase	64
	Brain	Increase	64	Brain	Increase	64
	Heart	Increase	64	Heart	NS	64
	Spleen	NS	64	Spleen	NS	64
	Skeletal muscle	Increase	65	Skeletal muscle	Increase	65
Mitochondrial protein	Liver	Increase	62			
Cytosol protein	Liver	NS	62			
Mitochondrial lipid	Liver	NS	63	Liver	Decrease	63
Cytoplasmic lipid	Liver	NS	63	Liver	Decrease	63
Mitochondrial function						
Respiratory rate						
Complex I substrates						
State 3	Liver	NS	62			
State 4	Liver	NS	62			
RCR[a]	Liver	Decrease	62			
Complex II substrates						
State 3	Liver	NS	62			
State 3	Liver	Decrease	63	Liver	NS	63
State 4	Liver	NS	62			
State 4	Liver	NS	63	Liver	NS	63
RCR	Liver	Decrease	62			
RCR	Liver	Decrease	63	Liver	NS	63
Complex III substrates						
State 3	Liver	Decrease	62			
State 4	Liver	NS	62			
RCR	Liver	Decrease	62			
Mitochondrial membrane potential	Liver	Decrease	63	Liver	Decrease	63
Enzyme activity						
Aconitase	Liver	Decrease	62			
Complex I	Liver	Decrease	62	Liver	Increase	63
Complex I	Skeletal muscle	Decrease	65	Skeletal muscle	NS	65
Complex II				Liver	Increase	63
Complex III				Liver	NS	63
Complex II + III				Liver	Increase	63
Complex IV				Liver	Increase	63
Complex IV	Skeletal muscle	NS	65	Skeletal muscle	NS	65
Complex V	Skeletal muscle	Decrease	65	Skeletal muscle	Decrease	65
ATP synthesis	Skeletal muscle	Decrease	65	Skeletal muscle	NS	65
Apoptosis						
Mitochondrial permeability transition	Liver	Increase	62, 63	Liver	Increase	63
TUNEL-positive cells (%)[a]				Liver	Increase	63
Biomarkers of aging						
Cataract formation					NS	64
Immune response					NS	64
Skin collagen changes					NS	64
Life span (mean survival)					NS	64
Life span (maximum survival)					NS	64
Incidence of lymphoma					Increase	64
Tumor-bearing mice					Increase	64
Mice with multiple tumors					Increase	64

[a] NS, no significant difference; RCR, respiratory control ratio; TUNEL, terminal deoxynucleotidyl transferase nick-end labeling.

age in human tissues and cultured human skin fibroblasts (73, 75).

Due to the multiple-copy nature of mtDNA in the human cell, mutant mtDNA molecules may co-exist with wild-type mtDNA, a condition termed "heteroplasmy" (87). In general, mtDNA mutations cannot cause mitochondrial dysfunction until their amounts accumulate above a critical threshold. Although most studies have shown that the overall proportion of mutant mtDNAs is low (55), we argue that the observed or detectable mutations may be just the tip of the iceberg of the aging-associated alterations of mtDNA. Moreover, most investigators examined whole tissue to screen for mtDNA mutations rather than individual cells. The mutated mtDNA molecules may be unevenly distributed and accumulate clonally in certain cells, causing a mosaic pattern of respiratory chain deficiency in tissues during aging. The mitochondrial respiratory function of skeletal muscle was severely impaired in the fiber segments harboring high proportions of mutated mtDNAs (85, 88–90). Rearrangements of mtDNA were also found to be abundant in COX-negative fibers in the skeletal muscle of elderly subjects (85, 88–90), and the proportion of mutated mtDNA was correlated with the decrease in COX activity (85, 88–90). Recent studies on dissected substantia nigra of postmortem human brains further revealed that very high levels of mtDNA deletions accumulate in individual dopaminergic neurons, but the proportion of mtDNA with deletions was not high in neurons from the hippocampus of aged individuals (91, 92). Importantly, the proportion of mtDNA with deletions increased significantly with age, and neurons containing more than 60% of deleted mtDNA molecules revealed a striking loss of COX activity (91, 92). These findings suggest that accumulation of mtDNA mutations is correlated with the decrease of mitochondrial OXPHOS function observed in aging tissue cells.

Although the origins of mtDNA deletions have remained unclear, oxidative damage–associated single- or double-stranded DNA breaks might be involved in their formation. It has been shown that the proportion of mtDNA with deletions correlates with the oxidative modification (8-OHdG) content of mtDNA (33). It has been demonstrated that treatment of human skin fibroblasts with a sublethal dose of oxidative stress results in the formation and accumulation of the common 4977-bp deletion in mtDNA (93). Moreover, the proportion of mtDNA with the 4977-bp deletion can be increased by environmental insults, such as ultraviolet (UV) irradiation (94–96), cigarette smoking (97, 98), and betel quid chewing (99). These studies have provided evidence to support the notion that ROS and free radicals are involved in the mechanisms underlying aging-associated somatic mutations in mtDNA. In summary, ROS and free radicals generated by aerobic metabolism and environmental insults can lead to the formation and accumulation of mtDNA mutations in human tissues during the aging process (Fig. 1).

Role of Acquired mtDNA Mutations in Aging

In humans, mtDNA codes for 13 polypeptides that are crucial components of OXPHOS and codes for two rRNAs (12S and 16S) and 22 tRNAs essential for protein synthesis in mitochondria (87). Because of the crucial role of mtDNA-encoded proteins in energy metabolism, mutations and/or loss of these proteins can result in mitochondrial dysfunction, ROS production, and cell death. Several recent studies have revealed that human cells harboring mutated mtDNA were defective in respiratory function, exhibited higher rates of ROS production, and were more susceptible to apoptotic stimuli (100, 101).

Evidence for the aging-associated decline in mtDNA function was also provided by the observations that steady-state levels of mtRNA were decreased in old *D. melanogaster* (102) and in various aging tissues of humans and animals (103–106). It has also been reported that the mtDNA/nuclear DNA ratio of the cybrids established from skin fibroblasts of old donors was significantly lower than those of young donors (107). These changes in the quality and quantity of mtDNA may affect, in a synergistic manner, the bioenergetic function of human mitochondria in the aging process (53–55). Moreover, an age-dependent decline in the rate of protein synthesis has been observed in *D. melanogaster*, mouse liver and kidney (108, 109), and human skeletal muscle (110) and skin fibroblasts (18). A recent study revealed that the content of mtDNA, mitochondrial gene transcripts and proteins, and ATP production in human skeletal muscle all declined with age, whereas the level of oxidative DNA lesions increased (23). These findings suggest a possible mechanism by which the decrease in the rates of mitochondrial transcription and protein synthesis may contribute to the age-related decline in the mitochondrial OXPHOS function.

Recently, the causal role of mtDNA mutations in mammalian aging was supported by studies using mice with a homozygous knock-in mutant subunit gene that expresses a proofreading-deficient version of mtDNA polymerase γ subunit α, the nucleus-encoded catalytic subunit of mtDNA polymerase (111, 112). The knock-in mice developed an mtDNA mutator phenotype with a 3–5-fold increase in the levels of point mutations, as well as increased amounts of deleted mtDNA. This increase in somatic mtDNA mutations has been associated with reduced life span and premature onset of aging-related phenotypes such as weight loss, reduced subcutaneous fat, alopecia (hair loss), kyphosis (curvature of the spine), osteoporosis, anemia, reduced fertility, and heart enlargement (111).

Similar mice expressing a proofreading-deficient version of DNA polymerase γ were used by other investigators (112) to evaluate the cellular mechanisms by which mtDNA mutations contribute to aging. These mtDNA mutator mice also accumulated mtDNA mutations and displayed features of accelerated aging. Importantly, the results revealed that

the accumulation of mtDNA mutations is not associated with increased markers of oxidative stress or a defect in cellular proliferation but is correlated with the induction of apoptotic markers, particularly in tissues characterized by rapid cellular turnover (112). The levels of apoptotic markers (e.g., caspase 3 activation) were also found to increase during aging in normal mice. Thus, these results suggest that accumulation of mtDNA mutations may promote apoptosis and play an important role in mammalian aging.

Although the mtDNA mutator mice accumulate mtDNA mutations and the presence of a severe dysfunction in the respiratory chain, it was found that mouse embryonic fibroblasts from mtDNA mutator mice exhibited normal rates of ROS production and did not show increased sensitivity to oxidative stress–induced cell death (113). The levels of antioxidant enzymes, protein carbonyls, and aconitase enzyme activity indicated no or only minor oxidative stress in tissues from mice with the mtDNA mutator genotype (113). Therefore, accelerated development of aging phenotype through mtDNA mutations can occur in the absence of increased ROS production or oxidative stress.

However, it should be noted that the oxygen consumption was 95% reduced in embryonic fibroblasts from mice with mtDNA mutator genotype (113). The error-prone DNA polymerase γ may cause extensive mutations throughout the mitochondrial genome, which may prevent the generation of ROS. These experiments have provided evidence to support the causal relationship between the loss in mtDNA integrity and mitochondrial dysfunction in aging, but these findings could not rule out the role of mitochondrial overproduction of ROS in the aging process (114).

Mitochondrial Role in Apoptosis During Aging

Apoptosis is a programmed process of cell death that has a tightly regulated initiation and execution. Evidence has been accumulating to suggest that dysregulation of apoptosis may contribute to age-associated changes such as progressive decline of physiologic function and significant increases in the incidence of cancer and degenerative diseases (115). However, it is still debatable whether aging suppresses or enhances apoptosis *in vivo* and how aging modulates the regulatory machinery of apoptosis in tissue cells.

Progressive cell loss mediated by apoptosis is linked to age-related decline in physiologic function or age-related disorders. The loss of neurons is closely associated with functional impairments such as dementia and motor neuron disability in neurodegenerative diseases such as Alzheimer disease, amyotrophic lateral sclerosis, and Parkinson disease (116). The aging process that occurs in the heart is characterized in animals and humans by a loss of cardiomyocytes and reactive hypertrophy of the remaining cells, which ultimately results in impairment of cardiac function in advanced age (115). Cell loss in these tissues can cause functional deterioration, thereby leading to aging (Fig. 1).

Many studies have demonstrated that apoptosis is up-regulated during aging in various cells such as those of the central nervous system, cardiomyocytes, hepatocytes, lymphocytes, and several types of cells in the reproductive system (115, 117–125). These observations suggest that aging enhances apoptosis under physiologic conditions and increases the susceptibility to apoptosis triggered by challenges. On the other hand, some investigators have reported that aging attenuates apoptosis in the colonic mucosa of Fischer 344 rats (126) and that senescent cells are less susceptible to apoptosis under the condition of oxidative stress or bioenergetic breakdown (127, 128). These discrepancies are thought to be due to different species, different strains, or different cell types (115) that were used in different studies.

Apoptosis can be triggered by both intrinsic and extrinsic pathways. Fas ligand/Fas receptor activation–induced apoptosis is an example of an extrinsic pathway. A wide variety of stimuli such as oxidative stress and DNA-damaging agents can induce the intrinsic pathway of apoptosis. Age-enhanced apoptosis with DNA fragmentation in various types of cells is usually associated with higher expression of p53 and Fas ligand/receptor (115). The p53 tumor suppressor is a universal sensor of DNA damage, and it regulates the transcription of genes required for cell-cycle arrest and apoptosis (129). Activation of the p53 protein and up-regulation of Fas receptor/Fas ligand expression have been demonstrated to induce apoptosis (130). Thus, the activation of p53 protein and up-regulation of Fas receptor/Fas ligand expression observed in aging tissues may be induced, at least in part, by aging-enhanced oxidative stress and/or DNA damage. In tissue cells that are exposed to oxidative stress over a long period of time, oxidative damage to DNA and/or subcellular components accumulates and the cells undergo cell death through the p53 or Fas ligand/receptor pathway (Fig. 1). Thus, the incidence of apoptosis in advanced age may depend on the level of accumulated injury.

In addition, mitochondrial regulation of apoptosis occurs during the initiation and regulation of the intrinsic pathway and during the cross-talk with the extrinsic apoptotic pathway in mammalian cells by mechanisms that are conserved through evolution (131, 132). Lethal agents or death signals that target the mitochondria and cause the release of cytochrome *c* and other pro-apoptotic proteins into the cytoplasm lead to activation of apoptosis (131, 132). Induction of mitochondrial permeability transition (MPT) and regulation of the Bcl-2 family proteins (anti-apoptotic and pro-apoptotic proteins) in the mitochondrial outer membrane play important roles by which mitochondria regulate and execute apoptosis in response to intrinsic and/or extrinsic stimuli (131–133). It was found that the

cytosolic content of cytochrome *c*, which is released from mitochondria, was significantly elevated in heart cells of 16- and 24-month-old male Fischer 344 rats when compared with that of 6-month-old rats (134). This indicates that mitochondrial regulation of apoptosis is involved in the age-associated increase in apoptosis. Moreover, the content of the anti-apoptotic protein Bcl-2 decreases with age, but the amount of the pro-apoptotic protein Bax remains unchanged in mitochondrial membranes (135). The decrease in Bcl-2 may result in the opening of MPT pores and release of cytochrome *c* from mitochondria (131–133). The alterations in the relative amounts of apoptotic and anti-apoptotic proteins on the mitochondrial membrane may be involved in the increase of apoptosis in aging human tissues.

As mentioned above, aging-associated accumulation of oxidative damage to macromolecules in mitochondria results in mitochondrial dysfunction. Apoptosis occurring in aging may be caused, in part, by the progressive decline in mitochondrial function. Studies on mice with a knockout of the mitochondrial transcription factor showed that defects in the respiratory chain are associated with massive apoptosis of affected cells in the heart (135). In addition, pathogenic A3243G and A8344G mutations as well as the 4977-bp deletion in mtDNA render human cells more susceptible to apoptosis stimuli such as UV irradiation (136, 137). Moreover, chronic oxidative stress in mice with a partial deficiency in MnSOD resulted in an increased sensitization of opening of MPT pores and premature induction of apoptosis (63). Therefore, aging-enhanced oxidative stress and inadequate supply of energy from mitochondria may contribute to an increased susceptibility of aging human and animal cells to apoptosis (Fig. 1).

Mitochondrial oxidative stress has been implicated in cell death (138). High levels of pro-oxidants produced by mitochondria can induce apoptosis by changing cellular redox status, depleting GSH, reducing ATP levels, and decreasing reducing equivalents such as NADH and NADPH (132). Pro-oxidants and ROS result in lipid peroxidation and opening of MPT pores in mitochondria (133). Several studies have shown that peroxidation of cardiolipin may weaken its association with cytochrome *c* and thus facilitate the detachment of cytochrome *c* from the outer surface of the inner mitochondrial membrane and its subsequent release into the cytoplasm (41, 139). Moreover, increased mitochondrial oxidant production is associated with elevated intracellular levels of calcium ions in myocytes of old animals and human subjects (140). The threshold for calcium-induced MPT decreases with age in mouse lymphocytes, brain, and liver (141), which in turn leads to a lower threshold for the release of apoptogenic proteins into the cytosol. Furthermore, it has been shown that oxidative damage to ANT in mitochondria increases with age in the flight muscle of houseflies (47). Oxidative stress markedly sensitizes mitochondria toward MPT induction, which correlates with oxidation of SH groups in ANT, one of the basic components of the MPT pore (VDAC-ANT complex) (133). These events contribute to the age-related increase in the tendency of opening of MPT pores and release of pro-apoptotic proteins from the intermembrane space of mitochondria. Thus, age-dependent elevation of mitochondrial oxidative stress may lead to the increase of apoptosis in aging tissues.

Moreover, mtDNA mutations are gradually accumulated and the activity/efficiency of energy metabolism declines in aging tissue cells that often exhibit a higher susceptibility to apoptosis (63, 138). It is conceivable that impairment of mitochondrial ATP production and the resulting energy depletion can lead to apoptosis. Recent studies have revealed that both apoptosis and necrosis occur in the cell death of senescent human skin fibroblasts *in vitro* (142) and human skin fibroblasts from older individuals (22). Treatment of the cells with inhibitors of ATP synthesis rendered them more susceptible to cell death and led to a switch in the death mode from apoptosis to necrosis (22). This may be due to the fact that apoptosis is an energy-dependent process; if the energy depletion is above a critical threshold level, then necrosis occurs (143). Some studies have suggested that age-related apoptosis and/or necrosis in response to energy depletion may occur through activation of the mitochondria-mediated signaling pathway (144). However, the detailed mechanism by which ATP depletion regulates the apoptotic pathway remains unclear.

Necrotic cell death is known to be associated with inflammatory reactions that cause tissue damage in several human diseases. Evidence has revealed that oxidative stress treatment increases necrosis in the cells from older donors (>60 years) and promotes greater enhancement of the release of inflammatory cytokines (22). This suggests that oxidative stress–induced necrosis in aging cells has a relatively higher potential to induce inflammatory reactions, which will affect neighboring live cells (Fig. 1).

In contrast, mutation of the p53 gene and altered p53 expression have frequently been found in the early stages of tumorigenesis. The point mutations of the tumor suppression gene might play an important role in the inactivation of apoptotic machinery. During aging suppressed apoptotic machinery can result in progressive accumulation of genetic damages and mutations, thereby promoting tumorigenesis. Therefore, aging-enhanced apoptosis may be one of the mechanisms to protect tissue cells from aging-associated tumorigenesis.

Aging-Associated Alterations in Gene Expression

Aging-associated declines in mitochondrial respiratory function can lead to lower ATP production and higher oxidative stress. Lower ATP levels can decrease the efficiency of energy-dependent processes and ATP-mediated signal transductions. More ROS can increase stress responses and oxidant-mediated signaling pathways.

To identify the molecular events associated with aging, a number of investigators have examined the age-related

Table 2. Aging-Associated Alterations in Gene Expression in Mammalian Tissues

Genes	Tissues	Alteration	References
Stress response			
Heat shock response	Skeletal muscle (mouse, monkey), neocortex (mouse), liver (mouse)	Increase	146, 147, 152, 154
Heat shock response	Heart (mouse), cortex (mouse)	Decrease	148, 151
DNA damage inducible	Skeletal muscle (mouse), neocortex (mouse)	Increase	146, 147
Oxidative stress inducible	Skeletal muscle (mouse, monkey), neocortex (mouse)	Increase	146, 147, 154
Lysosomal protease	Neocortex and cerebellum (mouse), liver (mouse)	Increase	147, 152
Inflammatory response			
Complement cascade	Neocortex and cerebellum (mouse), liver (mouse), skeletal muscle (mouse, monkey)	Increase	146, 147, 152, 154
Major histocompatibility complex molecule	Neocortex and cerebellum (mouse), skeletal muscle (monkey)	Increase	147, 154
Microglia activation factor	Neocortex (mouse), skeletal muscle (monkey)	Increase	147, 154
Inflammatory peptide	Neocortex and cerebellum (mouse), skeletal muscle (monkey)	Increase	147, 154
Energy metabolism			
Glycolysis	Skeletal muscle (mouse)	Decrease	146
Glycolysis	Heart (mouse), neocortex (mouse), liver (rat)	Increase	147, 149, 153
Mitochondrial OXPHOS	Skeletal muscle (mouse, monkey), heart (mouse), liver (rat)	Decrease	146, 147, 148, 154
Mitochondrial OXPHOS	Hypothalamus (mouse)	Increase	148
Fatty acid transport	Heart (mouse)	Decrease	149
Mitochondrial β-oxidation	Heart (mouse), skeletal muscle (monkey)	Decrease	149, 154
Mitochondrial β-oxidation	Liver (rat)	Increase	153
Creatine kinase	Skeletal muscle (mouse), neocortex (mouse), heart (mouse)	Increase	146, 147, 149
Protein turnover			
Protein degradation	Skeletal muscle (mouse), neocortex, cerebellum (mouse)	Decrease	146, 147
Protein degradation	Heart (mouse), hypothalamus, cortex (mouse)	Increase	148, 149
Protein degradation	Liver (rat)	No effect	153
Protein synthesis	Heart (mouse), cerebellum (mouse)	Decrease	147, 149

genome-wide changes in the gene expression profile in *D. melanogaster* (145); in skeletal muscle (146), brain (147, 148), heart (149–151), and liver (152) of the mouse; in liver of the rat (153); and in skeletal muscle of the rhesus monkey (154). It is noteworthy that the majority of the aging-related changes in the gene expression profiles in the tissues of animals can be reversed by caloric restriction (147, 149, 152, 154).

Studies using oligonucleotide-based microarrays to analyze the transcriptional alteration in the aging process in gastrocnemius muscle, neocortex, and cerebellum of the mouse (146, 147) revealed that aging results in a differential gene expression pattern indicative of a marked stress response and lower expression of metabolic and biosynthetic genes in skeletal muscle. It has thus been proposed that induction of stress response genes during aging is associated with an increase of damage to proteins and other biomolecules (146, 147). A functional decline in the enzyme systems required for the turnover of damaged molecules can result from an energetic deficiency in aged cells. The observed alterations in the transcription of genes associated with energy metabolism and mitochondrial function clearly indicate a decrease in mitochondrial biogenesis or turnover secondary to cumulative ROS-induced mitochondrial damage (Table 2).

Evidence from the gene expression profile of aging brain tissues (147) also indicated an increase in inflammatory response and oxidative stress and a reduction of neurotrophic factors in the neocortex and cerebellum of the mouse. 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. Interestingly, a stress response characterized by the induction of heat shock proteins and other oxidative stress–induced transcripts was found to occur in aged brain and skeletal muscle of the mouse (Table 2).

Furthermore, in the skeletal muscle of the mouse most age-related alterations of gene expression could be completely or partially prevented by caloric restriction (146). Similarly, caloric restriction selectively attenuated the aging-associated induction of genes encoding proteins involved in inflammatory and stress responses in the mouse brain (147). Indeed, 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, decrease the age-associated loss of dendritic spines, and reduce neuronal degeneration in the animal models of Parkinson disease (149). Therefore, the finding that caloric restriction attenuates both the increase

and decrease of gene expression in aging animals has validated the use of cDNA microarrays for analysis of aging-associated transcriptional alterations. These results have provided further support for the hypothesis that oxidative stress is an important cause of the aging of postmitotic tissues.

By comparing the gene expression profiles for various tissues of mice (146–149, 151–154), it was found that most of the aging-associated changes in gene expression profiles are tissue specific (Table 2). It was noted that aging induces the expression of a number of stress-response genes in skeletal muscle, neocortex, cerebellum, and liver of laboratory animals (146, 147, 152). In contrast, aging reduces the expression of other stress-response genes in the cortex and hypothalamus (148). Likewise, it was found that aging induced expression of several inflammatory genes in the neocortex, cerebellum, and liver but not in skeletal muscle, cortex, and hypothalamus. These results also suggest that tissues are subjected to different stresses during aging and that all the results indicate that oxidative stress plays a critical role in the aging process (Fig. 1). In addition, the analysis of aging-associated alterations in gene expression profiles in the mouse and fruit fly has led to the important conclusion that aging reduces expression of the genes involved in energy metabolism and mitochondrial respiratory function (145–154). This may be a result of oxidative damage and mutations of mtDNA accumulating in tissue cells during the aging process, which leads to a reduction of mitochondrial function or biogenesis. Moreover, the notion that the aging-associated reduction in the efficiency and capacity of OXPHOS is caused by altered mitochondrial gene expression was supported by the induction of free radical scavenging enzymes and inflammatory response proteins in tissue cells or cultured cells of old animals. However, it is not clear whether the observed aging-related changes in the mRNA levels are a causal factor or a consequence of aging. Further functional studies of the proteins encoded by aging-related genes and their roles in the biology of aging are warranted.

Concluding Remarks

Aging is a natural, complex, and multifactorial biologic process. Many of the studies conducted on cultured human cells and animals have revealed that aging is associated with impairment of bioenergetic functions, increased oxidative stress, attenuated ability to respond to stresses, and increased risk of contracting cancers and age-associated diseases (138). Most of these characteristics and phenomena gradually occur in advanced age in organs and tissue cells, which are usually correlated with mitochondrial ROS production, oxidative damage, accumulation of mtDNA mutations, mitochondrial dysfunction, activation of apoptosis/necrosis, and altered expression of specific clusters of genes. During aging the expression levels of the genes that increase normally in response to DNA damage and oxidative stress are increased, whereas those involved in energy metabolism, biosynthesis, and protein turnover are decreased. Interestingly, it has been shown that caloric restriction can reverse or retard these changes in the gene expression during aging, extend life span, slow down aging-associated physiologic changes, and reduce cancer incidence in rodents and primates. These findings suggest that modulating the rate of energy metabolism may bring about changes in the redox status, mitochondrial function, and genomic integrity of animal cells.

Recently evidence from studies of transgenic animals suggested that mtDNA mutations contribute to premature aging but may not correlate with higher oxidative stress (112, 113). Moreover, enhanced mitochondrial oxidative stress can result from extensive accumulations of oxidative damage and mutations in mtDNA (62–65) but are not associated with premature aging (64, 65). On the other hand, mitochondria-targeting ROS scavenger enzymes can reduce oxidative damage to mitochondria and prolong the life spans of animals (69). These observations have lent support to the notion that the endogenous and exogenous factors that result in mitochondrial oxidative stress and accumulation of oxidative damage and mutations to mtDNA may contribute, in a synergistic manner, to aging-associated phenotypes.

Although abundant experimental data have been gathered in the past decade to support the concept that mitochondrial dysfunction, ROS overproduction, and accumulation of mtDNA mutations in tissue cells are important contributors to human aging, the detailed mechanisms by which these biochemical events cause human aging remain to be established. The functional genomics and proteomics approaches to study aging on a genome-wide basis will provide novel information so we may gain a deeper understanding of the age-related alterations in the structure and function of mitochondria in the aging process. This is critical for the elucidation of the molecular mechanism of aging and for better management of aging and age-related diseases in humans.

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