**Reveiw Article**

# Oxidative Stress in Human Aging and Mitochondrial Disease−Consequences of Defective Mitochondrial Respiration and Impaired Antioxidant Enzyme System

**Yau-Huei Wei, Ching-You Lu, Chia-Yu Wei, Yi-Shing Ma and Hsin-Chen Lee**

*Department of Biochemistry*
*National Yang-Ming University*
*Taipei 112, Taiwan, ROC*

## Abstract

**Respiratory function of mitochondria is compromised in aging human tissues and severely impaired in the patients with mitochondrial disease. A wide spectrum of mitochondrial DNA (mtDNA) mutations has been established to associate with mitochondrial diseases. Some of these mtDNA mutations also occur in various human tissues in an age-dependent manner. These mtDNA mutations cause defects in the respiratory chain due to impairment of the gene expression and structure of respiratory chain polypeptides that are encoded by the mitochondrial genome. Since defective mitochondria generate more reactive oxygen species (ROS) such as $O_2^-$ and $H_2O_2$ *via* electron leak, we hypothesized that oxidative stress is a contributory factor for aging and mitochondrial disease. This hypothesis has been supported by the findings that oxidative stress and oxidative damage in tissues and culture cells are increased in elderly subjects and patients with mitochondrial diseases. Another line of supporting evidence is our recent finding that the enzyme activities of Cu,Zn-SOD, catalase and glutathione peroxidase (GPx) decrease with age in skin fibroblasts. By contrast, Mn-SOD activity increases up to 65 years of age and then slightly declines thereafter. On the other hand, we observed that the RNA, protein and activity levels of Mn-SOD are increased two- to three-fold in skin fibroblasts of the patients with CPEO syndrome but are dramatically decreased in patients with MELAS or MERRF syndrome. However, the other antioxidant enzymes did not change in the same manner. The imbalance in the expression of these antioxidant enzymes indicates that the production of ROS is in excess of their removal, which in turn may elicit an elevation of oxidative stress in the fibroblasts. Indeed, it was found that intracellular levels of $H_2O_2$ and oxidative damage to DNA and lipids in skin fibroblasts from elderly subjects or patients with mitochondrial diseases are significantly increased as compared to those of age-matched controls. Furthermore, Mn-SOD or GPx-1 gene knockout mice were found to display neurological disorders and enhanced oxidative damage similar to those observed in the patients with mitochondrial disease. These observations are reviewed in this article to support that oxidative stress elicited by defective respiratory function and impaired antioxidant enzyme system plays a key role in the pathophysiology of mitochondrial disease and human aging.**

**Key Words: oxidative stress, mitochondrial respiration, reactive oxygen species, free radicals, superoxide dismutase, catalase, glutathione peroxidase, mitochondrial DNA, 8-hydroxy 2'-deoxyguanosine, lipid peroxidation, antioxidant enzymes, aging, mitochondrial disease**

## Introduction

Mitochondria are the power plant of the animal and human cells. They utilize more than 90% of molecular oxygen consumed by tissue cells and a small fraction of which become reactive oxygen

Corresponding author: Dr. Yau-Huei Wei, Department of Biochemistry, School of Life Science, National Yang-Ming University, Taipei 112, Taiwan, ROC. Tel: 886-2-28267118, Fax: 886-2-28264843, E-mail: joeman@ym.edu.tw
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species (ROS) due to incomplete reduction in aerobic metabolism (1). An increase in the production or inefficient disposal of ROS will increase the odds that intracellular components get oxidatively modified or mutated as generally seen in nuclear and mitochondrial DNA (mtDNA). In the past decade, a number of human diseases have been found to associate with mutations in mtDNA (2-5). Among them, A8344G and A3243G transitions are detected in approximately 80% of the patients with myoclonic epilepsy and ragged-red fibers (MERRF) (6) and mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) (7) syndromes, respectively. Large-scale deletions of mtDNA frequently occur in the affected tissues of patients with mitochondrial myopathies such as chronic progressive external ophthalmoplegia (CPEO) and Kearns-Sayre syndromes (2, 8). Both point mutations and large-scale deletions of mtDNA significantly affect mitochondrial gene expression (9, 10) and result in defects in the structure and function of respiratory enzymes. Impairment of mitochondrial electron transport elicits an increased production of ROS and free radicals in the mitochondria (11, 12). Indeed, human cells harboring mutant mtDNA or defective mitochondria were found to accumulate higher levels of ROS and oxidative damage (13-15). It has been documented that patients with mitochondrial diseases exhibit premature aging (16) and their clinical symptoms are progressively worsening with time (17-19). This age-related progression of the disease is quite similar to the natural course of some types of neurodegenerative diseases (20, 21). However, it remains unclear as to how the age-dependent decline of the bioenergetic functions in the affected tissues of the patient leads to mitochondrial disorders or neurodegenerative diseases.

Under normal physiological conditions, superoxide anion ($\dot{O_2^-}$), hydroxyl radical (HO·) and hydrogen peroxide ($H_2O_2$) are continuously produced in tissue cells as by-products of aerobic metabolism (1, 4). More than 500 liters of oxygen is utilized daily by tissue cells of a normal human subject, and 1-5% of the oxygen consumed by the respiratory chain is incompletely reduced to $\dot{O_2^-}$ and $H_2O_2$ (1, 22). If not efficiently removed as that often occurs in the aging process, HO· may be produced from $H_2O_2$ *via* Fenton reaction in the presence of $Fe^{2+}$ or $Cu^+$ and cause oxidative damage to cellular components, including nucleic acids (23), proteins (24) and lipids (25).

To cope with the oxidative stress elicited by aerobic metabolism, animal and human cells have developed a ubiquitous antioxidant defense system, which consists of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase together with a number of low-molecular-weight antioxidants such as ascorbate, α-tocopherol and glutathione (26, 27). However, this antioxidant defense system may be overwhelmed by various pathological or environmental factors so that a fraction of ROS may escape destruction and form the far more reactive hydroxyl radicals (26, 27). An increase in ROS-elicited oxidative damage to DNA and other biomolecules may impair normal functions of tissue cells and lead to human aging and disease (23, 26).

## Mitochondria are the Major Intracellular Producer of ROS

Mitochondria are the intracellular organelles responsible for biological oxidation of various fuel molecules at the final stage of aerobic metabolism in the animal and human cells. They are also the major producers of ROS *via* incomplete reduction of $O_2$ by the electrons leaked out of the electron transport chain. NADH-coenzyme Q oxidoreductase (Complex I) and ubiquinol-cytochrome *c* reductase (Complex III) of the respiratory chain are the major sites that generate ROS in mitochondria (22, 28). Ubisemiquinone and flavosemiquinone radicals and ROS are continually generated and maintained at relatively high steady-state levels in mitochondria. Respiratory inhibitors that block the electron transport at Complex I or Complex III have been demonstrated to increase intracellular levels of $\dot{O_2^-}$ and $H_2O_2$ (29). It has been demonstrated that the rate of production of $\dot{O_2^-}$ and $H_2O_2$ in mitochondria increases with age in various tissues of the animal (30). In addition, the mitochondria isolated from skeletal muscle, heart and brain of mice lacking the heart/muscle isoform of the adenine nucleotide translocase produced larger amounts of ROS (31). These results support the general idea that impairment of the respiratory function leads to an increase in the amounts of ROS and free radicals generated by mitochondria (32, 33). Although antioxidant enzymes together with other antioxidants can dispose of ROS and free radicals, a fraction of them may escape these defense mechanisms and cause oxidative damage to various cellular constituents in the aging or disease tissues (34). An excess production of ROS is harmful to human cells, and any defects that lead to the overproduction of ROS will cause a catastrophe culminating in cell dysfunction or cell death. We have thus proposed that mtDNA mutation-elicited defects in the respiration and oxidative phosphorylation system result in an increase of oxidative stress and oxidative damage in the affected tissues of elderly subjects or the patients with mitochondrial disease (Figure 1).

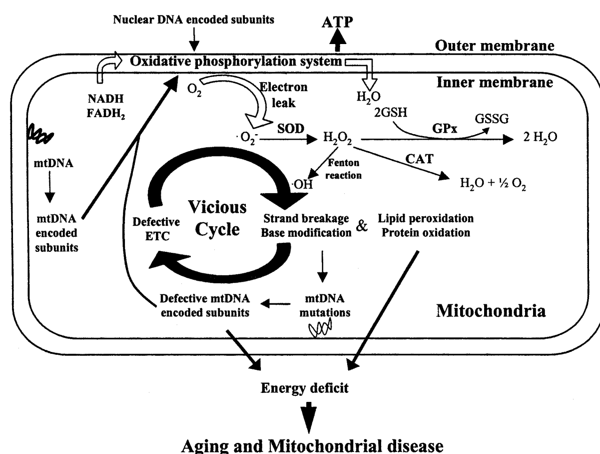


Fig. 1. A schematic illustration of the mitochondrial role in human aging and mitochondrial disease. Under normal physiological condition, 1-5% of the oxygen is converted to the reactive oxygen species (ROS) and free radicals due to incomplete reduction by one-electron transfer reactions in mitochondria. They are usually disposed of by the coordinate function of the antioxidant defense system consisting of free radical scavenging enzymes SOD, GPx and CAT together with a number of small-molecular-weight antioxidants. If escaped, they may cause oxidative damage (strand breakage and base modification) and mutation to mtDNA molecules that are attached, at least transiently, to the inner membranes. The mtDNAs with oxidative damage or mutation are transcribed and translated to produce defective protein subunits, which are assembled to form defective electron transport chain (ETC). The impaired ETC is not only inefficient in ATP synthesis but also generates more ROS *via* electron leak, which may further enhance oxidative damage to various biomolecules in mitochondria. This "vicious cycle" is being operated and accelerated in an age-dependent manner, and results in the widely observed aging-associated accumulation of oxidative damage and mutation of mtDNA, which ultimately results in progressive decline in the bioenergetic function of tissue cells in the aging process. On the other hand, the functions of free radical scavenging enzymes and other repair systems are progressively declined and thus oxidative damage to various biomolecules are gradually accumulated during aging in somatic tissues. As a result, aging and mitochondrial diseases will be manifested in the affected tissues of the elderly subjects or mitochondrial disease patients due to insufficient energy supply, enhanced oxidative stress and profound oxidative damage.

## Oxidative Damage to Mitochondria

While utilizing more than 90% of the oxygen uptake of the animal and human cells, mitochondria are subjected to direct attack by ROS generated by the respiratory chain during aerobic metabolism. Ames and coworkers (35) first demonstrated that the oxidative damage in mtDNA is much more extensive than that in nuclear DNA. They showed that the content of 8-hydroxy 2'-deoxyguanosine (8-OHdG), an index product of oxidative damage to DNA, in mtDNA was significantly higher than that in nuclear DNA in the rat liver. The 8-OHdG level in mtDNA of 24-month old rats was about three times higher than that of 3-month young rats. Other investigators showed that the levels of 8-OHdG in mtDNA and oxidized glutathione of various animal tissues are concurrently increased with age (36, 37). Moreover, the 8-OHdG content in mtDNA also increases in an age-dependent manner in human heart and brain (38, 39). Recently, we showed that the 8-OHdG levels in the skin and lung tissues and skin fibroblasts of elderly subjects were significantly higher than the corresponding values of young subjects (40, 41).

Being very rich in polyunsaturated fatty acids, mitochondrial inner membranes are prone to lipid peroxidation, which has been shown to increase with age in mitochondria of the somatic tissues of rodents and humans (42, 43). Lipid peroxidation is one of the major biochemical events leading to the deleterious effects of ROS and free radicals. We have demonstrated that mitochondrial lipid peroxidation is enhanced in various human tissues in the aging process (40, 43, 44). This may alter the fluidity and other biophysical properties of mitochondrial membranes and impair biochemical functions of various transporters and respiratory enzymes in the inner and outer membranes of mitochondria. It has been shown that cardiolipin, a unique phospholipid localized almost exclusively in the inner membrane of mitochondria, is particularly vulnerable to peroxidative damage due to its high content of unsaturated fatty acids (45). Since cardiolipin is essential for the normal function of cytochrome *c* oxidase and several anion carriers of the inner mitochondrial membrane, peroxidative damage to this phospholipid will inevitably impair the bioenergetic function of mitochondria. Indeed, it was found that a loss of cardiolipin and decline of cytochrome *c* oxidase activity occur concurrently with lipid peroxidation of the mitochondrial membrane (46). Recently, cardiolipin was demonstrated to be required for the specific binding of cytochrome *c* to mitochondrial inner membrane (47). Oxidative modification of cardiolipin abolishes its binding to cytochrome *c*, which is then detached and released from mitochondria. This oxidative alteration not only impairs electron transport function of the mitochondria but also induces apoptosis of the cell under oxidative stress (48).

On the other hand, mitochondrial proteins are also vulnerable to oxidative modification in aging. It was demonstrated that aconitase, a Kreb cycle enzyme in the mitochondrial matrix, is a specific target of ROS (49) and that the protein carbonyl content of adenine nucleotide translocase in the mitochondrial inner membrane of muscle tissues is increased with age of the housefly (50). In addition, the iron-sulfur centers of the respiratory enzymes (e.g., succinate dehydrogenase and NADH dehydrogenase) are prone

to oxidative modification and the electron transport function of mitochondria may be impaired by ROS under oxidative stress.

## Mitochondrial Role in Aging

The fact that mitochondria are the major generators and direct targets of ROS in the tissue cells has led us and other investigators to foster the idea that oxidative stress and oxidative damage in mitochondria are contributory factors to aging. In recent years, abundant experimental data have been accumulated to support the idea that mitochondrial function declines in human aging and degenerative diseases (33, 51). The bioenergetic function of mitochondria is decreased with age in the postmitotic cells (e.g., brain, heart and muscle) of the human and animals (33, 52). In 1989, we first reported that glutamate-malate-supported respiration and the electron transport activities of Complexes I and IV were more dramatically declined with age (53). Since ten polypeptides constituting these two respiratory enzyme complexes are encoded by mtDNA, we conjectured that mutations in mtDNA cause the age-dependent decline in the respiratory function of mitochondria. This notion was supported by the fact that an increasing number of point mutations, deletions and tandem duplications of mtDNA increase with age (54-56). The mtDNA molecules with 4,977 bp and other deletions have been found to increase exponentially with age in various human tissues (57-59). Two point mutations in mtDNA have also been reported to accumulate in aging human muscle (55, 60), although Pallotti et al. (61) failed to find a causal correlation between point mutations of mtDNA and age in the group of their study subjects. It is now generally accepted that mtDNA mutations are increased with age, but the proportion of each of the aging-associated mutated mtDNAs is generally less than five percent of total mtDNA in a cell (62). Thus, it was argued as to how such low levels of mutated mtDNA bring about significant decline of the bioenergetic function of mitochondria (63, 64). Several investigators have suggested that the reported mtDNA mutations are just the tip of the iceberg of the aging-associated alterations in the mitochondrial genome (51, 54). In fact, it has been shown that a wide spectrum of deletions of mtDNA occurs and accumulates in the muscle of the aged individual (65). Recently, Michikawa et al. (66) reported that a T414G transversion in the D-loop of mtDNA is accumulated at 20-50% of the total mtDNA in the skin fibroblasts from elderly subjects. This mutation is located at the control region of mtDNA and may impair the replication and transcription of mtDNA in tissue cells of the elderly human subjects. Furthermore, Hayakawa et al. (67) reported a wide array of fragmentation of mtDNA into 358 different sizes in human heart muscle. It is worth noting that a host of large-scale deletions in mtDNA is accumulated in aging skeletal muscle and that the amount of full-length mtDNA amplifiable by extra-long PCR markedly decreased with age (68). Therefore, the total amount of mtDNA molecules with a deletion may reach such a high level that mitochondrial respiration and oxidative phosphorylation are severely impaired (69). Indeed, muscle fibers with very low activity of cytochrome c oxidase were found in skeletal and heart muscles from elderly subjects (70, 71). Moreover, high levels of mutant mtDNA molecules were found in the cytochrome *c* oxidase-deficient muscle fibers (65, 71, 72). Accumulation of multiple mtDNA deletions, along with a concurrent decrease of the wild-type mtDNA, was found to correlate with the decrease of cytochrome *c* oxidase activity in aging skeletal muscle (72). Recently, we found that mtDNA mutation and oxidative DNA damage are concurrently increased in the aging human lung and skin (40, 41). Taken together, these findings clearly suggest a strong correlation between the decline of bioenergetic function of mitochondria and mutation of mtDNA in aging human tissues.

## Age-associated Alterations in Antioxidant Defense System

A number of investigators has reported that endogenous levels of antioxidant enzymes (except for Mn-SOD) and small-molecular-weight antioxidants in tissues are negatively correlated with the maximum lifespan potential of mammals and primates (73, 74). It is generally established that the gradual loss of the capability of animals to cope with oxidative stress is one of the characteristics of aging (75). Orr and Sohal (76) reported that transgenic *Drosophila melanogaster* overexpressing both CAT and Cu,Zn-SOD had about 33% longer lifespan as compared to the control. However, overexpression of Cu,Zn-SOD or CAT alone had only a minimal effect on the average lifespan and no effect on the maximum lifespan of the fruit fly (77). Recently, it was found that ubiquitous overexpression of Cu,Zn-SOD alone in mice does not extend the lifespan of the animal (78). This implies that efficient removal of ROS by coordinate expression of antioxidant enzymes is essential for longevity of *D. melanogaster*. It is well established that the animals that have lower rates of metabolism and mitochondrial production of ROS usually live longer (30, 75-77). However, past investigations on the influence of aging on antioxidant enzymes in the human and animals have resulted in conflicting results (73, 79-81) (Table 1). Aging-

**Table 1. The Changes between Free Radical Scavenging Enzymes in Different Tissues of Patients with Mitochondrial Diseases and Elderly Subjects**

| Subjects | Tissues | Cu, Zn–SOD | Mn-SOD | CAT | GPx | References |
|---|---|---|---|---|---|---|
| CPEO | Muscle | 0 | ↑ | – | – | 85[c] |
| CPEO | Skeletal muscle | ↑ | ↑ | – | – | 86[c] |
| CPEO | Skin fibroblast | – | ↑ | 0 | 0 | 93[a,c] |
| KSS | Skeletal muscle | ↑ | ↑ | – | – | 86[c] |
| MELAS | Muscle | 0 | ↑ | – | – | 85[c] |
| MELAS | Myoblast | ↑ | ↑ | ↑ | – | 91[c] |
| MERRF | Blood | ↓ | ↓ | – | – | 89[c] |
| CC | Skin fibroblast | – | ↑ | – | – | 14[b,c] |
| FILA | Skin fibroblast | – | ↑ | – | – | 80[c] |
| Elderly subjects | Lung | 0 | 0 | ↑ | ↓ | 80[c] |
| | Liver | ↑ | ↑ | 0 | ↓ | 80[c] |
| | Skeletal muscle | ↓ | ↑ | 0 | 0 | 34[c] |
| | Skin fibroblast | ↓ | ↑ | ↓ | ↓ | 41[c], 93[a,c] |

Symbols: ↑, increase; ↓, decrease; 0, no change; –, not determined. Abbreviations: CC, cardiomyopathy with cataract; CPEO, chronic progressive external ophthalmoplegia; FILA, fatal infantile lactic acidosis; MELAS, mitochondrial encephalomyopathy, lactic acidosis with stroke-like episodes syndrome; MERRF, myoclonic epilepsy with ragged-red fibers syndrome; KSS, Kearns-Sayre syndrome. The superscripts *a*, *b*, and *c* indicate the changes at the mRNA, protein, and activity levels.

associated changes in antioxidant defense systems appear to depend on species, sex and the type of the tissue examined (73, 79, 82). It was argued that age-associated increases in the activities of some free radical scavenging enzymes of tissue cells can be counterbalanced by the decreases of some others, so that the overall antioxidant capacity of the tissue cells may not be significantly affected in aging of the animal and human (27). This explanation seems attractive but is not supported by our recent findings described below.

The abilities of human cells to respond to endogenous and exogenous oxidative stress may be compromised by alterations of gene expression of antioxidant enzymes (83, 84). An imbalance of the free radical scavenging enzymes is thought to enhance oxidative stress and elicit oxidative damage to tissue cells during the aging process (83). Recently, we demonstrated that the activities of Cu,Zn-SOD, CAT and GPx in human skin fibroblasts were decreased significantly with age (41). However, the activity of Mn-SOD was increased with age before 65 years but was mildly decreased thereafter. These non-parallel changes may induce an imbalance in the intracellular levels of prooxidants and antioxidants, which in turn elevates oxidative stress and increases oxidative damage in skin fibroblasts. Indeed, we found that the specific contents of lipid peroxides (measured as malondialdehyde) and 8-OHdG in human tissues are increased in an age-dependent manner (40, 41, 44). This is consistent with the findings that reduced glutathione level is declined (36) and ROS and 8-OHdG levels are increased in animal mitochondria in an age-dependent manner (30, 36). In another study, we found that Mn-SOD activity was increased with age but GPx and CAT activities did not show significant changes in human skeletal muscle (84). These results support our working hypothesis that alterations in the activities of free radical scavenging enzymes play an important role in the age-related increase of oxidative stress in human tissues. It is worth pointing out that the generally observed age-

**Table 2. Comparison of the Activities and RNA Levels of Free Radical Scavenging Enzymes in the Skin Fibroblasts from CPEO and MERRF Patients with Those of the Age-matched Healthy Subjects**

| Fibroblasts | MnSOD | | Catalase | | GPx | |
|---|---|---|---|---|---|---|
| | Enzyme activity | RNA level | Enzyme activity | RNA level | Enzyme activity | RNA level |
| CPEO (n=6) | 267.8±54.2* | 0.63±0.13* | 14.3±3.3 | 0.68±0.17 | 39.6±6.2 | 0.54±0.02 |
| MERRF (n=3) | 32.2±15.3* | 0.05±0.02* | 7.7±1.3* | 0.19±0.13* | 63.9±9.7 | 0.63±0.13 |
| Control (n=18) | 130.9±38.9 | 0.23±0.08 | 13.5±3.20 | 0.73± 0.12 | 47.3±10.8 | 0.51±0.11 |

The data for the enzyme activities were obtained from three independent experiments and are expressed as mean±SD. The superscript "*" indicates significant difference ($p < 0.01$) in the free radical scavenging enzyme activity and RNA levels of skin fibroblasts between the patients and control. Values for mRNAs of free radical scavengers Mn-SOD, catalase and GPx were normalized with the level of $\beta$-actin RNA. Control data were obtained from the skin fibroblasts of 18 healthy subjects between 25 and 70 years old, who provided skin tissues for cell culture. The fibroblasts examined were between three and six population doublings. The fibroblasts were established from the skin biopsies of the CPEO and MERRF patients and subjects who were ruled out of having any of the known mitochondrial diseases. The figures presented in this table are compiled from the original data in the Ph.D. thesis of Ching-You Lu (ref. 93), and part of which has been published (ref. 41).

dependent decline in small-molecular-weight antioxidants in the plasma and tissues may further aggravate the oxidative stress in the tissues of elderly subjects.

## Role of ROS in Mitochondrial Diseases

Mitochondrial diseases are a unique group of human diseases that are mostly caused by defects in the respiration and oxidative phosphorylation system (3, 4). They dominantly affect the tissues or organs of high energy-demand, such as brain, heart and skeletal muscle. More than one hundred mtDNA mutations have been established to associate with a wide spectrum of mitochondrial disease (4, 21). Some of these mutations have been demonstrated to cause dysfunction of the respiratory chain (9, 10) and in turn lead to an increase of electron leak and over-production of ROS in mitochondria (13-15). Thus, both impairment of respiratory function and increased production of ROS have been attributed to the pathophysiology of mitochondrial disease (19, 31). In an early study, Piccolo et al. (17) found that lipid peroxides and fluorescent adducts of organic aldehyde with plasma proteins are elevated in blood cells of the patients with CPEO syndrome. Moreover, the skeletal muscle with a significant increase of 8-OHdG content and higher immunohistochemical staining for Mn-SOD displayed more pronounced appearance of ragged-red fibers (mainly caused by over-proliferation of abnormal mitochondria) in the patients with KSS or CPEO syndrome (85). Luo et al. (15) demonstrated that there was an increase of hydroxyl radicals and aldehydic lipid peroxidation products in skin fibroblasts from patients with Complex I deficiency. It has been established that enhanced oxidative stress, caused by impairment of the respiratory function of mitochondria (85, 86) or by targeted disruption of the Mn-SOD gene (87), may cause neuromuscular disorders that are similar to those seen in the patients with mitochondrial diseases (88). Recent studies in this and other laboratories have provided evidence to support that increase of endogenous oxidative stress elicited by impairment of the respiratory chain in the affected tissues of the patients plays an important role in the pathogenesis and progression of mitochondrial diseases (19, 41, 86, 88).

## Alteration of Antioxidant Enzyme System in Mitochondrial Diseases

It has been known for some time that the activities of free radical scavenging enzymes are altered in the affected tissues of patients with mitochondrial diseases (Table 1). Antioxidant defense system is impaired and oxidative damage to DNA is enhanced in skeletal muscle of the patients with mitochondrial encephalomyopathies (85, 86, 89). The skeletal muscle displaying ragged-red fibers in the patients with CPEO and MELAS syndromes showed an increase in the expression of Mn-SOD but not of Cu,Zn-SOD (85). In the patients with CPEO syndrome, it was found that Mn-SOD-positive muscle fibers predominantly exhibited decreased activity of cytochrome *c* oxidase. On the other hand, Mitsui et al. (86) found that the cytochrome *c* oxidase-negative ragged-red fibers had elevated levels of Mn-SOD and Cu,Zn-SOD in the patients with KSS and CPEO

syndromes. The 8-OHdG level in cellular DNA of these muscle fibers was significantly higher than that of the normal control. Defects in Complex I have been identified in some patients with fatal infantile lactic acidosis, cardiomyopathy, hepatopathy with tubulopathy, Leigh syndrome and those with lactic acidemia (14, 16, 90). It was found that the severity of cytochrome *c* oxidase deficiency was correlated with the increase of production of $O_2^-$ and induction of mRNA of the MnSOD gene, and that the rate of $O_2^-$ production was decreased by the induction of Mn-SOD gene expression (14). This kind of adaptive (or compensatory) response of antioxidant enzymes was also observed in cultured myoblasts from three MELAS patients harboring 20% to 40% of mtDNA with the A3243G mutation (91). Barrientos and Moraes (92) developed several xenomitochondrial cybrids with 40% deficiency in Complex I and 143B cells with impaired Complex I activity caused by different concentrations of rotenone, an inhibitor acting at Complex I. They found that the respiration-deficient cybrids and rotenone-treated 143B cells all exhibited retarded growth, declined respiratory function and lower mitochondrial membrane potential accompanied by an elevation of ROS production and increased lipid peroxidation.

Recently, we cultured skin fibroblasts from nine patients with CPEO syndrome for the study of free radical scavenging enzymes. We found that the skin fibroblasts from the patients all had significantly higher enzyme activity and mRNA level of Mn-SOD but those of CAT and GPx were not increased or even decreased (ref. 93 and Table 2). Western blot analysis revealed similar imbalance in protein levels of these antioxidant enzymes in fibroblasts. These results indicate an imbalance between the $H_2O_2$ generation and disposal systems in the fibroblasts from the patients with CPEO syndrome. We confirmed this by the finding that fibroblasts from CPEO patients contained 2-3 fold higher levels of $H_2O_2$, which was measured on a flow cytometer after staining the fibroblasts with DCFH-DA. Moreover, we found that such an imbalance of free radical scavenging enzymes is more pronounced in myoblasts than in skin fibroblasts of the CPEO patients. It is important to note that the oxidative damage to DNA in muscle of CPEO patients is much more extensive than that of patients with other types of diseases that are not associated with neuromuscular disorders (93). The mean 8-OHdG/$10^6$dG ratio in the muscle DNA of the CPEO patients was 50.4±23.8 (N=3), and that for the age-matched healthy subjects was 6.3±0.2 (N=8). The difference between the two groups was found to be significant ($p<0.05$, Student t test). On the other hand, we also found that oxidative stress in skin fibroblasts of the patients with MERRF syndrome is higher than that of the age-matched controls (Table 2). Brambilla et al. (94) reported that the Se-dependent and -independent GPx activities are increased in response to deficiency of respiratory enzymes in human myeloid leukemia U937 cells after treatment with chloramphenicol or ethidium bromide. Taken together, these observations suggest that an impairment or functional imbalance of free radical scavenging enzymes plays an important role in the pathogenesis and age-dependent progression of CPEO, MERRF and MELAS syndromes and possibly of the other mitochondrial diseases.

## Pathological Consequences of Imbalance in Antioxidant Enzymes

A fine balance between free radical scavenging enzymes is important for the cellular resistance to oxidative stress (83). A low SOD relative to GPx and/or CAT could lead to the accumulation of superoxide anions. On the other hand, a high SOD relative to GPx and/or CAT may lead to an increased production of $H_2O_2$. Chen and Ames (95) found that senescence-like growth arrest could be induced by $H_2O_2$ in human diploid fibroblasts. Therefore, any significant increase in the SOD activity must be accompanied by a comparable increase in CAT and/or GPx activity to prevent excessive buildup of $H_2O_2$ in the cell. Several investigators studied the effects of overexpression of SOD in several cell types including mouse L cells, neuroblastoma cells, murine fibroblasts, mouse epidermal cells and NIH/3T3 fibroblasts transfected with the cDNA of human Cu,Zn-SOD (96-99). The transfectants overexpressing Cu,Zn-SOD alone were more susceptible to DNA strand breaks, growth retardation, easy killing by an extracellular burst of cell $O_2^-$ and $H_2O_2$, and exhibited the feature of cell senescence (98, 99). Some clones showed adaptation to Cu, Zn-SOD overproduction by an increase in GPx or CAT activity and the double transfectants of CAT and Cu,Zn-SOD or GPx were better protected from oxidative damage. Moreover, Li et al. (99) obtained two Mn-SOD overexpressing clones by transfection of NIH/3T3 mouse fibroblasts with the Mn-SOD cDNA. The two clones showed different sensitivities to $H_2O_2$ and menadione and altered cell cycle progression, resulting in an accumulation of cells in G2/M phase and a decrease of cells in the mitotic phase (99). Furthermore, the cells overexpressing Mn-SOD had higher intracellular level of $H_2O_2$ and showed a 9.5-fold induction of mRNA of the matrix-degrading metalloprotease-1, which has been shown to be involved in the process of carcinogenesis and aging (100).

The most striking pathological consequences of elevation of ROS elicited by defects in the free radical

scavenging enzymes were observed in the gene-knock-out rodents (31, 101-104). Melvo et al. (101, 102) first discovered that mice lacking Mn-SOD developed neurological disorders similar to those found in the patients with certain mitochondrial diseases. This group has further demonstrated that genetic disruption of the GPx-1 gene developed respiratory function defects and enhanced oxidative stress in mice (104). Recently, it was reported that the mean lifespan of wild-type *Caenorhabditis elegans* was extended 44% by treatment with two small synthetic antioxidants, superoxide dismutase/catalase mimetics (105). The mimetics also normalized the lifespan of prematurely aging worms harboring a mutation in the *mev-1* gene that encodes a mitochondrial electron transport protein (culminating in a 67% increase of lifespan). Since the genes that are involved in the disposal of ROS are highly conserved among eukaryotes, the biochemical basis by which antioxidant enzymes affect lifespan in invertebrates may also apply to higher animals and humans (106). These observations clearly indicate that a defect or imbalance in any of the free radical scavenging enzymes may cause pathological changes, and that lifespan may be significantly shortened by decreasing the antioxidant capacity of the human and animals.

On the other hand, we have observed that the intracellular levels of $H_2O_2$ in the skin fibroblasts of the patients with CPEO or MERRF syndrome are significantly higher than that of the normal fibroblasts (93, 94). This is easily explained by the findings that Mn-SOD is up-regulated in CPEO skin fibroblasts but is down-regulated in the fibroblasts of MERRF patients. Moreover, it was reported that serum levels of vitamin E and coenzyme $Q_{10}$ and SOD activity were decreased in the patients with MELAS or MERRF syndrome (89). These and other lines of evidence have led us to suggest that imbalance or defect in antioxidant enzyme systems is an important causal factor and contributes to premature aging and the development of oxidative stress-elicited pathologies of mitochondrial disorders (84).

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**Abbreviations used:** CPEO, chronic progressive external ophthalmoplegia; CAT, catalase; Cu,Zn-SOD, copper, zinc-dependent superoxide dismutase; GPx, glutathione peroxidase; MELAS, mitochondrial encephalopathy, myopathy with stroke-like episodes syndrome; MERRF, myoclonic epilepsy with ragged-red fibers syndrome; Mn-SOD, manganese-dependent superoxide dismutase; mtDNA, mitochondrial DNA; 8-OHdG, 8-hydroxy 2'-deoxyguanosine; ROS, reactive oxygen species.

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