The Role of Mitochondrial Superoxide Anion (O$_2^-$) on Physiological Aging in C57BL/6J Mice

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Mitochondrial superoxide anion (O$_2^-$)/Mitochondrial SOD/Oxidative damage/Apoptosis/Physiological aging.

Much attention has been focused on the mitochondrial superoxide anion (O$_2^-$), which is also a critical free radical produced by ionizing radiation. The specific role of the mitochondrial O$_2^-$ on physiological aging in mammals is still unclear despite widespread evidence that oxidative stress is involved in aging and age-related diseases. The major endogenous source of O$_2^-$ is generated as a byproduct of energy metabolism from mitochondria. In order to better understand how O$_2^-$ relates to metazoan aging, we have comprehensively examined age-related changes in the levels of oxidative damage, mitochondrial O$_2^-$ production, mitochondrial antioxidant enzyme activity and apoptosis induction in key organs of an inbred mouse strain (C57BL/6J). Oxidative damage accumulated and excess apoptosis occurred in the brain, ocular and kidney with aging, but comparatively little occurred in the heart and muscle. These rates are correlated with O$_2^-$ levels. Mitochondrial O$_2^-$ production levels increased with aging in the brain, ocular and kidney, and did not significantly increased in the heart and muscle. In contrast to O$_2^-$ production, mitochondrial SOD activities increased in heart and muscle, and remained unchanged in the brain, ocular and kidney with aging. These results suggest that O$_2^-$ production has high organ specificity, and oxidative damage by O$_2^-$ from mitochondria mediated apoptosis can lead to organ atrophy and physiological dysfunction. In addition, O$_2^-$ from mitochondria plays a core role in physiological aging.

INTRODUCTION

Recent research using genetically modified animals have elucidated that aging can be defined as organ and physiological dysfunctions caused by a gradual decline of homeostatic function. It is known that radiation leads to premature aging.$^{1,2}$ Much attention has recently focused on the hypothesis that inflammatory processes, some of which are mediated through free-radical production, may contribute to cellular and organismal aging.$^{3,4}$ Many radiation researchers are also interesting in the putative contributions that radiation exposure plays in a variety of age-related pathologies, including but not limited to cancer. In particular, inflammatory responses may factor into some of these diseases.$^{6}$ All of this suggests that the biological effects of ionizing radiation and free radicals are tightly linked. In fact, pre-exposure of oxidative stress confers protective effect against the lethality imposed by subsequent X-irradiation.$^{5-8}$ It has been known that exposure to ionizing radiation (IR) results in the formation of free radicals in various cells and organisms. In the presence of oxygen, the radiation-induced free radicals including the hydroxyl radical (OH·), superoxide anion (O$_2^-$) and organic radicals (R·) are thought to initially form. The oxygen is indispensable for biosynthesis of ATP in most organisms. However, oxygen can be converted to reactive oxygen species superoxide anion (O$_2^-$) mainly from electron leakage from complexes I and III of the electron transport chain. O$_2^-$ can be metabolized and transformed to hydrogen peroxide (H$_2$O$_2$) and (OH·).$^{9,10}$ Such endogenously generated reactive oxygen species (ROS) readily attack a wide variety of cellular entities, resulting in damage that compromises cell integrity and function.$^{9,11,12}$ O$_2^-$ produced from mitochondria is usually converted to H$_2$O$_2$ by superoxi-
ide dismutase (SOD), which is an antioxidant enzyme, and H₂O₂ is then metabolized to harmless H₂O and O₂ by catalase and glutathione peroxidase (GSH-Px). Cells maintain homeostasis by effectively dealing with the ROS that they produce. Disruptions of the balance between O₂⁻ production and anti-oxidant levels can lead to premature aging or geriatric diseases. While this relationship is known in general terms, the tissue-specific changes in mammals have not yet been completely elucidated. The causal association between O₂⁻ and physiological aging cannot be evaluated critically without studying the comprehensive alterations as animal age.

We now present data that helps clarify the aging alterations of oxidative damage, mitochondrial O₂⁻ production, mitochondrial antioxidant enzyme activities (mitochondrial SOD activities), mitochondrial electron transfer system complex activities (cytochrome c oxidase activities), apoptosis induction and body temperature in heart, brain, muscle, ocularus and kidney of 2, 6, 12 and 24 month-old inbred mice. These data support the hypothesis that mitochondrial O₂⁻ contributes to physiological aging, triggering oxidative damage and apoptosis.

**Materials and Methods**

**Animals**

C57BL/6j mice of 2, 6, 12, 24 month-old were used and kept under a SPF condition: 22 ± 1°C, approximately 40% humidity, 12/12-h light/dark cycle, a standard diet CE2 (9.3% water, 25.8% crude protein, 4.5% crude fat, 4.5% crude fiber, 6.7% crude ash)(CREA Japan, Inc.) and sterile water ad libitum. Newborn mice were separated from their mothers on day 28 and grouped as 4 males or 4 females per cage, and they were checked daily. Mice were euthanized shortly before they died to reduce suffering. All experimental procedures were designed in accordance with Guidelines for Animal Experimentation of Tokai University and approved by the Tokai University Animal Care and Use Committee.

**Isolation of Mitochondria**

Mitochondria were isolated from mouse tissues using a standard procedure by differential centrifugation. The mice fasted for 24 hr and euthanized by a cervical dislocation under Nembutal anesthesia. The organs were obtained by laparotomy just after beheading and exsanguinations. After washed by ice-cold PBS, the organs were minced in a volume of isolation buffer (210 mM mannitol, 70 mM sucrose, 0.1 mM EDTA, 5 mM Tris- HCl (pH 7.4)) that was ninfold greater than the tissue weight. The minced organs were homogenized in isolation buffer at 300–600 rpm and with 10–30 strokes using a teflon homogenizer. The homogenate was centrifuged at 350 × g for 10 min, 4°C. The supernatant was transferred to a fresh tube, and centrifuged at 17,500 × g for 10 min, 4°C. The mitochondria-containing pellet was suspended in TE buffer (50 mM Tris-HCl (pH 7.4), 0.1 mM EDTA), and the supernatant was also kept as a cytoplasmic fraction. The protein concentration was determined by BCA protein assay kit (PIERCE, ROCKFORD, IL).

**Measurement of 8-OHdG**

Immediately after isolation, the tissues were immersed in an optimal cutting temperature (OCT) compound (Lab-Tek) and frozen in dry ice-ethanol for immunohistochemical staining. For fluorescent labeling, frozen sections (7 μm) were fixed in ice-cold acetone and blocked with goat serum. The sections were reacted and stained at 4°C for 12 hr with mouse Ab to 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Nikken SEIL, Shizuoka, Japan) conjugated with secondary Abs of anti-Ig Alexa Fluor 488 (Molecular Probes) in advance. Fluorescence was measured with a confocal laser scanning microscope (LMS510 META) (Carl Zeiss, Inc.).

**Measurement of Carbonylated Protein**

Carbonylated protein as an indicator of oxidized protein was evaluated by Enzyme Linked Immunosorbent Assay (ELISA) technique. Mitochondrial proteins were treated with 10 mM DNPH. 250 ng mitochondrial proteins in 50 mM NaHCO₃ was coated on enhanced protein-binding ELISA plate (Caster) by incubating at 4°C for 8 hr. Nonspecific binding to the plate was minimized by blocking the wells with 100 μl blocking buffer (3% BSA, 0.1% NaN₃ in PBS) at 37°C for 1 hr. After the supernatant was removed, 100 μl of anti-DNP antibody diluted with buffer G (0.1% BSA, 0.1% gelatin, 0.1% NaN₃, 1 mM MgCl₂ in PBS) was added to each well and incubated at 37°C, 1 hr. After the supernatant was removed, the plate was washed four times with PBS and incubated 100 μl of horseradish peroxidase-conjugated secondary antibody diluted with 0.05% Tween 20 in PBS was added to the plate, followed by incubation at 37°C for 1 hr. The plate was washed four times to remove the unbound secondary antibody. After 100 μl of ELISA coloring solution (0.0156 M C₆H₈O₇, 0.1 M Na₂HPO₄-12H₂O, 0.4 mg/ml o-Phenylenediainime dihydrochloride, 0.2 μl/ml 30% H₂O₂) was added per well, the reaction was terminated by the addition of 100 μl of 1 M H₂SO₄. The absorbance was measured using a computer-controlled spectrophotometric plate reader (Spectra Max 250) (Molecular Devices) at a wavelength of 492 nm.

**Measurement of Cytochrome c Release**

The cytoplasmic fractions were suspended in an equal amount of 2x sample buffer (0.125 M Tris-HCl (pH 6.8), 10% 2-mercaptoethanol, 4% SDS, 10% sucrose, 0.004% bromophenol blue) and were electrophoresed on 12.5% polyacrylamide gels. The gels were transferred to polyvinyliden difluoride membranes (ATTO CO., Tokyo, Japan),
Measurement of superoxide anion \((O_2^-)\)

\(O_2^-\) production was measured using the chemiluminescent probe MPEC (2-methyl-6-p-methoxyphenylethynyl-imidazopyrazinone) (ATTO Co., Tokyo, Japan). MPEC has an advantage of low background relative to MCLA (3, 7-dihydro-2-methyl-6-(4-methoxyphenol) imidazol[1, 2-a] pyrazin-3-one) that is generally used.\(^{15,22,23}\) \(60 \mu\)g of intact mitochondrial fraction was added to 1 ml assay buffer (50 mM HEPES-NaOH (pH 7.4), 2 mM EDTA) containing 0.7 mM of MPEC. The solutions were placed into a photon counter with an AB-2200 type Luminescencer-PSN (ATTO Co., Tokyo, Japan) and measured at 37°C. The rates of \(O_2^-\) were expressed as counts per second.

Measurement of activities of mitochondrial superoxide dismutase \((SOD)\)

Mitochondrial SOD activity was determined by using a SOD assay kit-WST (DOJINDO MOLECULAR TECHNOLOGIES, INC.). The SOD Assay Kit-WST allowed for very convenient SOD assays by utilizing Dojindo’s highly water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt), which produced a water-soluble formazan dye upon reduction with a \(O_2^-\).\(^{24,25}\) 40 \(\mu\)g of intact mitochondrial fraction was used.

Measurement of activities of cytochrome c oxidase \((COX)\)

A conventional method to measure cytochrome c oxidase (COX) activity is by monitoring oxidation of reduced cytochrome c at 550 nm.\(^{26}\) Attempting similar measurements using intact mitochondrial fractions in 96-well plates, we encountered several problems as follows: high rates of spontaneous oxidation of cytochrome c, nonlinear kinetics, and irreproducibility between measurements. Consequently, to measure COX activity, the DAB (3, 3’-Diaminobenzidine Tetrahydrochloride) technique\(^{27}\) employed the principal that COX oxidizes reduced cytochrome c, which is then reduced by DAB. This resulted in an oxidized DAB polymer that was detectable using a spectrophotometer at 450 nm, with readings directly proportional to the activity of COX. 40 \(\mu\)g of the intact mitochondrial fraction was added to 50 \(\mu\)l assay buffer (100 mM potassium phosphate buffer (pH 7.4) containing 0.02% laurylmaltoside). The solutions were added to 100 \(\mu\)l of substrate medium (4 mM DAB, 100 \(\mu\)M reduced cytochrome c, 2 \(\mu\)g/ml catalase). Absorbance was immediately measured for 20 minutes using a spectrophotometer (Spectra Max 250) (Molecular Devices) at 450 nm, 37°C. Control incubations were performed which included mitochondrial fraction plus 0.3 mM cyanide to show specificity of DAB oxidation by COX.

Measurement of body temperature

Colonic temperature as an indicator of body temperature was determined by using a rectal probe (Microprobe thermometer BAT-12, Muromachi Kikai CO., LTD.). To minimize their discomfort and stress, the measurement was performed once a day, and the mouse was given at least a three-day rest interval on occasions when the same individual was measured several times. Furthermore, the measurements were performed at the same time of day.

RESULTS

Oxidative damage: 8-hydroxy-2’-deoxyguanosine (8-OHdG)

Using immunohistological staining, 8-OHdG was observed as a marker of oxidative damage in DNA. 8-OHdG accumulation with aging was observed using this approach.\(^{28}\) 8-OHdG levels increased in the cortex of cerebrum and hippocampus in brain (Fig. 1). They also increased in the cerebellum (data not shown). The levels slightly increased in heart and skeletal muscle (biceps femoris) (Fig. 1). In the oculus, the accumulation rate varied from tissue to tissue; specifically, it increased substantially in choroids, retinal pigment epithelium and sensory retina of retina and epithelium of cornea. Finally, levels also increased in the kidneys (Fig. 1).

Oxidative damage: carbonylated protein

Carbonylated protein was measured as a marker of oxidized protein in the intact mitochondrial fractions from various mouse tissues. Mitochondria were selected because they are as the major endogenous source of \(O_2^-\) production.\(^{9,29,30}\) The carbonylated protein in the oculus and kidney of 2 month-old mice increased to twice the levels in the brain, heart and muscle (data not shown). The protein levels increased with aging in brain and heart but the increases were statistically insignificant (Fig. 2). On the other hand, it increased 1.2 fold in the muscle, 2.3 fold in the oculus and 1.5 fold in the kidney of 24 month-old mice compared to 2 month-old mice (Fig. 2).

Apoptosis

The leakage of cytochrome c from mitochondria to the cytoplasm was quantified as a marker of apoptosis. In normal cells, cytochrome c is located primarily in mitochondria,
but cellular damage induces apoptotic signals that result in cytochrome c release from mitochondria to the cytoplasm. The cytochrome c then activates a caspase cascade to induce apoptosis.\textsuperscript{31,32} The amount of cytochrome c increased 2.2 fold in brain, 13.3 fold in oculus and 3.5 fold in kidney of 24 month-old compared to 2 month-old mice but remained unchanged in heart and muscle (Fig. 3).

\textit{O$_2^-$ level and mitochondrial SOD activity}

The relative mitochondrial mass per cell could influence O$_2^-$ production and could vary from organ to organ. Therefore, VDAC1 proteins, which are used as a marker of mito-
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While the amount of VDAC1 did vary from organ to organ, it did not change with aging (data not shown). Thus, any differences in $O_2^-$ reflect age-dependent changes in production rather than simply changes in mitochondrial concentration. Mitochondrial $O_2^-$ production level was measured using an $O_2^-$-specific chemiluminescent probe (2-methyl-6-p-methoxyphenylethynylimidazopyrazinone: MPEC). $O_2^-$ production increased in the brains of 6, 12, and 24 month-old mice compared to 2 month-old mice. The levels increased in the oculus of 24 month-old mice and kidney of 12 month-old mice. In contrast, the levels remained unchanged in heart with aging and actually decreased in the muscle of 24 month-old mice (Fig. 4A).

In contrast to $O_2^-$ production, mitochondrial SOD (a mitochondrial antioxidant enzyme) activities increased in the

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**Fig. 3.** Cytochrome c release to cytoplasm. *(Top figures)* Western blotting. *(Bottom columns)* Each value shows the comparison to the value of each organ of 2 month-old mice (mean ± S.D, n = 4, *P < 0.04, **P < 0.001 according to the Student t-test.).

**Fig. 4.** Superoxide anion ($O_2^-$) production and Mn-SOD activity. *(A)* $O_2^-$ production (mean ± S.D, n ≥ 6, *P < 0.03, **P < 0.004 according to post hoc tests using Dunnett's test. The post hoc tests were executed only if *P < 0.05 in one-way ANOVA in each organ.), *(B)* Mn-SOD activity (mean ± S.D., n ≥ 6, *P < 0.02, **P < 0.001 according to post hoc tests using Dunnett's test. The post hoc tests were executed only if *P < 0.05 in one-way ANOVA in each organ.).
hearts of 24 month-old mice and the muscles of 12 and 24 month-old mice as compared to 2 month-old mice. The activity remained unchanged in the brains, oculus and kidney as mice aged (Fig. 4B).

**Cytochrome c oxidase (COX) activity**

As a marker of mitochondrial function, cytochrome c oxidase (COX) activity was measured. There are five complexes that constitute the electron transport chain and reside in the mitochondrial inner membrane. The electrons are finally sent to cytochrome c oxidase (COX) in electron transport chain complex IV and ATP is synthesized in complex V. Thus, COX activity is one important index of mitochondrial function.14) The activity was correlated with the amount of VDAC1 protein levels (data not shown). The activity remained unchanged in brain, heart and muscle (Fig. 5). In contrast, the activity decreased significantly in oculus and kidney and experienced a 20% reduction in 24 month-old mice as compared to 2 month-old mice (Fig. 5).

**Body temperature**

The body temperature significantly decreased with aging, with a 0.75°C decline in 12 month-old mice and a 1.30°C decline in 24 month-old mice as compared to 2 month-old mice (Table 1).

**DISCUSSION**

To clarify whether physiological aging results from a disruption in the balance between O$_2$ production and antioxidant levels, we examined oxidative damage caused by ROS in several organs in an inbred mouse strain (C57BL/6J) as they progressed through their lifespan under normal laboratory conditions. ROS enhances aging owing to their ability to attack cellular constituents such as DNA, protein and lipids. Increases in some aging markers (e.g., 8-OHdG, carbonylated protein and lipid peroxide) have been observed with aging in a variety of animal species.15,18,34–36 We also observed increases in 8-OHdG levels. In particular, levels rapidly accumulated in the brain, oculus and kidney (Fig. 1).
In the oculus, 8-OHdG showed a remarkable accumulation in the choroids, retinal pigment epithelium, sensory retina of the amphibliestrodte and epithelium of the cornea. This result is in close accord with the studies using a dry-eye model mouse or Cu/Zn-SOD gene knockout mouse.37,38 Carbonylated proteins showed a tendency to increase in all organs with aging, with substantial increases in the oculus and kidney (Fig. 2). Even in 2-month-old mice, carbonylated proteins in the oculus and kidney increased twice as much as in the brain, heart and muscle (data not shown). This shows that oculus and kidney are hypersensitive organs to oxidative stress and tend to accumulate oxidative damage, perhaps because the rate of cell division is lower in these as compared with the other organs. The cells that are damaged and have lost function are then removed from organs by apoptosis to maintain homeostasis.31 The apoptotic induction increased significantly in the brain, oculus and kidney with aging (Fig. 3). These data suggest that oxidative damage associated with aging mediates some of its deleterious effects through the induction of apoptosis.

It has been estimated that the generation of O$_2^-$ and its dismutated product H$_2$O$_2$ may constitute as much as 1–2% of total electron flow,39 although others have placed this value at 0.1%.40 It is known that oxygen is initially converted to O$_2^-$ by electron leakage from complexes I and mainly complex III of mitochondria.9,10,41,42 There are reports of O$_2^-$ production as a function of aging in the housefly and C. elegans.23,43 We have previously demonstrated that O$_2^-$ production in C. elegans mitochondria remained unchanged with aging.23 In mammalian organs, there are few reports that measured O$_2^-$ production levels from mitochondria directly. Our data indicate that O$_2^-$ production levels from mitochondria increased in brain, oculus and kidney while production remained unchanged in heart and decreased in muscle (Fig. 4). Because they are highly vascularized, blood flows through the tissues of the brain, oculus and kidney at particularly high rates, which may explain the elevated production of O$_2^-$ in these organs.44–46 As already described, the oculus is particularly hypersensitive to oxidative stress. Indeed, hyperoxia causes premature retinopathy in premature fetuses of animals including humans.57 In addition, O$_2^-$ is produced by ultraviolet light (UV) from sunlight in the oculus. This UV exposure to the oculus increases age-related oxidative stress accumulation in these organs.58–60 As already described, the oculus is particularly hypersensitive to oxidative stress. Indeed, hyperoxia causes premature retinopathy in premature fetuses of animals including humans.57 In addition, O$_2^-$ is produced by ultraviolet light (UV) from sunlight in the oculus. This UV exposure to the oculus increases age-related oxidative stress accumulation in these organs.58–60

Mitochondrial SOD is an important antioxidant enzyme because most O$_2^-$ is produced from mitochondria as a byproduct of energy metabolism.9,10 There are contra-
unchanged.\textsuperscript{23}) To examine the change of energy metabolism in mouse organs with aging, cytochrome c oxidase (COX) activity is often used as a marker of mitochondrial function.\textsuperscript{14)} We showed here that COX activities significantly decreased in the oculus and kidney, which are the same organs in which O$_2^-$ significantly increased (Fig. 5). These organs may fall in a vicious cycle in which O$_2^-$ damages components of the electron transport system and the damage leads to further mitochondrial dysfunction. The dysfunction then causes even more O$_2^-$ overproduction. On the other hand, our data showed a contradictory result in the brain; namely, that O$_2^-$ production increased with aging but COX activity remained unchanged (Fig. 5). There are two previous reports of COX activity in the brain, but these results are contradictory and may be explained by region-specific difference in the brain.\textsuperscript{57,58)} This suggests that more experimentation is necessary in which different regions of the brain are examined. Our results are summarized in Table 2.

O$_2^-$ production decreased in muscles (biceps femoris and soleus muscles) of 24 month-old mice, which also experienced a 33\% reduction in wet weight of the muscles compared to 2 month-old mice ($p < 0.001$)(data not shown). This suggests that decreases in O$_2^-$ may be due to a reduction in energy metabolism that correlates or is caused by reductions in muscle mass. These results are in accord with some previous reports.\textsuperscript{59)} This supposition is consistent with our observation of decreased body temperature as a function of aging (Table 1).

There are many reports that examine the relationship between mitochondrial oxidative stress and aging in the whole body or individual organs. In this paper, we systematically examined the altered response of oxidative stress with aging in some of the main animal organs. Comparisons were made among organs so that the physiological aging of individual organism can be comprehensively clarified. Our data showed that physiological aging correlates with and presumably results from the accumulation of oxidative damage caused by increasing O$_2^-$ production from mitochondria, apoptotic induction, organ atrophy and dysfunction of organs by oxidative damage, and finally decrease of cellular response. Decreases in body temperature suggest decreases of energy metabolism with aging not only in muscle but also other organs. More studies are necessary in order to understand the relationship between energy metabolism, oxidative stress from mitochondria, and oxidative damage. Nonetheless, our present study provides information that should enable this goal.

We have not examined the relation between specific age-related diseases and oxidative stress in this study, but found that O$_2^-$ overproduction from mitochondria in mice leads to premature aging with several phenotypes such as decreasing muscular power and lost of eyesight (unpublished data). Among other things, this study could serve as an important baseline to comprehensively enumerate the various pathologies associated with radiation exposure in mice. Specifically, it would be interesting to similarly examine a group of mice after various exposures to ionizing radiation. This would be a particularly relevant comparison given the importance in free radical damages in both normal aging and after radiation exposure. This study may also help address the question of whether the various age-related diseases in atomic bomb survivors are due to endogenous attack by mitochondrially generated free radicals or direct effects of radiation exposure.

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