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Review

Mitochondria, Oxidative Stress and Aging*

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In the eighties, Miquel and Fleming suggested that mitochondria play a key role in cellular aging. Mitochondria, and specially mitochondrial DNA (mtDNA), are major targets of free radical attack. At present, it is well established that mitochondrial deficits accumulate upon aging due to oxidative damage. Thus, oxidative lesions to mtDNA accumulate with age in human and rodent tissues. Furthermore, levels of oxidative damage to mtDNA are several times higher than those of nuclear DNA. Mitochondrial size increases whereas mitochondrial membrane potential decreases with age in brain and liver.

Recently, we have shown that treatment with certain antioxidants, such as sulphur-containing antioxidants, vitamins C and E or the *Ginkgo biloba* extract EGb 761, protects against the age-associated oxidative damage to mtDNA and oxidation of mitochondrial glutathione. Moreover, the extract EGb 761 also prevents changes in mitochondrial morphology and function associated with aging of the brain and liver. Thus, mitochondrial aging may be prevented by antioxidants. Furthermore, late onset administration of certain antioxidants is also able to prevent the impairment in physiological performance, particularly motor co-ordination, that occurs upon aging.

Keywords: Mitochondrial DNA, glutathione, lipid peroxidation, *Ginkgo biloba*

1. THE FREE RADICAL THEORY OF AGING AND THE MITOCHONDRIAL THEORY OF AGING

One of the most relevant theories raised to explain aging is the free radical theory of aging, which was first proposed by Harman forty years ago.[1] According to this theory, oxygen-derived free radicals are responsible for the age-associated impairment at the cellular and tissue levels. The free radical theory of aging assumes that cellular aging is associated with oxidative stress, which was defined by Sies as a disturbance in the balance between pro-oxidants and antioxidants, in favor of the former.[2]

A great deal of experimental evidence supports the free radical theory of aging, especially the extension of life span obtained by increasing the antioxidant defense and the involvement of reactive oxygen species (ROS) in age-associated degenerative diseases.[3–6] Thus, administration of antioxidants can increase the mean lifespan

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of flies.[7,8] Orr and Sohal have recently found that simultaneous overexpression of copper–zinc superoxide dismutase and catalase genes in transgenic *Drosophila* extends their mean and maximum life span. Furthermore, these transgenic flies exhibited a delayed loss of physical performance and a lower amount of protein oxidative damage.[9]

Age-related declines of cognitive function and motor skills are associated with oxidative protein damage within different regions of the brain and oxidative stress in brain mitochondria.[10,11] Moreover, administration of the spin-trapping agent N-tert-butyl-α-phenylnitrone[12] or dietary restriction[13] decreased the oxidative damage to protein in the brain of rodents with a concurrent improvement in age-related behavioral deficits.

Oxygen free radicals and peroxides are generated continuously in the mitochondrial respiratory chain.[14,15] Indeed, about 1–2% of oxygen used by mammalian mitochondria in state 4 does not form water but oxygen-activated species.[14,15]

On this basis, Miquel and coworkers proposed the mitochondrial theory of cell aging.[16] This theory suggests that senescence is a by-product of oxy-radical attack to the mitochondrial genome of fixed postmitotic cells.[16] Mitochondria from postmitotic cells use O_2 at a high rate, hence releasing oxygen radicals which exceed the cellular antioxidant defence.[17] The role of old mitochondria in cell aging has been outlined by the degeneration induced in cells microinjected with mitochondria isolated from fibroblasts of old rats.[18]

2. MITOCHONDRIAL PRODUCTION OF REACTIVE OXYGEN SPECIES AS A DETERMINANT OF MAXIMUM LIFE SPAN

At the beginning of this century, Rubner pointed out the inverse relationship between the rate of oxygen consumption and the maximum life span of species. Much later, Harman suggested that mitochondria might be the biological clock in aging since the rate of oxygen consumption should determine the rate of accumulation of mitochondrial damage produced by free radical reactions.[19] Rubner's theory explains the differences in maximal life span potential among numerous but not all species. Exceptions to this theory are birds and primates, who exhibit at the same time high oxygen consumption and high longevity.[20] The explanation for this paradox is that mitochondrial production of ROS is not proportional to oxygen consumption.[14,20,21] ROS production by mitochondria is lower in pigeon than in rat, whereas oxygen consumption is higher in pigeon than in rat.[20–22] Thus, mitochondria from birds use oxygen more efficiently and exhibit less free radical leak through the respiratory chain. Studying up to five species, Sohal and coworkers found that mitochondria from shorter-lived species produce relatively higher amounts of ROS than those from the longer-lived species.[23–25] Hence, the rate of ROS production, and not merely the rate of oxygen consumption, appears to determine the maximal life span potential.

3. MITOCHONDRIAL AGING AS A MODEL OF CHRONIC OXIDATIVE STRESS

The continuous generation of ROS by mitochondria throughout cell life produces an age-related "chronic" oxidative stress which plays a key role in cellular aging (see Figure 1). A number of studies have shown that oxidative damage to mitochondrial DNA (mtDNA), proteins and lipids occurs upon aging.[26–30]

DNA damage has been observed in a wide range of mammalian cell types exposed to oxidative stress.[31] This damage includes single and double strand breaks, deletions, base changes, oxidative damage and even chromosomal aberrations. The major molecular mechanisms involved

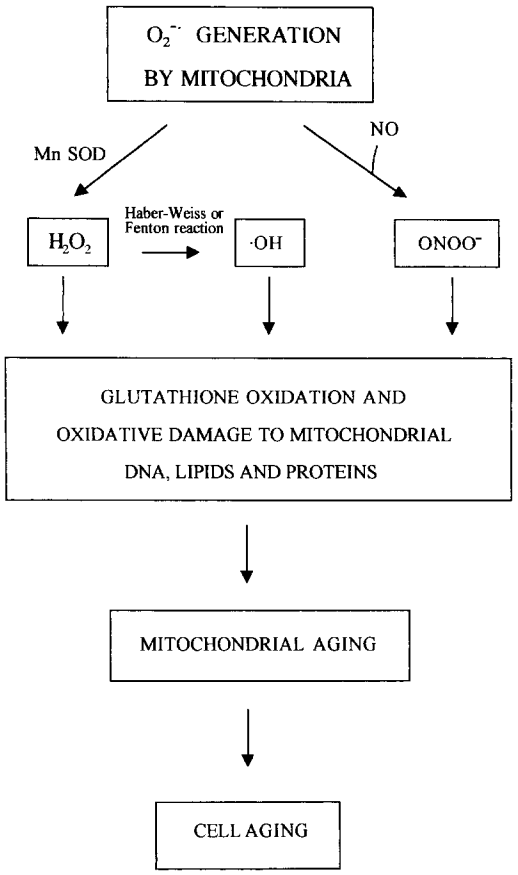

FIGURE 1 Mitochondrial aging as a model of chronic oxidative stress.

are direct reaction of hydroxyl radicals and carbonyl compounds with DNA and activation of nucleases.[31] Superoxide and H_2O_2 do not react with DNA unless transition metal ions are present to allow hydroxyl radical formation. The hydroxyl radical may attack deoxyribose, purines and pyrimidines, giving rise to numerous products.[31]

Mitochondrial DNA is specially susceptible to oxidative damage and mutation because it lacks protective histones[32] and is close to the ROS generated continuously by mitochondria. Indeed, levels of oxidative damage to mtDNA are several times higher than those of nuclear DNA, and mtDNA mutates several times more frequently than nuclear DNA.[26,33,34] Moreover, Suter and Richter have recently reported that oxidized bases are present to a moderate extent in the 16.3 kb mtDNA molecules but in high levels – around 50 times the value of nuclear DNA – in the mtDNA fragments.[34] These results together with the finding of mitochondrial oxidative damage endonucleases demonstrate the existence of a mtDNA repair system.[34–36]

Oxidative lesions of mtDNA accumulate with age in human and rodent tissues.[28,31,37,38] Thus, the mtDNA repair system cannot cope with the ROS generated throughout cell life in mitochondria. Point mutations and deletions in mtDNA occur in tissues from old animals.[39–42] Furthermore, point mutations and aberrant forms in mtDNA of postmitotic cells are associated with age-related degenerative diseases.[43,44] The impairment of mtDNA may affect transcription of mitochondrial genes.[45] Indeed, an age-related decrease in the levels of mitochondrial transcripts in some rat tissues and in *Drosophila* have been reported.[46,47] Furthermore, since mtDNA has no introns, any mutation affects a coding DNA sequence.[32] Thus, it was suggested that mtDNA mutations may be important contributors to aging and neurodegenerative diseases.[17,33,48]

Recently, we have found that oxidative damage to mtDNA is several times higher in brain than in liver, both in young and in old animals.[30] Hence, it appears that brain mitochondria are more exposed to oxidative stress than liver mitochondria. This increased oxidative stress may contribute to the well-known fact that neurons suffer more impairment upon aging than hepatocytes.

Mitochondrial reduced glutathione (GSH) plays a key role in the protection against the oxidative damage to mtDNA. Indeed, the oxidative damage to mtDNA which occurs upon aging is directly related to an oxidation of mitochondrial glutathione.[28] Glutathione oxidation increases with age in mitochondria from liver, kidney and brain of rats.[28] It is worth noting that this increase was much higher in mitochondria than

in whole cells. These results support the idea that mitochondria are a major source of free radicals in aging[16,21,24,49] and emphasize the relevance of mitochondria as primary targets of damage associated with aging.[16]

A change in the GSH redox status would indicate that mitochondrial antioxidant systems cannot cope with the oxidant species generated throughout the cell life. Therefore, GSH oxidation may occur prior to oxidative damage to other mitochondrial components, and it might be an early event in the chronic oxidative stress associated with mitochondrial aging. This points out the importance of maintaining an adequate GSH status to protect cells against oxidative damage of important molecules such as DNA.

The role of protein damage in cell aging became apparent when it was found that catalytically less active or inactive forms of some enzymes accumulate during aging.[50–52] Post-translational modifications seem to be responsible for this accumulation of inactive proteins.[53] Most of these modifications may be due to oxygen radical-mediated oxidation of enzymes, which is a marking step in protein turnover.[54–56] Oxidative damage appears to occur selectively in certain mitochondrial proteins. Thus, it has been reported recently that mitochondrial aconitase, an enzyme of the citric acid cycle, is a specific target of oxidative damage during aging of houseflies.[57]

Regarding peroxidation of mitochondrial lipids upon aging, part of it appears to be due to changes in membrane lipid composition which enhance its susceptibility to oxidative damage.[26] Thus, a progressive decline in the amount of linoleic acid together with an increase in the amount of long-chain polyunsaturated fatty acids, which are more sensitive to oxidation, was reported.[26,58] Furthermore, the rate of the aging process may depend on the sensitivity of mitochondrial lipids to oxidative damage. Thus, the fatty acids analysis of liver mitochondria from eight mammalian species has revealed that the total number of double bonds and the peroxidizability index of mitochondrial membrane lipids are inversely correlated with maximum life span.[59]

An important change in mitochondrial lipid composition is the age-related decrease found in cardiolipin content. Indeed, it decreases with age in heart, liver and nonsynaptic brain mitochondria.[60–62] Since cardiolipin is required for optimal catalytic activity of inner mitochondrial enzymes,[63] modifications in cardiolipin composition may be involved in age-related changes of certain activities, such as those of the respiratory chain.[26,62]

As pointed out recently by Wei,[64] a vicious cycle would operate in mitochondria upon aging. The concurrent enhancement of lipid peroxidation and oxidative modification of proteins in mitochondria further increases mutations and oxidative damage to mtDNA in the aging process.[64] The respiratory enzymes containing the defective mtDNA-encoded protein subunits may increase the ROS production, which in turn would aggravate the oxidative damage to mitochondria.[64]

On the other hand, superoxide radical produced during mitochondrial respiration reacts inside mitochondria with nitric oxide to yield damaging peroxynitrite.[65,66] Furthermore, mitochondria are a source of NO, which may increase superoxide radical and hydrogen peroxide formation by mitochondria.[67] Future research is needed to elucidate the role of mitochondrial nitric oxide and peroxynitrite in the age-associated oxidative stress.

4. OXIDATIVE STRESS CAUSES CHANGES IN MITOCHONDRIAL FUNCTION AND MORPHOLOGY UPON AGING

Oxidative stress may be responsible for age-associated deficits in mitochondrial function as well as changes in mitochondrial morphology,[26,30] and experimental evidence supports this hypothesis, as explained below.

Age-related decreases in membrane potential of brain and liver mitochondria have been reported.[29,30,68] This may reduce the energy supply in old cells since the mitochondrial membrane potential is the driving force for ATP synthesis.

An acute oxidative stress causes an inhibition of mitochondrial respiration,[69] which affects the mitochondrial membrane potential. Moreover, hyperoxia reduces the mitochondrial membrane potential in microvascular cells.[70] Hence, the oxidative stress associated with aging may be responsible, at least in part, for the age-related impairment in mitochondrial membrane potential and respiratory activity. Indeed, intracellular peroxide levels increase with age in whole cells,[29,68] which correlates with parallel changes in peroxide generation by isolated mitochondria.[29,30,71] It is likely that the accumulation of peroxides in whole cells upon aging comes from the continuous peroxide generation by mitochondria throughout the cell life, although we cannot rule out that other structures, such as peroxisomes, may also have a role.

On the other hand, mitochondrial morphology is important because changes in mitochondrial ultrastructure modulate mitochondrial function.[72] Indeed, volume-dependent regulation of matrix protein packing modulates metabolite diffusion and, in turn, mitochondrial metabolism.[72] Enlargement, matrix vacuolization and altered cristae have been evidenced in mitochondria from old animals by electron microscopy and flow cytometry.[29,30,73,74] Alterations of mitochondrial crests which occur in old mitochondria may be responsible for the age-related impairment in mitochondrial membrane potential that we have found.

It is well known that acute oxidative stress causes mitochondrial swelling.[75] Thus, age-associated chronic oxidative stress may be the cause, at least in part, of mitochondrial swelling. Furthermore, a correlation between changes in mitochondrial morphology and function seems to occur upon aging.

Several studies have shown a decline in activities of complexes I, II and especially IV.[27] Moreover, the respiratory activity of isolated mitochondria decreases with age in liver, skeletal muscle and brain.[76–78] Age-related decreases in the activities of mitochondrial anion carrier proteins – such as the phosphate carrier and ATP/ADP translocation in liver mitochondria[79,80] and Ca^{++}, adenine nucleotide and pyruvate carriers in heart mitochondria[60,81–84] – have also been reported.

We studied biochemical pathways which depend on mitochondrial function in isolated hepatocytes and found that gluconeogenesis from lactate plus pyruvate, but not from glycerol or fructose, decreases upon aging.[29] Gluconeogenesis from lactate involves mitochondria, whereas from glycerol or fructose it does not. The lower rate of gluconeogenesis from lactate plus pyruvate is due to an impaired transport of malate across mitochondrial membrane using the dicarboxylate carrier.[29] Furthermore, post-transcriptional modifications appear to be involved in the age-related impairment of such carrier, since its gene expression does not change with age.[29]

Nevertheless, the fact that the respiratory activity and some mitochondrial carriers are impaired upon aging does not necessarily mean that all mitochondrial functions are affected by aging. Thus, the rate of urea synthesis in hepatocytes does not change with age.[29]

An increased generation of oxygen free radicals may be responsible for the decline in the activity of mitochondrial membrane proteins, such as metabolite carriers and respiratory chain complexes. In fact, it is known that exposure of mitochondria to free radicals causes impairment of the mitochondrial inner-membrane proteins[75] and an inhibition of mitochondrial respiration.[18]

Damage to mitochondrial electron transport may be an important factor in the pathogenesis of neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis.[85] Activation of excitatory amino acid receptors causes enhanced NO and

superoxide production, which can lead to the generation of peroxynitrite.[86–88] It is suggested that NO and specially peroxynitrite mediate mitochondrial damage in neurodegenerative disorders, since they can inhibit components of the neuronal mitochondrial respiratory chain.[85,89]

ROS and NO are important physiological modulators of mitochondrial functions, but may damage mitochondria when present in excessive amounts.[90,91] Nitric oxide inhibits respiration reversibly at cytochrome c oxidase,[92,93] but peroxynitrite inhibits irreversibly at complexes I–III[94] and also at cytochrome oxidase.[95] Peroxynitrite would be the reactive intermediate accounting for nitric oxide-dependent inactivation of electron transport components and ATPase in living cells and tissues.[96] Further research is needed to clarify the role of peroxynitrite on mitochondrial aging, specially on the age-related decrease in cytochrome oxidase activity.

Mitochondria are mediators of apoptosis. Mitochondria are at the same time the target and the source of oxidative stress, nitric oxide and Ca^{2+}.[97] Mitochondrial dysfunction induced by superoxide, NO and the consequent peroxynitrite production play a key role in neuronal apoptosis or neurotoxicity induced by several insults.[66,86,92,98,99] Indeed, cytochrome c release by mitochondria is involved in NO-induced neuronal apoptosis[100] and prevention of mitochondrial permeability transition by cyclosporine A protected cells against apoptosis induced by amyloid β-peptide or nitric oxide-generating agents.[66] Exposure to the parkinsonian neurotoxin 1-methyl-4-phenylpyridium (MPP+) and nitric oxide simultaneously causes cyclosporin A-sensitive mitochondrial calcium efflux and depolarization.[101] Thus, nitric oxide may induce apoptosis via triggering mitochondrial permeability transition in several cell types, such as neurons and myeloid cells.[102,103] Neuronal apoptosis induced by amyloid β-peptide or NO was prevented by antioxidants, such as GSH or overexpression of mitochondria-localized manganese superoxide dismutase.[66]

5. ANTIOXIDANTS PREVENT AGE-ASSOCIATED MITOCHONDRIAL OXIDATIVE STRESS

The free radical theory of aging proposed by Harman[1] is specially attractive because it provides a rationale for intervention, i.e. antioxidant administration may slow the aging process. In 1979, Miquel and Economos were the first to show that administration of thiazolidine carboxylate increases the vitality and life span of mice.[7] Later, Furukawa *et al.*[104] reported that oral administration of GSH protects against the age-associated decline in immune responsiveness. More recently, we found that administration of some sulphur-containing antioxidants protects against the age-associated GSH depletion in mouse tissues as well as partially prevented the age-related decline in neuromuscular co-ordination.[8] These antioxidants also increased the mean life span of *Drosophila*.[8]

Recently, we have investigated the protective effect of a standardized extract from dried leaves of *Ginkgo biloba* (EGb 761) on the age-associated oxidative damage to mtDNA.[30] EGb 761 is a mixture of flavonoids, heterosides and terpenes.[105] The antioxidant action of this *Ginkgo biloba* extract is due to its components, the flavonol glycosides, which are known for scavenging superoxide anions as well as hydroxyl and peroxyl radicals.[106,107] Flavonoids also prevent lipid peroxidation in the membranes, especially due to their ability to interact with and penetrate the lipid bilayers.[108]

Oral administration of EGb 761 to rats for three months is able to prevent the oxidative damage to mtDNA that occurs in liver and brain upon aging.[30] Treatment with EGb 761 also protects against the oxidation of mitochondrial GSH and the age-related increase in peroxide generation by mitochondria.[30] Hence, EGb 761 prevents the chronic oxidative stress associated with mitochondrial aging in rats.

In addition, treatment with EGb 761 prevented age-associated impairments in mitochondrial

morphology and respiratory function. Indeed, this treatment prevented the changes in size and structural complexity that occurs in brain and liver mitochondria upon aging.[30]

Moreover, it prevents the decrease in energy status under state 4 that occurs in liver and brain mitochondria from old rats.[30] Our results suggest that EGb 761 exhibits beneficial effects on mitochondrial aging by preventing the chronic oxidative stress associated with this process.

We have also found that certain antioxidants, such as thiazolidine carboxylate derivatives or vitamins C and E, protect against mitochondrial GSH oxidation and mtDNA oxidative damage associated with aging.[28] Moreover, late onset administration of certain sulphur-containing antioxidants, such as GSH or a thiazolidine carboxylate derivative, is able to prevent not only the age-related oxidative damage to mtDNA in brain, but also the impairment in physiological performance, particularly motor co-ordination, that occurs upon aging.[11] Thus, we found an inverse relationship between motor co-ordination and oxidative damage to brain mtDNA in mice. To pursue studies in humans, the practical importance of an effective antioxidant treatment which started late in life should be emphasized.

The facts reported here underline the role of oxidative stress, and particularly oxidative damage to mtDNA, in aging at tissue and whole organism levels. Hence, experimental evidences again give support to Miquel's hypothesis of the key role of mitochondrial oxidative damage in the aging process[17] as well as to Sohal's hypothesis of the rate of pro-oxidant generation as a key factor in the rate of aging.[24]

In conclusion, administration of certain antioxidants – such as GSH, thiazolidine carboxylate derivatives, vitamins C and E or the *Ginkgo biloba* extract EGb 761 – may prevent or delay the oxidative stress and the physiological impairment associated with aging. Nevertheless, further studies on dietary supplementation with antioxidants need to be carried out, especially epidemiological studies.

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