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Review

Oxidative damage and mitochondrial decay in aging

(bioenergetics/mitochondrial DNA/cardiolipin/acetyl-L-carnitine/neurodegeneration)

*Mark K. Shigenaga, Tory M. Hagen, and Bruce N. Ames**

Division of Biochemistry and Molecular Biology, 401 Barker Hall, University of California, Berkeley, CA 94720

Contributed by Bruce N. Ames, July 27, 1994

ABSTRACT **We argue for the critical role of oxidative damage in causing the mitochondrial dysfunction of aging. Oxidants generated by mitochondria appear to be the major source of the oxidative lesions that accumulate with age. Several mitochondrial functions decline with age. The contributing factors include the intrinsic rate of proton leakage across the inner mitochondrial membrane (a correlate of oxidant formation), decreased membrane fluidity, and decreased levels and function of cardiolipin, which supports the function of many of the proteins of the inner mitochondrial membrane. Acetyl-L-carnitine, a high-energy mitochondrial substrate, appears to reverse many age-associated deficits in cellular function, in part by increasing cellular ATP production. Such evidence supports the suggestion that age-associated accumulation of mitochondrial deficits due to oxidative damage is likely to be a major contributor to cellular, tissue, and organismal aging.**

Aging, an inevitable biological process, is characterized by a general decline in physiological function that leads to morbidity and mortality. Specific causes of this decline are not known, although various lines of evidence implicate stochastic events as being a fundamental driving force behind this process (1). We review the evidence that sustained damage inflicted by endogenously produced oxidants is the likely cause of the age-related deficits in mitochondrial function. This decline is associated with a generalized physiological decline that is common to all aging organisms. In a companion review (2) we discussed the evidence that oxidation is a major contributor to cellular aging and the degenerative diseases that accompany aging such as cancer, cardiovascular disease, immune-system decline, brain dysfunction, and cataracts. Also reviewed was the evidence that dietary antioxidants, such as ascorbate, tocopherol, and carotenoids, the main source of which are fruits and vegetables, protect against these degenerative diseases.

Oxidants are produced continuously at a high rate as a by-product of aerobic metabolism. These oxidants include superoxide ($O_2^{\cdot-}$), H_2O_2, and hydroxyl radicals ($HO^{\cdot}$) (the same oxidants produced by radiation) and possibly singlet oxygen ($^1O_2$). They damage cellular macromolecules, including DNA (3), protein (4), and lipid (5). Accumulation of such damage may contribute to aging and age-associated degenerative diseases.

The continuous threat of oxidant damage to the cell, tissue, and organism as a whole is underscored by the existence of an impressive array of cellular defenses that have evolved to battle these reactive oxidants (6). However, these defenses are not perfect and, consequently, cellular macromolecules become oxidatively damaged. The accumulation of these damaged macromolecules is proposed to contribute significantly to aging (2).

Mitochondria constitute the greatest source of oxidants on the basis of the following evidence. (*i*) The mitochondrial electron transport system consumes approximately 85% of the oxygen utilized by the cell. (*ii*) In contrast with other oxidant-producing systems of the cell (cytochrome P450, various cytosolic oxidases, β-oxidation of fatty acids in peroxisomes, etc.), mitochondria are required for the production of ATP and are present in relatively high numbers in essentially all cells of the body. Cellular energy deficits caused by declines in mitochondrial function can impair normal cellular activities and compromise the cell's ability to adapt to various physiological stresses. We argue that this oxidative damage, and in particular oxidative damage to mitochondria, is a major factor in aging.

Age-Related Oxidative Damage to Mitochondrial Macromolecules

Oxidative Damage to Mitochondrial DNA. Levels of oxidative damage to mtDNA isolated from rat liver or various human brain regions are at least 10-fold higher than those of nuclear DNA (7–9). This increase correlates with the 17-fold higher evolutionary mutation rate in mtDNA compared with nuclear DNA (10). These higher levels of oxidative damage and mutation in mtDNA have been ascribed to location of the DNA near the inner mitochondrial membrane sites where oxidants are formed, lack of protective histones, and lack of DNA repair activity. Oxidative lesions in mtDNA accumulate as a function of age in human diaphragm muscle (11), human brain (8), and rat liver (2). The amount of 8-oxo-2′-deoxyguanosine (oxo^8dG), a biomarker of oxidative DNA damage, in mtDNA in human diaphragm muscle is reported in an 85-year-old individual to reach levels of approximately 0.5% of the dG residues in mtDNA. Comparisons of this mtDNA with mtDNA isolated from younger individuals indicate an approximate 25-fold increase with age. A high level of oxo^8dG (0.87% of dG residues) is also observed in mtDNA isolated from regions of the human brain from one individual 90 years of age (8). The level of oxo^8dG in mtDNA of rat liver shows a 2- to 3-fold increase in 24-month-old rats (less than their maximal lifespan of 30 months) (2). This less impressive elevation presumably reflects a decreased accumulation of damage in mitotic versus postmitotic cells. The age-associated accumulation of oxidative damage to mtDNA correlates with the level of mtDNA deletions seen in a number of tissues composed of postmitotic cells (see below; ref. 11). It is argued that this damage leads to mutations that results in dysfunctional mitochondria. Oxidative damage to brain mtDNA may contribute to the age-dependent increase in the incidence of neurodegenerative diseases (8).

Oxidative Damage to Mitochondrial Protein. The accumulation of oxidatively damaged proteins, the extent of which varies within and among tissues, in-

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Abbreviations: ALCAR, acetyl-L-carnitine; NMDA, *N*-methyl-D-aspartate.

*To whom reprint requests should be addressed.

creases markedly with age (4). As in the case of oxidative damage to DNA, an age-associated increase in oxidative damage to mitochondrial protein is observed (12). The accumulation of oxidized dysfunctional protein with reactive carbonyl groups could lead to inter- and intramolecular cross-links with protein amino groups and cause loss of biochemical and physiological function in mitochondria. Thus the age-related accumulation of protein oxidation products in mitochondria may also lead to loss of energy production and increased production of oxidants.

Oxidative Damage to Mitochondrial Lipids. The fluidity of cellular membranes decreases with age (13), a change that may be attributed in part to oxidation of plasma and mitochondrial membrane lipid components. Part of this increased sensitivity to oxidants appears to be due to changes in membrane lipid composition. For example, in the liver microsomal and mitochondrial membrane fractions isolated from rodents, there appears to be a progressive decline in the amount of linoleic acid (18:2). This change is roughly paralleled by an increase in the amount of long-chain polyunsaturated fatty acids (22:4 and 22:5), a subclass of lipids that exhibit a higher degree of unsaturation and are more sensitive to oxidation reactions than linoleic acid (14). Most of these substitutions (18:2 to 22:4 and 22:5) appear to occur in the fatty acid composition of cardiolipin. Because cardiolipin plays a pivotal role in facilitating the activities of key mitochondrial inner membrane enzymes (see below), it would be expected that changes that increase its susceptibility to oxidative damage would be deleterious to normal mitochondrial function.

The age-dependent accumulation of lipids that are more prone to peroxidation may also, following peroxidation, increase the rigidity (or decrease the fluidity) of cell membranes. Mitochondria appear to account for essentially all the net loss of water that occurs with age in certain tissues (liver and heart) (15), which is consistent with the age-associated increase in membrane rigidity observed in this organelle. Similarly, decreases in lateral diffusion of plasma membrane proteins (e.g., receptors) appear to be associated with a general decline in signal transduction that is commonly observed in aging organisms.

Phospholipase A_2 appears to be important for repair of oxidatively damaged lipids (16). Phospholipase A_2 activity in the inner mitochondrial membrane increases in response to conditions associated with increased oxidant production, such as bacterial endotoxin treatments (17). Increases in inner mitochondrial phospholipase A_2 activity are also observed in mitochondria isolated from rats fed fish oil (18) or given insufficient vitamin E (19), dietary treatments associated with increased lipid peroxidation. Efficient membrane antioxidants such as ubiquinol and its synthetic derivatives inhibit release of fatty acids catalyzed by phospholipase A_2 (20), presumably by inhibiting oxidation of lipids. Physiological conditions such as hypothyroidism or hibernation, which lead to reduced mitochondrial oxygen consumption, are associated with a marked decline in phospholipase A_2 activity (21). These observations support the suggestion that phospholipase A_2 is a repair enzyme that catalyzes the removal of oxidized lipids in membranes. Without such a repair activity peroxidized lipids could accumulate, the consequence of which might include increased membrane permeability and loss of mitochondrial respiratory control.

Age-Related Changes in Mitochondria

Bioenergetics. The components of the electron transport chain, which catalyze the phosphorylation of ADP to ATP, work as an integrated system composed of a total of five protein complexes. mtDNA encodes 13 of the proteins and nuclear DNA encodes approximately 60. Complexes I–IV are involved in the oxidation of NADH, electron transport, and the generation of an electrochemical gradient. This electrochemical gradient, which is created by pumping protons across the inner mitochondrial membrane, is utilized by ATP synthase (complex V) as a source of energy. Relevant to mitochondrial function is the efficiency of electron movement through the electron transport chain and its coupling to oxidative phosphorylation to produce ATP. The coupling efficiency can be measured experimentally by determining the ratio of ATP production to molecular oxygen consumed (ADP/O), and whether the mitochondria are in state 3 or state 4. State 3 represents a condition where the rate of oxidative phosphorylation is not limited by ADP concentration. State 4, a condition where the level of ADP limits oxidative phosphorylation, is associated with a reduced respiratory chain, leading to increased formation of O_2^- byproduct.

Temporary or sustained loss of mitochondrial function and ATP production can have a major impact on the fidelity of cellular defenses and repair processes. This may result in increased mutational load, increased accumulation of dysfunctional cellular macromolecules, and a decreased capacity to mount an appropriate stress response when challenged. Probable age-associated loss of function in mitochondria is suggested (Table 1) by the evidence of increased mtDNA deletions (26, 29) and point mutations (31, 32), increased oxidative damage to mtDNA (2, 8, 11), increased levels of aberrant forms of mtDNA (30, 33, 34), formation of mtDNA-protein crosslinks (35), increased production of mitochondrially derived oxidants (22–25, 50, 51), decreased state 3/state 4 ratio (47, 48), decline in activities of complexes I, II, and IV (36–39), and age-related decreases of mitochondrial cytochrome oxidase in postmitotic tissues (49). Marked changes in mitochondria with age have been observed histologically, including enlargement, matrix vacuolization, shortened cristae, and loss of dense granules (46). As only about half of these enlarged mitochondria can be recovered from old animals (46) it is quite possible that differences in the function of mitochondria isolated from old versus young animals are underestimated by this selective loss and may be one reason for the apparent lack of age-associated biochemical changes in this organelle (reviewed in ref. 47). Along with the histological changes cited above, the potential for lipid peroxidation (the "peroxidizability index") in the inner mitochondrial membrane increases (44), making the mitochondria more susceptible to damage by oxidants. Furthermore, the decreased content of 18:2-containing lipids, which are optimal for cardiolipin interactions with proteins of the inner mitochondrial membrane (52), may account for the decreased state 3/state 4 ratio, and increased O_2^- and H_2O_2 formation that has been observed in some tissues with age. These changes, in turn, can contribute to increased loss of efficiency in mitochondrial function.

Species-Specific Differences in Longevity Correlate Inversely with Metabolic Rate. The metabolic rate is a function of the total amount of oxygen consumed by the organism per unit time. This rate is dependent on the amount of metabolically active organs plus their respective tissue specific rates of oxygen consumption, which differ depending on mitochondrial content and workload. Thus, changes in the metabolic rate of a specific tissue per unit mass appear to correlate positively with the content of mitochondria (53). Metabolic rate correlates inversely with maximum life-span potential and correlates directly with the cytochrome oxidase content per cell; larger, longer-lived animals contain less cytochrome oxidase per cell (54). The total body content of cytochrome *c* and cytochrome oxidase is inversely correlated with body size (55, 56). Rates of protein synthesis increase as a function of metabolic rate, and the increase may be due to increased protein turnover rates that are stimulated in part by endogenous oxidative damage. This suggestion is supported by the consistent inverse correlation of protein half-lives and body size with the rate of oxygen consumption (57).

Phylogenetic Differences in Mitochondrial Proton Leakage: Relationship to Spe-

Table 1. Age-related changes in mitochondria

Parameter measured	Effect	Organ	Animal	Ref.
Oxidant production and damage				
O_2^- and H_2O_2 production	Increase	Heart	Rat	22
O_2^- and H_2O_2 production	Increase	Liver	Various	23, 24
O_2^- and H_2O_2 production	Increase	Kidney, heart	Various	25
Oxidative damage to mtDNA	Increase	Brain, diaphragm muscle	Human	8, 11
Oxidative damage to mtDNA	Increase	Liver	Rat	2
Mitochondrial DNA aging				
mtDNA deletions	Increase	Various brain regions	Human	26–28
mtDNA deletions	Increase	Diaphragm muscle, various organs	Human	11, 29
mtDNA additions/deletions	Increase	Liver	Mouse, rat	30
mtDNA point mutations	Increase	Extraocular muscle	Human	31, 32
Circular dimer mtDNA	Increase	Brain	Mouse	33
Circular dimer mtDNA	Increase	Kidney, heart	Mouse, rat	34
mtDNA-protein crosslinks	Increase	Liver	Rat	35
Membrane and electrolyte changes				
Complex I	Decrease	Brain	Monkey	36
Complexes I and IV	Decrease	Brain	Monkey	37
Complexes I and IV	Decrease	Respiratory muscle	Rat	38
Complexes I, II, and IV	Decrease	Skeletal muscle	Human	39
Cardiolipin levels	Decrease	Heart, nonsynaptic neurons	Rat	40, 41
Cardiolipin levels	No change	Liver mitochondria, microsomes	Rat	14
Carnitine–acylcarnitine exchanger	Decrease	Heart	Rat	42
Phosphate translocator	Decrease	Heart	Rat	43
Pyruvate translocator	Decrease	Heart	Rat	40
Membrane cholesterol/phospholipid ratio	Increase	Heart	Rat	40
Membrane cholesterol/phospholipid ratio	Increase	Lymphocyte	Human	13
Peroxidizability index	Increase	Liver mitochondria microsomes	Rat	14
Membrane fluidity	Decrease	Liver	Rat	44
Water content	Decrease	Heart	Rat	15
Membrane potential	Decrease	Lymphocyte	Mouse	45
Recovery of damaged mitochondria	Decrease	Liver	Mouse	46
Mitochondrial bioenergetics				
State 3 respiration	General decrease	Various	Various	Reviewed in 47
State 4 respiration	General decrease	Various	Various	Reviewed in 48
State 4 respiration	No change	Various	Various	Reviewed in 47
ADP/O	No change	Various	Various	Reviewed in 47
Cytochrome oxidase immunoreactivity	Decrease	Limb/diaphragm muscle	Human	49

Peroxidizability index, the potential of membrane lipids to undergo peroxidation; ADP/O, ratio of ATP production to molecular oxygen consumed, an index of oxidative phosphorylation.

cies-Specific Longevity. Porter and Brand (58) reviewed phylogenetic differences in the extent of proton leakage across the inner mitochondrial membrane. This proton leakage, which is inversely correlated with species-specific body weight and life-span, could be an important factor governing the rate at which mitochondrially derived oxidants are produced. Thus, animals with a high metabolic rate and short life-span, such as rodents, exhibit a significantly higher rate of proton leakage as compared with larger mammals. Though there is no direct evidence linking proton leakage to oxidant production, conditions associated with increased proton leakage are associated with an increased production of mitochondrially derived oxidants. Thyroid hormones, for instance, increase both proton leakage and state 4 respiration; this change accompanies increases in mitochondrial respiration, oxygen consumption, production of oxidants, and formation of lipofuscin, a marker of oxidative damage. Conversely, hypothyroidism is associated with decreased proton leakage and less state 4 respiration (59). The rate of proton leakage may explain the difference in basal metabolic rate and life-span between the very long-lived reptile *Amphibolurus vitticeps* and the short-lived rat, two species with the same body size and body temperature. The proton leakage and metabolic rate of this reptile are both about 20% those of the rat (60). Phylogenetic differences in species-specific metabolic rate have been shown to positively correlate with state 4 respiration and O_2^- formation (25). Thus, it is quite plausible that the species-specific rate of proton leakage, which is associated with increased state 4 respiration, could be a major factor in the species-specific rate of oxidant production.

Mitochondrial DNA Mutations and Aging. mtDNA defects can lead to mitochondrial dysfunction; some of these defects are genetically inherited and have been shown in some instances to be associated with an extensive amount of mtDNA deletions (30–80% of all mtDNA) or point mutations resulting in energy deficits and compromised tissue function (61). mtDNA deletions, many of which are produced because of illegitimate recombinational events at direct repeat sequences, are particularly prevalent in postmitotic tissues (62). Associated with these deletions are myopathies and increased susceptibility to neurodegenerative disorders.

The type of deletions and point mutations in mtDNA that cause inherited myopathies are also observed to increase with age (63). The age-associated increase in the level of any of the common deletions (e.g., mtDNA 4977, mtDNA 7436,

and mtDNA 10422) produced spontaneously is low (<0.1% versus 30–80% for inherited cases). While the effect of this low level of deletions may not be significant, it is postulated that these deletions represent only a small portion, "the tip of the iceberg" (62), of the multitude of deletions and point mutations that might exist and accumulate with age. It is plausible that the accumulation of all mtDNA defects could account for the age-related deficits in mitochondrial bioenergetic capacity and function.

The role of oxidants in the formation of mtDNA deletions is supported by the observation that doxorubicin, a compound that stimulates mitochondrial oxidant production, creates a marked elevation in mtDNA deletions in cardiac tissue; this effect is blocked by ubiquinone (coenzyme Q_{10}) (64), a key component of the mitochondrial electron transport system whose reduced form, ubiquinol, exhibits antioxidant properties (65). The age-associated accumulation of the common deletion mtDNA 4977 also appears to correlate with oxygen consumption (27, 62) as well as functional workload (66). This and other mtDNA deletions have been postulated to be responsible for the degeneration of neurological function, cardiovascular function, and muscle movement that are common in older individuals (63).

Studies that have examined the content of cytochrome oxidase in mitochondria show a progressive and random loss in this enzyme (49, 67) which correlates well with the age-associated decline in mtRNA synthesis (68). A study of human diaphragm muscle indicates that cytochrome *c* oxidase decreases markedly beyond the seventh decade of life (69). Examination of various muscle tissues (extraocular muscles, human diaphragm, skeletal muscles), brain, liver, heart, and lung (11, 26, 28, 29, 62, 69) reveals age-associated increases in mtDNA deletions. These deletions are proposed to create tissue bioenergy mosaics (29, 70) that may account for losses in bioenergetic capacity. This has been shown to occur with age in skeletal muscle (71) and in liver (72).

Mitochondrial Compensatory Mechanisms. The loss of functional mitochondria with age appears to be compensated in part by the increased workload of the remaining intact population of mitochondria (73). The increase in senescent tissue of mtDNA copy number (74) supports the idea of an adaptive mechanism designed to restore mitochondrial function. These changes may account for the apparent lack of effect of aging on the level of adenine nucleotide levels observed in cells of aged organisms.

Thus, the increases in either mtDNA copy number (74) or the expression of nucleus encoded proteins for oxidative phosphorylation (66) may be feedback mechanisms that compensate for mitochondria harboring defective proteins or mtDNA. The result of such a mechanism is to allow a cell to adapt to a localized loss of mitochondrial function.† Compensatory mechanisms in the fully functional cells mask the inefficiencies of their dysfunctional neighbors but in doing so increase their workload, their energy expenditures, and the probability of incurring damage and loss of function.

Imbalances in the Electron Transport Chain Produce Increased Superoxide and Hydrogen Peroxide. Damage to inner membrane proteins constituting the electron transport chain can alter the efficiency of electron transport. Imbalances in the stoichiometry of functional electron transport proteins is proposed to lead to a leakage in the flow of electrons to the terminal electron acceptor, cytochrome oxidase (75). The decreased age-related expression of cytochrome oxidase in tissues such as the heart, liver, and brain are of particular relevance. Furthermore, alteration in protein conformation due to direct oxidative damage or through DNA mutation may cause inefficient transfer of electrons through the electron transport chain. This would increase the likelihood of superoxide formation. Treatment of submitochondrial particles with glutaraldehyde increases O_2^- and H_2O_2 production, presumably by inducing crosslinks between proteins and lipids of the inner mitochondrial membrane (76). Crosslinks of inner mitochondrial membrane proteins by oxidants, or reactive aldehydes generated from lipid peroxidation, may also result in increased O_2^- and H_2O_2 production, thus further increasing the damage that can lead to mitochondrial dysfunction.

Mutation to Nuclear DNA-Encoded Mitochondrial Protein. Mutations in the many nuclear DNA-encoded proteins of the mitochondria could also lead to mitochondrial dysfunction. Such mutations, which are likely to be produced in part by endogenous oxidative damage, may result, for example, in lowered efficiencies of electron transport components, lowered efficiencies of substrate and phosphate transporters, and lowered rates of ATP synthesis. Nuclear genes encoding mitochondrial proteins are transcribed continuously and are therefore expected to be at an increased risk of mutation compared with other regions of the genome that are transcribed at a lower rate, if at all. Although nuclear DNA is considerably less susceptible to mutation than mtDNA, once formed, the products of such mutation would affect the bioenergetics of all of the mitochondria in the cell. Mutation in the nuclear gene encoding a protein product associated with cytochrome *c* oxidase activity is postulated to be responsible for the encephalomyelopathy of Leigh syndrome (77). Mutations to nuclear DNA that encode proteins of the mitochondrial electron transport chain will include lethal mutations that are cytotoxic and dominant mutations that cause mitochondrial dysfunction but allow the cell to remain viable.

Functional Consequences of the Age-Related Changes in Mitochondrial Cardiolipin. Cardiolipin, a diphosphatidyl glycerol derivative found principally in mitochondria, plays an important role in mitochondrial membrane structure and function. The decrease of cardiolipin with age is associated with a decrease in state 3/state 4 ratio. Cardiolipin interacts with various proteins of the inner mitochondrial membrane and plays a pivotal role in maintaining their activities (48). In addition, cardiolipin appears to play an important role in controlling the permeability of the inner mitochondrial membrane to small molecules as well as in establishing mitochondrial proton gradients.

Mitochondrial cardiolipin content has been reported to decrease with age in a number of tissues, including heart, liver, and nonsynaptic brain mitochondria (40, 41, 43). This loss, which may be due to a decline in mitochondrial cytidine triphosphate (CTP):phosphatidate cytidylyltransferase activity (78), could play a critically important role in the age-related decrements in mitochondrial function. The change in mitochondrial cardiolipin is paralleled in mitochondria by a decrease of the inner membrane surface area (79), smaller, sparser cristae (80), and increased fragility (81).

The functional changes in mitochondrial enzyme activities that accompany the modifications in cardiolipin composition include a decrease in the activity of cytochrome oxidase that, as mentioned above, appears to be involved in the age-related increases in the production of mitochondrially derived oxidants. Other proteins of the inner mitochondrial membrane that also require interaction with cardiolipin for optimal catalytic activity include the ADP/ATP translocator, phosphate translocator, mitochondrial ATP synthase, and mitochondrial substrate transporters, as well as the palmitoyl carnitine transferase and carnitine translocase systems (52). Under certain experimental conditions that strip cardiolipin off protein, denaturation and complete loss of activity of many of these proteins are observed. Cardiolipin ap-

†Analogous compensatory effects are proposed to account for the age-dependent loss of dopaminergic neurons. Functional deficits in dopaminergic neurons are not observed until approximately 80% of these cells are lost. This implies that with the attrition of these nerve cells, the remaining viable neurons increase their workload to the point of adequately compensating for this loss.

pears to be essential for the activity of the proteins it interacts with, because substitution with other mitochondrial phospholipids (e.g., phosphatidylcholine and phosphatidylethanolamine) has little or no effect in reconstituting activity. The age-related decrease in heart mitochondrial cardiolipin is correlated with an increased cholesterol-to-phospholipid ratio (82), a change that is associated with increased membrane rigidity. Acetyl-L-carnitine (ALCAR) fed to old rats increases the amount of cardiolipin to levels similar to that of young rats (83), suggesting that ALCAR administration may improve cellular bioenergetics in the aged rat.

Cardiolipin contains a higher ratio of unsaturated to saturated fatty acid residues compared with the other phospholipids of the inner mitochondrial membrane, a characteristic that increases its sensitivity to oxidation. The sensitivity of cardiolipin to peroxidation increases with age in rodents, an effect that appears to be attributable in large part to the replacement of 18:2 acyl side chains with more readily peroxidizable 22:4 and 22:5 acyl side chains (14). The mechanism underlying the age-related change in the composition of the acyl side chains of cardiolipin is not known. Interestingly, calorie restriction, a dietary regimen that extends life-span in rodents, maintains the level of 18:2 acyl side chains and inhibits the cardiolipin composition change to the 22:4 and 22:5 class of lipids (14). Calorie restriction does not appear to have a marked effect on cardiolipin levels (14).

Oxidative stress conditions decrease cardiolipin levels by inducing oxidation of its unsaturated fatty acyl side chains. After episodes of ischemia–reperfusion, cardiolipin appears to be destroyed selectively by oxidants (84–86). The extensive lipid peroxidation that occurs at the inner mitochondrial membrane as a result of this challenge appears to be catastrophic to the integrity of cardiolipin. This in turn leads to the inactivation of cytochrome *c* and mitochondrial enzymes (86) and increases the permeability of the inner mitochondrial membrane (85). Because cardiolipin is important in precursor protein import into mitochondria (87), changes in its level or acyl side chain composition could adversely affect targeted insertion of nucleus-encoded mitochondrial proteins.

Mitochondrial Damage: Effects on Mitogenesis. Exposure of HeLa cells to 80% O_2 for 2 days inhibits mitochondrial respiration and is associated with growth inhibition and loss of mitogen responsiveness (88). This treatment leads to the inactivation of the thiol-containing mitochondrial enzymes NADH dehydrogenase, succinate dehydrogenase, and α-ketoglutarate dehydrogenase. Studies in mammalian cell culture show that oxidative stress can adversely affect the activity of key mitochondrial enzymes and subsequently lead to a decline in ATP production (89). The important lesions that lead to decline in mitochondrial enzyme activities are not known but could be derived from mutations to mitochondrial or nuclear genes (90). Epigenetic effects such as direct protein damage (e.g., oxidation of vicinal dithiol and monothiol-containing enzymes of the mitochondrial inner membrane) may also create a condition that indirectly leads to genetic damage of these key genetic loci. For example, oxidant-induced damage to inner mitochondrial membrane proteins can lead to increased leakage of O_2^- and H_2O_2 that then may cause mtDNA mutations. Oxidative inactivation of mitochondrial proteins that leads to decreased mitochondrial efficiency may be related to the age-associated depression of mitochondrial protonmotive force observed in human fibroblasts (91) and murine lymphocytes (45). A decline in mitochondrial membrane potential can lead to lower ATP production and decrease the efficiency of energy-dependent processes such as signal transduction. The loss of mitogen responsiveness in lymphocytes isolated from elderly individuals has been attributed in part to the decrease in mitochondrial and plasma membrane potentials (see below).

ALCAR and Mitochondrial Function. The β-oxidation of fatty acids serves as a key source of energy for many tissues. For these tissues the activity of carnitine–acylcarnitine exchange across the inner mitochondrial membrane is of great importance. Investigations of heart mitochondria indicate that the activity of this exchange reaction, which is mediated by a thiol-containing and mersalyl-sensitive carrier protein (92), is decreased significantly with age (42). It has been suggested that the lower intramitochondrial pool of carnitine is in part responsible for this age effect.

A rapidly growing body of evidence suggests that the apparent age-related deficits in mitochondrial function can be slowed or reversed by ALCAR, a normal component of the inner mitochondrial membrane that serves as a precursor for acetyl-CoA as well as the neurotransmitter acetylcholine. Once deacetylated, L-carnitine, which remains in the inner mitochondrial membrane, can be reacylated and further serve to shuttle lipid substrates into mitochondria for β-oxidation. ALCAR has been shown to reverse the age-related decrease in the levels of mitochondrial membrane phospholipid cardiolipin and the activity of the phosphate carrier in rat heart mitochondria (83). Furthermore, the age-associated decrease in mtDNA transcription is reversed rapidly by ALCAR (93). Chronic administration of this compound to rats is associated with a reduction in the accumulation of lipofuscin in Purkinje neurons (94) and pyramidal neurons of the prefrontal cortex and hippocampus (95). It has also been shown to attenuate the age-related decrements in active avoidance learning (96). ALCAR's effect on mitochondrial function in the aging brain is supported by its ability to create a shift in ATP production from glycolytic pathways to mitochondria (97). *In vitro* studies indicate that ALCAR increases the number of *N*-methyl-D-aspartate (NMDA) receptors of cultured cerebellar granule cells, prevents age-associated reduction of nerve growth factor binding to PC12 cells (98), and attenuates the rate of mortality in rat dorsal root ganglia neurons (99). In aged mice treated with ALCAR for 3 months, dopamine release is enhanced compared with untreated control animals. The age-associated loss of the D1 subclass of striatal dopamine receptors is also attenuated by this treatment (100). In addition, ALCAR reverses the age-associated decrease in mitogen-induced lymphocyte proliferation and protects lymphocytes isolated from old donors from cytotoxicity following a challenge with oxidants (101). ALCAR appears to completely protect canine frontal cortex proteins from oxidation after cardiac arrest and restoration of circulation (102). The multiplicative effects of ALCAR in reversing the age-related decline in various physiological parameters associated with mitochondrial function may be attributable to its ability to deliver acetyl-CoA equivalents to the tricarboxylic acid cycle and to facilitate the mitochondrial β-oxidation of fatty acids, thereby increasing the production of ATP. It is plausible that ALCAR can increase the metabolic efficiency of compromised subpopulations of mitochondria and cause a redistribution of the metabolic workload, resulting in increased cellular efficiency and possibly a decrease in the rate at which mitochondria-derived oxidants are produced.

Effects of Calorie Restriction on Mitochondrial Function and Oxidant Production

It has been suggested that Darwinian fitness in animals is increased by the delay of reproductive function during periods of low food availability (103) and that the saved resources are invested in maintenance of the body until food resources are available for successful reproduction (104). For example, during the winter months when food is scarce, Syrian hamsters undergo a process of gonadal regression that is accompanied by patterns of daily torpor, a metabolically depressed state. These two important physiological adaptations minimize consumption of metabolic fuel by divert-

ing resources away from reproductive function and by transiently lowering metabolic rate. When animals that had already undergone gonadal regression are treated with testosterone, daily torpor is not observed (105). This suggests a potentially important link between neuroendocrine modulation of reproductive function and whole body aerobic metabolism. Another example of the dramatic life-extending effects of lowered oxygen consumption rates is the unusually long 8-year life-span of the pocket mouse *Perognathus longimembris*, a rodent with a low waking metabolic rate and the ability to undergo daily torpor (106).

During torpor or hibernation, a condition of extended torpor, mitochondrial respiration of liver drops markedly. This decrease in cellular respiration is associated with increased microviscosity and decreased permeability of the inner mitochondrial membrane, a change that leads to decreased transport of substrates for intramitochondrial energy production. Phospholipase A_2 activity also decreases (21), indicating that oxidation of lipids of the inner mitochondrial membrane is reduced. Specifically, electron transfer through the ubiquinol:cytochrome *c*1 segment of the respiratory chain of liver mitochondria isolated from hibernating ground squirrels (*Citellus undulatus*) is inhibited by 70–80% compared with mitochondria isolated from nonhibernating control animals (107).

The physiological mechanisms that control the conservation of energy during periods of low food availability, as observed experimentally with calorie restriction, may temporarily but profoundly affect the metabolic rate of organisms capable of entering this state. Therefore, daily torpor, by lowering the metabolic rate and oxidant production that can exert pro-aging effects, is likely to be responsible for at least some of the life-prolonging effects of calorie restriction. These findings are not necessarily inconsistent with the observation of total lean body O_2 consumption being unchanged when the effects of calorie restriction and *ad libitum* feeding are compared (108). We suggest that while whole body metabolic rate may remain constant, significant shifts in the proportion of oxygen consumed by the different organs will occur, as would be predicted on the basis of principles of physiological adaptation. When food consumption is low (i.e., calories are restricted), workload is reduced in organs (stomach, intestines, colon, liver, and kidney) that participate in food absorption and digestion. In contrast, spontaneous motor activity *increases* in calorie-restricted rodents. Female $B6C3F_1$ mice (28 months of age) or male and female F344 rats reared on this dietary regimen exhibit an approximately 50% higher spontaneous motor activity than control animals fed *ad libitum* (109–111). This increase in motor activity is expected to elevate the amount of oxygen consumed by the muscle tissues involved. Thus, when metabolic energy is limiting, a paradoxical rise in spontaneous movement is observed, presumably as an evolutionary adaptation that signals the organism to seek food. Although this behavior requires expenditures of metabolic energy that are limiting, it improves survival by increasing the probability of the animal finding food. The extent to which this shift in tissue-specific oxygen consumption occurs is not known. However, it is reasonable to speculate that the drop in oxygen consumption by organs of the digestive system (as well as the reproductive system) will be partially compensated by increases in oxygen consumption due to motor activity.

Neuronal and Neuroendocrine Aging and Mitochondrial Dysfunction

Oxidants and mitochondrial deficits may lead to increased neuronal loss through excitotoxic mechanisms. Because central nervous system function is critical in homeostasis, the attrition of a sufficient number of neurons can lead to age-associated disability and loss of various physiological functions such as receptor-mediated signal transduction.

The loss of sensitivity in central neuronal receptors to agonist stimulation is a hallmark of the aging process (112). This appears to be particularly true in central (hippocampal, striatal) muscarinic cholinergic systems and in the striatal dopamine systems. Decreased receptor numbers and less efficient signal transduction seem to be responsible for a marked decline in cognitive and motor functions. Oxidation has been implicated in membrane (112) and mitochondrial damage in neurons. The central nervous system is enriched in both unsaturated lipids and nonheme iron, two ingredients that could cooperate to produce oxidant damage and cell death (113). A functional consequence of oxidant damage is increased membrane rigidity that can lead to a decline in receptor-mediated signaling, possibly explaining the age-associated decline in responsiveness of the β-adrenergic, dopaminergic, and muscarinic receptor systems to agonist stimulation. The effective age-related loss in signal transduction can be mimicked in younger animals by treatments such as kainic acid or ionizing radiation (112) that produce oxidative damage to these receptors.

The decline in mitochondrial function that is proposed to occur with age in terminally differentiated neurons appears to increase their sensitivity to cell death by excitatory factors. It has been shown *in vitro* (114) and *in vivo* (115) that deficits in mitochondrial function can lead to neuronal degeneration and death by sensitizing neurons to the excitotoxic effects of endogenous glutamate, a neurotramitter that binds to the NMDA receptor and under normal physiological conditions is excitatory. The neurotoxic effects of excitatory amino acids have been implicated in a number of neurodegenerative processes such as Huntington disease, Parkinson disease, amyotrophic lateral sclerosis, Alzheimer disease, and the AIDS dementia complex and may also be involved in a number of neurological disorders, including stroke, epilepsy, trauma, and neuropathic pain (116–118). It is suggested that voltage-dependent NMDA receptor ion channels will become increasingly sensitized to the neurotoxic effects of glutamate when cell membranes become partially depolarized because of decreased mitochondrial energy production (119). The decline in the plasma membrane potential would thus permit the release of the voltage-dependent blockade of the NMDA receptor channel by Mg^{2+}. This release allows persistent and uncontrolled receptor activation that leads to calcium mobilization from intracellular stores, oxidant production, neuronal damage, and cell death (120). Such a mechanism could account for the progressive age-associated loss of these neuronal populations.

The prooxidant effect of glutamate receptor activation, which appears to be amplified markedly when mitochondrial function is compromised (121, 122), is revealed in studies that show increases in the formation of lipid peroxidation products and lipofuscin by the NMDA receptor agonists NMDA and kainic acid (123). Studies on the effect of NMDA-induced neuronal death suggest reactive byproducts of nitric oxide as one of the principal cytotoxic species (124). NMDA receptor activation triggers a rapid influx of calcium into the cell that stimulates calmodulin-dependent nitric oxide synthase activity, creating a marked elevation in nitric oxide production and subsequent cellular damage. This effect of nitric oxide appears to be mediated by peroxynitrite, a powerful oxidant formed from nitric oxide and superoxide (125). Under physiological conditions peroxynitrite rapidly decomposes to generate oxidants with a reactivity similar to that of hydroxyl radical. Inhibitors of nitric oxide synthase effectively protect neuronal cells in culture from the cytotoxic effects of NMDA and glutamate (126). Antioxidant treatments with 21-aminosteroids appear to be somewhat effective in attenuating the neurotoxic effects of these excitatory agents *in vitro* (127). In addition, both glutathione (GSH) and oxidized glutathione (GSSG) are protective against the acute neuronal toxicity, but they act by distinct pathways; GSH appears to act as an antioxi-

dant, while GSSG binds to the vicinal thiol residues of the NMDA receptor, thus blocking receptor activation (128). The prophylactic effects of antioxidant treatments on age-associated, glutamate-dependent neuronal losses have yet to be tested. Long-term treatment of rats with ALCAR also attenuates the age-associated reduction in the density of hippocampal NMDA receptors (129). Ubiquinone also protects neuronal cells in culture from glutamate toxicity (130). It is not clear, however, whether the protection afforded by ubiquinone in this cell culture model is due to its role as a component of mitochondrial electron transport or as an antioxidant. Coadministration of nicotinamide (a precursor of NADH) and ubiquinone is more protective against the neuronal injury caused by the mitochondrial toxin malonate than either compound alone; this treatment prevents depletion of ATP that is postulated to sensitize neurons to the cytotoxic effects of excitatory amino acids (131). The available evidence suggests that acute neurological damage or chronic neuronal degeneration that accompanies aging may be provoked in part by mitochondrial dysfunction and the oxidants produced by the NMDA receptor-mediated pathway.

Mitochondrial Dysfunction and Immune System Decay

Age-associated deficits in mitochondrial bioenergetics are suggested to play an important role in T-cell function. Plasma membranes of T cells isolated from old individuals appear to be partially depolarized compared with plasma membranes of T cells isolated from young individuals. This effect, which reflects a depressed resting plasma membrane potential (132), may be secondary to deficits in mitochondrial function. Decreased mitochondrial membrane potential also appears to correlate with decreased mitogen responsiveness (45). Interventions such as ALCAR and ubiquinone, both of which are known to increase the efficiency of mitochondrial ATP production, have been reported to improve cell-mediated immunity in human subjects (101, 133).

Clinical studies in elderly humans indicate that various dietary antioxidants such as glutathione, β-carotene, and α-tocopherol improve cell-mediated immunity (see references cited in ref. 2). Increasing the intracellular antioxidant levels, improving cell surface thiol status, improving mitochondrial function, and decreasing oxidant-induced membrane rigidity could all be mechanisms by which dietary antioxidants serve to boost cell-mediated immunity.

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