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THE ROLE OF OXIDATIVE DAMAGE IN MITOCHONDRIA DURING AGING: A REVIEW

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1. ABSTRACT

Aging is a complex process (or series of processes). Recent evidence suggests that several of its most important mechanisms are linked by means of cellular damage caused by reactive oxygen species (ROS). Oxidative damage may be a major factor in the loss of physiological functions that occur in degenerative diseases and aging. This is because, in aerobic organisms, the mitochondrial electron transport chain plays an important role in energy production and is a significant source of ROS that damage DNA, RNA, and proteins in cells. While oxidative events in other cell organelles are likely to contribute to the pathobiology of aging, this review highlights alterations in mitochondrial function that, due to accumulated oxidative damage, occur with age.

2. INTRODUCTION

Oxidative damage is hypothesized to be one cause of aging in metazoans (1-7). Oxidatively damaged macromolecules accumulate with age in every organism examined; thus, oxidative damage is implicated in many human age-related diseases. We will review studies of oxidative damage in mitochondria during aging and arguments for or against oxidative damage as a major mechanism of aging (7).

3. THE CHEMISTRY OF REACTIVE OXYGEN SPECIES

ROS are produced as a byproduct of normal cellular metabolism. Aerobic organisms are constantly subjected to ROS, which include:

I. Species derived from reduction of molecular oxygen:

1. Superoxide
2. Hydrogen peroxide
3. Hydroxyl radical

II. Species derived from reaction of carbon-centered radicals with molecular oxygen:

1. Peroxyl radicals
2. Alkoxyl radicals
3. Organic hydroperoxides

III. Other oxidants that can result in free radical formation:

1. Hypochlorous acid
2. Peroxynitrite
3. Singlet oxygen

A major ROS produced by the cell is superoxide, which is converted by SOD to hydrogen peroxide (H_2O_2).

Catalase and superoxide dismutase (SOD) are enzymes defending against ROS in all cells; Catalase converts H_2O_2 to molecular oxygen and water, however their defense is not always completely effective.

SOD exists in two major forms in the eukaryotic cell: Cu,ZnSOD in cytoplasm and MnSOD in mitochondria, which are thought to be the primary cellular source of ROS and which accumulate oxidative damage. Thus, MnSOD may be an important defense against oxidative damage. In mice MnSOD appears to be more critical because its mutations have more severe consequences than Cu,ZnSOD mutations (8,5).

Ozone, nitric oxide, and nitrogen dioxide are also ROS from exogenous sources. The latter two are also produced endogenously (9). Possibly the most prolific source of ROS, particularly superoxide and hydrogen peroxide, is leakage from the mitochondrial electron transport chain (10). Superoxide is formed in various autoxidation reactions and by enzymes such as peroxidases, cytochrome P450, and xanthine oxidase (11). By means of the respiratory burst oxidase, as a defense mechanism against infectious agents, phagocytes also produce ROS (12-14).

Some experimental and epidemiological studies suggest that administration of antioxidants may prevent development of age-associated disorders such as cancer (15) or cardiovascular and neurodegenerative diseases (16,17) (e.g., Parkinson's and Alzheimer's disease (18,19). But the Beta-Carotine and Retinal Efficacy Trial (CARET study by Omenn et al., [20]) showed that after an average of four years of supplementation, the combination of beta-carotene and vitamin A had no benefit - and may have had an adverse effect - on the incidence of lung cancer and the risk of death from lung cancer, cardiovascular disease, and any other cause in smokers and workers exposed to asbestos. Three other studies (Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study [ATBC] [21]), Physicians' Health Study [22] and the Linxian Study in China [23]), examining the effects of supplementary vitamin A and beta-carotene, suggest hypotheses for the lack of effect.

In the CARET and ATBC studies, the study populations were at high risk. In these studies the administration of vitamin A appeared to have an adverse effect on populations who presumably had already suffered cellular damage leading to morbidity and death. In the Physicians study, where there was a mix of high and low risk groups (smokers and non-smokers), there was no elevation (RR=1.01) of risk which could plausibly be the average of an elevated risk in smokers and a lower risk in supplemented non-smokers. In the Chinese study a benefit (RR=0.91) was discovered in a population with a presumed deficiency of vitamin A. Consistent with this is the negative correlation of high cancer risks and baseline vitamin A serum levels in the CARET study.

Two basic explanations are suggested. First, is that once significant oxidative damage is incurred in high risk groups, vitamin A will not reverse it and, as a potent hormonal factor in cell growth and differentiation, could even promote late stages in a multi stage model of carcinogenesis. Second, the redox relations of vitamins A, C, and E are complex and might be distributed by super-supplementation of vitamin A. Thus the CARET study may reflect more on the use of vitamin A as a "treatment" rather than as a preventative anti-oxidant. A conclusive study would be to start supplementation before starting toxin exposure, (e.g., smoking).

4. NORMAL FUNCTIONS OF ROS

ROS formation takes place during normal physiological functioning (24,25). Under aerobic conditions in living cells, superoxide anions and hydrogen peroxide are produced and converted into hydroxyl radicals by the iron-catalyzed Fenton reaction. Excessive ROS production may overwhelm the antioxidant capacity of cells (such as intracellular nonprotein sulfhydryls [NPSH]) to modulate cytotoxicity, thereby initiating a pathogenic cascade of events (26). This is exemplified in mitochondria by interactions of free radicals with transition metal centers. Gerschman *et al* (27) recognized: 1.) ROS are the common mechanism of oxygen and radiation toxicity; 2.) an increase in prooxidants, or a decrease in antioxidants, will lead to cell damage; and 3.) oxygen toxicity is a continuous phenomenon.

5. CLASSES OF MACROMOLECULES UNDERGOING OXIDATIVE DAMAGE

Accumulation of molecular oxidative damage may be important in the senescence-related decline of the physiological fitness of organisms (28,29). Such damage is a random, rather than a controlled, process (30-32). Old animals have increased mitochondrial production of superoxide and hydrogen peroxide (33,34). Many studies demonstrate the sensitivity of mitochondrial protein, lipids, and DNA to oxidative stress (35-38).

Accumulation of proteins damaged by oxidative stress depends on tissue, cell, and protein type (39, 2, 40,32). The adenine nucleotide translocase (ANT) protein shows strong carbonylation during normal aging because it: a.) contains readily oxidized arginine, proline, and lysine residues and b.) is associated with cardiolipin containing unsaturated fatty acids (32). Accumulation of oxidized dysfunctional protein in carbonyl groups could lead to inter- and intra-molecular cross-links with other amino groups and, therefore, to loss of biochemical and physiological function. Age-related accumulation of protein oxidation products in mitochondria may disrupt the efficiency of energy production, thereby increasing ROS production (2).

Head *et al* (41), in a canine model, showed an increase in lipid peroxidation with age. Increasing oxidative damage to lipids was found in the brain and serum, but not in cerebrospinal fluid (CSF). The correlation of malondialdehyde (MDA) levels in brain and serum suggests that damage occurs both centrally and

systemically. MDA levels may reflect not only the extent of lipid peroxidation, but also the oxidative susceptibility of high- and low-density lipoproteins in serum (42). Age-dependent increases in MDA levels may be due to increases in serum protein (43). MDA can cross-link protein side-chains, slow protein degradation, and reduce protein turnover in rats and humans (44).

Mechanisms underlying the age-dependent increase in serum MDA may be linked to oxidative damage - which, in DNA, accumulates over the life of a cell (45,46). Generation of DNA strand breaks is necessary to trigger the p53-dependent cell cycle pathway (47), which mediates inhibition of proliferation and apoptosis with features observed in replicatively senescent normal diploid somatic cells (48). Whether or not strand breaks occur depends on stabilization of the p53 tumor suppressor gene product and induction of downstream mediators (49). There are conflicting data on the relation of oxidative stress and the type of proliferative senescence described by Hayflick (50,51). Several experiments demonstrated oxidative stress inhibits cell proliferation; others indicated inhibition of proliferation mediated by hyperoxia was reversible (52,53).

During aging, oxidative damage to macromolecules increases exponentially in a variety of tissues in different species (54). That such damage causes functional loss was found by *in vitro* models (55). Stadtman (56) demonstrated that oxidative damage to proteins causes loss of catalytic and structural integrity; altered proteins are preferentially hydrolyzed. Protein and lipid oxidation products may form cross-linked, undegradable products such as lipofuscin (57). Studies (58;59) indicate that oxidative damage to proteins is selective, implying that some, but not all, catalyzed reactions are impaired in aging. Specificity of protein damage provides a link between ROS and senescence-associated functional alterations (e.g., age-related carbonylation of mitochondrial aconitase and ANT in flies is associated with loss of protein activity [59]).

6. MITOCHONDRIA AS MAJOR SOURCES AND TARGETS OF ROS

Mitochondria are an important link between accumulation of oxidative damage caused by ROS and alterations in function associated with aging (60**).** Oxidative damage in mitochondria is the leading candidate for a unitary mechanism of aging in aerobic organisms (3). The mitochondrial theory of aging, a variant of the free radical theory of aging, suggests that accumulation of damage to mitochondria, mitochondrial DNA (mtDNA), and RNA leads to aging in both humans and animals (61-65). Because approximately 90% of cellular oxygen is consumed in mitochondria, and 3% of molecular oxygen reduced by mitochondria is not reduced to water, mitochondria may be the major intracellular contributor to superoxide (O_2^-) generation - and perhaps oxidative stress in general (34)

6.1. Structure of mitochondria

Mitochondria are the main sources of energy in the cell. They contain their own mtDNA, a small 16.5 kb circular molecule in humans coding for 13 polypeptides, 22 tRNAs, and 2 rRNAs - all of which are components of the respiratory-chain/oxidative phosphorylation system (66). Mitochondria have four structural compartments: matrix, inner membrane, intermembrane space, and outer membrane. In the inner membrane there are protein systems, such as the electron transfer chain complexes (20^+ discrete electron carriers and an unspecified number of "structural" proteins, all of which are organized into four multi-protein complexes [I-IV]) and the "fifth" complex, F_1F_o-ATP synthase, [67]).

Optimal oxidative phosphorylation requires energy-transducing membranes to have: a.) low proton conductance and b.) exchange carriers allowing metabolites to permeate while maintaining osmotic stability. Nicholls (68) suggested that, in the mitochondrial inner membrane, the high proportion of protein (50% integral protein, 25% peripheral protein, and 25% lipid) results in close packing of proteins. Recent x-ray studies indicate that much of the protein mass is in water outside the lipid bilayer so the mitochondrial bilayer may be more substantial than Nicholls suggests (69**)**.

6.2. Mitochondrial functions

Mitochondrial functions include ATP production, heme and cholesterol biosynthesis, and cellular calcium regulation (70). Although these functions are the most important intracellular sources of oxidants (71,72), it is uncertain how much O_2^- they form (73). The main sites where O_2^- is generated are the ubiquinone pool and nicotinamide adenine dinucleotide (NADH) dehydrogenase (Complex I). Electrons are transferred, individually, to form ubisemiquinone, which reacts with oxygen to form O_2^-. Mitochondrial macromolecules and mitochondria that are isolated from old animals may produce more H_2O_2 than younger animals (72-74). These oxidants ($O_2^-$and H_2O_2).damage cellular macromolecues, including DNA, protein and lipid. Accumulation of such damage may contribute to aging and age-associated degenerative diseases.

6.3. Mitochondrial DNA

Mitochondrial DNA (mtDNA) is located in the matrix and is attached to the inner membrane. mtDNA lacks introns, histones, and other DNA proteins and has less complete repair mechanisms than nuclear DNA (nDNA) (75-76). This makes mtDNA prone to oxidative damage. Damage in mtDNA is more extensive, rapid and persistent than damage in nDNA (31). ROS-promoted damage to mtDNA includes fragmentation and deletions. A marker for damage, 8-hydroxy-deoxyguanosine (8-OHDG) (77), increases with age and degenerative diseases. *In vitro* oxidative stress, as occurs during mitochondrial lipid peroxidation or during the transition, is accompanied by mtDNA fragmentation and increases in 8-OHDG. Because mtDNA encodes proteins involved in electron transport and oxidative phosphorylation, oxidative damage may lead to respiration and phosphorylation deficiencies (74).

6.4. MtDNA mutations

Since the sequencing of human mtDNA in 1981 (78), a number of mutations have been described and linked

to a heterogeneous class of age-related disorders (79). The incidence and abundance of mutant mtDNAs increase with age, particularly in tissues with high energy demands (80-88). A variety of point mutations are detectable (89). Khrapko *et al* (90) found that colon, lung, muscle, and tumors derived from these tissues share many mitochondrial "hotspot" point mutations. Tissue-specific characteristic mtDNA was discovered in Attardi's lab at CalTech (91). The levels of 8-OHDG mtDNA adducts and deletions increase exponentially with age. (92-96). In human skeletal muscle, fibers lacking cytochrome c oxidase (complex IV) activity accumulate with age (97,98).

Certain control region (CR) mtDNA mutations also accumulate with age (99). Mixtures of mutant and normal CR mtDNAs (heteroplasmy) have been reported in human brain (100), hair (101) and cancer (102,103) cells. Mitochondrial oxidative phosphorylation (OXPHOS) enzyme activities decline with age in human and primate muscle (86,104,105), liver (106), and brain (107) tissue. Cytochrome c oxidase (COX)-deficient muscle fibers accumulate with age in heart and skeletal muscles (108,109). COX-negative regions contain clonal expansions of individual mtDNA rearrangements (110). This increase correlates with accumulation of mtDNA mutations, including deletions (80,82-85,92,110-126) and base substitutions (123-126)

mtDNA damage is high in tissues prone to age-related dysfunction. Basal ganglia accumulate the most mtDNA damage, followed by various cortical regions. The cerebellum remains relatively free of mtDNA damage throughout life (127,119). This suggests that accumulation of mtDNA mutations may be important in the age-related decline of somatic tissues (111,128,129). As mutations accumulate, they exacerbate inherited OXPHOS defects until combined defects result in energetic failures (129).

A few hundred to a few thousand mtDNA molecules are present in an individual cell. Production of these molecules is not correlated with cell cycle parameters. During mitosis (or meiosis), mitochondria are randomly distributed to daughter cells. The existence of multiple DNA molecules, all susceptible to mutation, leads to heteroplasmy (130). mtDNA heteroplasmy is found in numerous types of animals (131). Phenotypic manifestations of mtDNA mutations are dependent on the levels of heteroplasmic mutant mtDNA in a cell, tissue, or organ (79, 132-137).

6.5. Role of mitochondria in telomere shortening

Human telomeres are specialized chromosomal end structures composed of TTAGGG repeats. These telomeres protect chromosomes from degradation, fusion and recombination (138). Immortal eukaryotic cells, including transformed human cells, use telomerase, an enzyme that elongates telomeres, to overcome incomplete replication. However, telomerase has not been detected in normal somatic cells, which lose telomere length as they replicate (139).

In telomere the TG strand is longer than its complement, leaving a region of single-stranded DNA of up to a few hundred nucleotides at the 3' end. In mammals, the single-stranded end is sequestered in a "T loop" (140), is folded back, and paired with its complement in the double-stranded portion of the telomere. Looped DNA is bound by proteins TRF1 and TRF2. T loops may protect the 3' ends of chromosomes, making them inaccessible to nuclease and enzymes that repair double-strand breaks (141). Data suggest that the G-rich strand is more vulnerable to oxidative damage (142). Opening the telomeric loop and exposing the single-stranded, G-rich overhang might signal arrest of the cell cycle. Short telomere length, single-strand breaks, low levels of loop-stabilizing proteins, or other factors may trigger opening of the loop (143,144).

6.6. Role of mitochondria in apoptosis: Relevance to aging

Mitochondria are central to the life and death of eukaryotic cells (145-152). Mitochondria contain and release apoptotic proteins (e.g., cytochrome c and apoptosis-inducing factor [153]). The mitochondrial permeability transition (PT) pore is critical in apoptosis. Opening of the PT pore releases cytochrome c and apoptosis-inducing factor (AIF) into cytosol (154). Mitochondrial oxidative stress occurs early in apoptosis - prior to DNA fragmentation - and may cause PT pore opening. Esteve (155) found decreases in mitochondrial membrane potential (and an increase in peroxide content) in apoptotic fibroblasts. Experiments show that change in mitochondrial activity is common in apoptosis (156-158). ROS-induced apoptosis may remove mitochondria when ROS production exceeds scavenging capacity (159).

Age-related alterations in the mitochondrial pathway of apoptosis can affect tissue function. These alterations can arise from oxidative damage to mitochondria. Common features of apoptotic cells and cells from old animals include increased mitochondrial peroxide production, oxidation of glutathione, and oxidation of mtDNA (160). mtDNA and respiration are not essential for apoptosis (161,162). However, the absence or impaired function of mtDNA can influence the rate of this process - probably by regulating ROS production (163). Therefore, it appears that the relation of aging and apoptosis is not established.

6.7. Functional decline of mitochondria with aging

With age, mitochondrial function declines and mtDNA mutations increase. Cell energy deficits (caused by declines in mitochondrial function) can impair cell activities and compromise adaptation to physiological stress (164). Age-related impairment in respiratory enzymes decreases ATP synthesis and enhances ROS production by increased electron leakage in the respiratory chain. Human mtDNA is susceptible to oxidative damage and mutation when exposed to high levels of ROS (64).

Aging is associated with declines in the capacity of various cell types, including neurons, to respond to metabolic stress due to impairment of mitochondrial function. Although some neurodegenerative diseases are associated with mitochondrial dysfunction, it is not clear if

changes are due to normal aging or to exposure to pathophysiologic agents (67).

Mitochondrial ROS generation has been studied in animal models of neuronal disease (165). De la Asuncion *et al* (166) found mitochondrial glutathione markedly oxidized with aging in rats and mice. The oxidized-to-reduced-glutathione ratio increases with age in the liver, kidney, and brain. In the liver, glutathione disulfide (GSSG) as a percent of glutathione (GSH) changed from 0.77 +/- 0.19% (n =5) in young rats to 2.47 +/-1.25% (n=5) in old ones. In the brain and kidney, values for old rats were higher than those for young rats. Oral antioxidants protected against glutathione oxidation and mtDNA damage in rats and mice.

Sohal (167) argues that accrual of molecular oxidative damage as a mechanism governing the rate of aging, is supported by: (*i*) life span of cold blooded animals and mammals with unstable basal metabolic rate (BMR) being extended and oxidative damage attenuated by a decrease in metabolic rate (Experimental life span extensions are accompanied by reduced oxidative damage. Both activity and regimen might affect life span. For example, elimination of flying prolongs the life span of flies by 3-fold and decreases accumulation of protein and DNA oxidative damage [168,169]. Regimens that extend life span of poikilotherms decrease metabolic rates and accumulation of oxidative damage [170]).; (*ii*) single gene mutations in Drosophila and *C. elegans* that extend life span slow physiological activities, albeit via different mechanisms, decreasing oxidative damage; and (*iii*) caloric restriction decreases body temperature and oxidative damage. He indicates that studies of transgenic over expression of antioxidant enzymes are supportive but ambiguous.

In rodents, life span is extended if caloric intake is decreased from the level of ad libitum fed animals. The temperature of calorically restricted animals is daily transiently lowered by as much as 4°C in rats and 13°C in mice, indicating that a BMR is responsive to caloric intake (171,172). Stadtman (173) found that protein carbonyls increase with age in insects with steady state concentration inversely related to life expectancy. This was confirmed in mammals. Macromolecular oxidative damage rates of mitochondrial ROS increase with age and are inversely related to species mean life span (MLS) (174).

7. THE ROLE OF ROS IN GERIATRIC DISORDERS

ROS are involved in many diseases of the elderly, including Alzheimer's disease (AD) and Parkinson's disease(PD), as well as conditions affecting vision, such as cataracts and macular degeneration (175). Neurodegenerative diseases have been linked to mutations in mtDNA and nDNA. Genetic and phenotypic variability of mitochondrial diseases is due to the number of mitochondrial genes involved and the cell pathways and functions in which mitochondria play a role.

Oxidative modification of proteins is important in aging and age-related neurodegenerative disorders (176, 2). Modification of amino acid side chains in proteins can lead to diminished function (176,2,177). Degradation of oxidized proteins by proteinases is possible, but oxidatively induced, proteinase-resistant, protein cross-linking can occur preventing removal (178,179).

7.1. Alzheimer's disease (AD)

Oxidative damage may play a critical role in AD neuropathology (180). Friede (181) noted alterations of oxidative metabolism in AD. This might precede and drive amyloid deposition in brains of affected subjects. Extensive evidence suggests that lipid peroxidation is important in AD. Indices of lipid peroxidation in AD include TBARS, phospholipid composition, levels of alpha- and beta-unsaturated aldehydes, activities of enzymes that clear lipid peroxidation products, and concentrations of isoprostanes (180,176).

The most abundant oxidized DNA base product from hydroxyl radical attack is 8-OHDG. mtDNA had a threefold increase of 8-OHDG in the AD parietal cortex compared with controls. A small, but noticeable, increase in 8-OHDG in nDNA in AD cases, compared to controls, was reported (182). Results, confirmed using gas chromatography/mass spectrometry (183), were expanded to frontal, parietal, and temporal lobes (184). Immunocytochemical detection of 8-OHDG and 8-hydroxyguanosine showed increased DNA and RNA oxidation is limited to vulnerable neurons in AD (185,186). mtDNA abnormalities in AD were confirmed by *in situ* hybridization studies (187).

The AD brain shows higher levels of oxidative damage to proteins (188) and lipids (189). This may be due to the deposition and accumulation of (Aβ) protein in senile plaques (190). Amyloid precursor protein (APP), from which Aβ is proteolytically cleaved (191), is vulnerable to oxidative damage and exposing APP to metabolic stress favors production of amyloidogenic fragments (192,193).

Epidemiological studies show a large reduction in AD risk due to consumption of Non Steroidal Anti-Inflammatory Drugs (NSAIDs) -- especially ibuprofen (194). Ibuprofen may reduce the risk of AD — not only through its anti-inflammatory properties but also due to direct effects on amyloid proteins. Other drugs showing promise in controlling AD are statins (possibly because of anti-inflammatory properties) and the effects of nicotine metabolites on amyloid proteins (195).

7.2. Parkinson's and other neurognerative diseases

Mitochondrial dysfunction occurs in Parkinson's disease (PD). Humans exposed to 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) developed PD (196,197). MPTP is converted to 1-methyl-4-phenylpyridine (MPP+) by monoamine oxidase in glial cells (198). MPP+ is taken up by cells possessing dopamine reuptake sites and concentrated in negatively charged mitochondrial matrices (199,200). Inside mitochondria, MPP+ inhibits Complex I (201) causing degeneration of catecholaminergic neurons in the *substantia nigra* and *locus ceruleus* (196,197,202,203).

Evidence of mitochondrial dysfunction also exists for other neurodegenerative diseases (e.g., degenerating and nondegenerating tissues of Amyotrophic lateral sclerosis (ALS) subjects [204] in progressive supranuclear palsy and multisystem atrophy [205-208] and Huntington's disease. Treatment of mitochondrial dysfunction is hampered by the difficulty of delivering bioactive molecules *in vivo*. Vitamin E has had only a modest effect on AD in epidemiologic studies. Smith *et al* (209) developed a strategy for targeting bioactive molecules to mitochondria by attaching them to the lipophilic triphenylphosphonium cation through an alkyl linker. These molecules rapidly permeate lipid bilayers and, because of the large mitochondrial membrane potential (negative inside), accumulate several hundredfold in isolated mitochondria and in mitochondria in cultured cells. For example, a triphenylphosphonium cation was coupled to coenzyme Q or vitamin E derivatives. Significant doses of these compounds could be fed safely to mice over long periods, coming to steady-state distributions within the heart, brain, liver, and muscle. Therefore, mitochondria-targeted bioactive molecules can be administered orally, leading to accumulation at potentially therapeutic concentrations in tissues affected by mitochondrial dysfunction. Targeting bioactive molecules to mitochondria can be adapted to any neutral, bioactive molecule. This complements approaches to target DNA and molecules related to mitochondria (210-212).

8. EVIDENCE FOR OR AGAINST OXIDATIVE DAMAGE AS A MAJOR MECHANISM OF AGING

The SOD mimetic EUK-8 is a research tool for investigating mechanisms of aging. It was reported to have extended lifespan in *C. elegans* (213). However, Keaney and Gems, (214), in five trials administering EUK-8 in liquid culture with *E. coli*, and two trials using defined liquid medium, found no increase in lifespan, but a dose-dependent reduction of lifespan and fertility. Extension of *C. elegans* lifespan by EUK-8 may occur only under specific culture conditions.

8.1. Drosophila studies

To test models of oxidative aging, creation of transgenic organisms complements traditional genetics. *Drosophila melanogaster* is a popular model because of transgenic and other genetic and molecular tools available, its short life span (1–2 months), and ease of culture (215-217).

In Drosophila engineered to overexpress MnSOD, Tower (218) used five approaches: two single component systems (transgenes with native [normal] promoters and transgenes with heterologous promoters) and three binary systems ("GAL4/UAS", "FLP-out", and "tet-on"). He concluded that over-expression of Cu,ZnSOD and MnSOD genes increases life span.

Several studies tested the oxidative stress hypothesis to determine if overexpression of antioxidative enzymes, either alone or in combination, increased life span in transgenic *Drosophila* (174). Three groups reported that overexpression of Cu,ZnSOD increased life span (219).

Orr and Sohal (220) reported that life span extension in *Drosophila* studies involving P element-mediated introduction of genomic fragments bearing the coding sequence and attendant regulatory sequences including promoter, intron and 3' flanking sequences of Cu,ZnSOD and catalase . No survival effects were found in transgenic flies possessing a single extra copy of either Cu,ZnSOD (221), or catalase (222). The life span of flies with extra copies of both Cu,ZnSOD and catalase increased by up to 34% over controls bearing two vector-only insertions. This was accompanied by increased physical activity, reduction in oxidative damage, and increased metabolism as measured by oxygen consumption. Because cis-regulatory sequences are present, overexpression of antioxidative genes is likely to follow normal physiological expression. Since P element insertion is a quasi-random process, expression can be impacted by regulatory regions near the insertion site — or the P element insertion can act to alter gene expression at (or near) the integration site. "Position effects" can impact longevity so it is important to use multiple lines (219).

Sun and Tower (223) used the FLP-out binary transgenic system, which yields transgene expression in adults in all tissues. One element of this system is the FLP recombinase transgene control led by a heat shock promoter. The second is the target gene under control of a constitutive promoter (Actin 5C), interrupted by a FLP recognition target (FRT) bounded sequence preventing expression. FRTs are target sequences for FLP recombinase. Consequently heat shock may liberate FRT-defined elements, permitting expression of the target gene. Advantages of this system are, a.) expression of the Cu,ZnSOD gene can be delayed until the adult phase avoiding negative consequences of over-expression on redox regulation during development and, b.) control is represented by genetically identical flies not subjected to heat shock. Since the two elements of this system are inserted by P element-transformation, position effects can affect expression as well as expression of neighboring genes, which can affect longevity.

The amount of MnSOD enzyme overexpression varied between six independent transgenic lines, with increases of up to 75%. Life span increased in proportion to increases in MnSOD enzyme. MLS increased up to 33%. Maximum life span, as measured by time to 90% mortality, increased by as much as 37%. Therefore, adult Drosophila life span is limited by MnSOD activity, analogous to results for Cu,ZnSOD (223,224). Both Cu,ZnSOD and MnSOD increased mean and maximum life span with no detectable negative effect on metabolic activity. Overexpression of catalase and MnSOD did not increase life span, consistent with catalase being found in excess in adult flies (223).

Sun and Tower (223) found, in most cases, no life span extension in female flies. With the exception of the SOD^{3A1} line, extensions in male flies were observed in short-lived lines (mean life spans <40 days). In a third experiment, male life span increases of 48% and 14% were noted, where control mean life spans were 25 and 36 days. The genetic component impacted life span increases only of male flies. Their graph of life span extension vs. control life span reveals a negative correlation, suggesting that

Cu,ZnSOD has beneficial effects only in a compromised genetic background. A rescue effect is notable, but its relevance to aging uncertain.

Parkes *et al* (224), to determine the consequences of SOD1 overexpression in motorneurons on normal longevity, generated transgenic *Drosophila* expressing human SOD1. Using the GAL4/UAS system, they demonstrated that overexpression of SOD1 motorneurons extends lifespan by up to 40% and rescues the lifespan of a short-lived SOD null mutant. This suggests that SOD activity in motorneurons is important to aging and longevity in *Drosophila*. Life span increase was sufficiently large, relative to its genetic variation, to be convincing.

However, Tower (218) indicated that increased longevity is not associated with decreased metabolism as assayed by O_2 consumption; therefore, both life span and metabolic potential might be increased. The pattern of expression produced by the D42-Gal4 "driver" was: broad during embryogenesis; in motorneurons, interneurons, some peripheral glial cells, and low level in fat body in larvae; a small number of cells within the central brain; and in motorneurons within the ventral ganglia in adults.

Expression in any or all of these tissues and stages could extend life span. Since, in adults, aging occurs primarily in motor neurons, these neurons are likely an important site of transgene action (218).

In *Drosophila*, overexpression of Cu,ZnSOD and catalase (but not of either alone) increased mean lifespan by 33% (220). This indicates that control of ROS and a need to balance SOD and catalase (225) are important for longevity. The balance differs over cell types because overexpression of human SOD1, without additional catalase in *Drosophila* motor neurons, extends lifespan (224).

8.2. *C. elegans* studies

The dauer is an alternative larval stage in *C. elegans* which allows animals to survive low food availability. Well-fed worms live three weeks. Dauer larvae can live for two months without affecting post-dauer lifespan (226). Mutations in *daf-2* and *age-1*, produce a dauer constitutive (Daf-C) phenotype and in *clk-1* mutations, which are believed to slow metabolism and markedly increase lifespan (227).

Taub *et al* (228) no longer have confidence in observations associating a reduction in *C. elegans* adult lifespan with a mutation in the catalase gene *ctl-1*. They confirmed that *C elegans* has multiple catalase genes and that the original strain, TU1061, had decreased transcription of *ctl-1* messenger RNA. They found several errors - one identifying a single nucleotide deletion as the defect in the *ctl-1* mutation and others in the identification of strains carrying mutations in multiple genes. They did not see the expected reduction in *ctl-1* mRNA in other strains. Longevity results obtained with these strains are therefore meaningless.

A handful of genes affect *C. elegans* lifespan through pathways downstream or parallel to the insulin signaling pathway. The feeding-defective *eat* mutants live slightly longer in a *daf-16*-independent manner. *eat* mutations might extend lifespan through a mechanism resembling caloric restriction in mammals (229). *clk* (biological timing abnormality) mutations also extend lifespan, though not as much as mutations in *daf-2* pathway genes, and slow physiological processes (230). *clk-1* encodes a protein in coenzyme Q synthesis, implicating mitochondria in lifespan determination (231,232). *clk-2* encodes a protein in DNA repair and telomere maintenance (233-235).

Lee *et al* (236) used 5,690 genes in an RNAi screen to identify genes that, when inactivated, extend lifespan. A number of genes essential for mitochondrial function affected *C. elegans* lifespan. In a screen for increased lifespan, they found a probable null mutation in a mitochondrial leucyl-tRNA synthetase gene (*lrs-2*) increased lifespan.

Long-lived worms with impaired mitochondria had lower ATP content and oxygen consumption, but different responses to ROS and other stress. Thus, the longer lifespan of *C. elegans* with compromised mitochrondria cannot be due simply to lower ROS production, suggesting a more complex coupling of metabolism and longevity.

8.3. Mouse studies

Van Remmen (235) found that oxidative damage occurs during aging of SOD2 knockout heterozygotes. Heart mitochondria from heterozygous (*Sod2*$^{-/+}$) knockout mice had 50% less MnSOD. The decrease was associated with increased oxidative damage (reduced activity of iron-sulfur proteins sensitive to oxygen stress [aconitase and Complex I]). Mitochondrial function was altered in *Sod2*$^{-/+}$ mice, as shown by decreased respiration by complex I and increased sensitivity of PT to induction by calcium and *t*-butylhydroperoxide. Induction of PT in heart mitochondria from *Sod2*$^{-/+}$ mice was associated with release of cytochrome *c* and DNA fragmentation. Cardiomyocytes from neonatal *Sod2*$^{-/+}$ and *Sod2*$^{-/-}$ mice were more sensitive to death than cardiomyocytes from *Sod2*$^{+/+}$ mice after *t*-butylhydroperoxide treatment. Sensitivity was decreased by inhibiting PT with cyclosporin A.

To understand the role of MnSOD in antioxidant defense and apoptosis in the heart, Remmen examined reduced MnSOD expression on oxidative damage, mitochondrial function and oxidative stress-induced apoptosis in hearts of *Sod2*$^{-/+}$ mice. MnSOD regulated the mitochondrial pathway of apoptosis as well as antioxidant defense. There was no change in life table parameters.

Diverse genes have evolved to protect cells from macromolecular damage. One class includes structural and regulatory genes for scavenger enzymes (e.g., SOD 1, 2, 3 and catalase). G.M. Martin (personal communication) found evidence supporting the theory in mice. Independent lines of transgenic mice overexpressing a cDNA for human catalase directed to mitochondria had ~17% increases in maximum life spans. Transgenic mouse lines

overexpressing human CAT in perioxisomes or nuclei did not show a significant extension in life span.

In a series of studies (236,237,28,29), effects of oral supplementation with acetyl-L-carnitine and alpha lipoic acid on the physiological and memory function of elderly mice were examined. The physical activity of elderly mice - and their performance in memory tests - improved to nearly the level of young mice. Acetyl-L-carnitine was hypothesized to increase mitochondrial energy production by affecting membrane function and alpha-lipoic acid to deal with increased production of ROS. Improvement in function was greater when both agents were administered than when each was administered independently.

Bluher *et al* (238) engineered mice to have the insulin receptor in adiocytes disabled. These were raised in an experiment with several other strains - all were fed *ad libitum* diets. Mice with the insulin receptor knocked out had an 18% higher mean and maximum life span even though they ate more food. Knockout mice were lean despite higher food consumption.

This study suggests the importance of hormonal factors in controlling metabolism and oxidative processes. IGF-1, insulin and GH have been studied in multiple species (239). As biological complexity increases, receptor complexity increases (e.g., from one insulin receptors in flies and worms to four in tetrapods). Metabolic functions and growth and development processes may separate, as suggested by results of the Baltimore longitudinal Study of Aging (240). This may be due to thyroid (T3) hormone which controls mitrochondrial function and mitrochondriogenesis under oxidative stress (241).

9. CONCLUSIONS

Numerous studies support the notion that mutations and oxidative damage to mtDNA (with associated decline in mitochondrial respiratory function) are important contributors to human aging. Many animal models provide insights into effects of oxidative damage to mitochondria in aging. Mitochondrial oxidative damage plays a major role in life span control in various systems. It is not clear if this represents an effect on pathology or is related to basic aging processes.

As the biological complexity of an organism increases, the competition of growth and reproductive processes with somatic cell maintenance for energy may be reduced (239). The caloric restriction model may not apply in the same way to humans as it does in simpler test systems (240). Respiratory function and longevity may simultaneously increase in humans as has been found in some test systems (241). The role of oxidative processes in human mitochondria may be more complex, with multiple inter-dependent tissue systems linked by hormonal systems (242) than in, at least, some test systems.

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