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Forum Review

Is the Mitochondrial Free Radical Theory of Aging Intact?

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ABSTRACT

The present state of the mitochondrial free radical theory of aging is reviewed. Available studies do not support the hypothesis that antioxidants control the rate of aging because: (a) they correlate inversely with maximum longevity in vertebrates, and (b) increasing their concentration by different methods does not increase maximum lifespan. On the other hand, comparative studies consistently show that long-lived mammals and birds have low rates of mitochondrial reactive oxygen species (ROS) production and low levels of oxidative damage in their mitochondrial DNA. Furthermore, caloric restriction, which extends longevity, also decreases mitochondrial ROS production at complex I and lowers mtDNA oxidative damage. Recent data show that these changes can also be obtained with protein restriction without strong caloric restriction. Another trait of long-lived mammals and birds is the possession of low degrees of unsaturation in their cellular membranes. This is mainly due to minimizing the presence of highly unsaturated fatty acids such as 22:6n-3 and emphasizing the presence of less unsaturated fatty acids such as 18:2n-6 in long-lived animals, without changing the total amount of polyunsaturated fatty acids. This leads to lower levels of lipid peroxidation and lipoxidation-derived protein modification in long-lived species. Taken together, available information is consistent with the predictions of the mitochondrial free radical theory of aging, although definitive proof and many mechanistic details are still lacking. *Antioxid. Redox Signal.* 8, 582–599.

INTRODUCTION

WHY AND HOW does aging occur? This is a most interesting scientific question. A large number of theories of aging have been proposed (86). However, any appropriate theory must explain four main characteristics of aging (144): it is progressive, endogenous, irreversible, and deleterious for the individual. Stressing the endogenous character of aging is useful to distinguish between maximum lifespan and survival, whose meanings are, unfortunately, too frequently mixed. Aging comes from endogenous sources, and the rate of aging of different animal species, and thus their maximum lifespan potentials (MLSP), are mainly determined by their genes, not by the environment. However, the mean lifespan is mainly determined by the environment and to a lesser extent by the genotype.

Today, the Free Radical Theory of Aging (FRTA; 46, 48) is the one most supported by experimental data (reviewed in 3), although dissenting opinions about it also exist (81). The FRTA fits well with the four characteristics of aging stated above. According to this theory, free radicals are endogenously produced at mitochondria under normal physiological conditions, they are produced continuously throughout life, and their deleterious effects on DNA are irreversible due to accumulation of somatic mutations during aging in postmitotic tissues. Furthermore, the FRTA can explain most of the observations upon which other theories are based, including the accumulation of crosslinks and waste products like lipofuscin during aging, the rate of living, and the somatic mutation theories of aging.

In this review we present available data concerning antioxidant defenses, mitochondrial oxygen radical generation, tissue fatty acid unsaturation, methionine content of proteins, and oxidative damage to macromolecules, in vertebrates with widely different maximum longevities and subjected or not to calorie restriction. Recent investigations finding similar

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mechanisms of decrease in mitochondrial oxidative stress in caloric and protein-restricted animals are also described. All these evidences are consistent with the predictions of the FRTA.

OXIDATIVE STRESS AS RESPONSIBLE FOR AGING RATE

Carl Wilhem Scheele was the first to describe the negative effects of oxygen on living beings back in the XVIIIth century. However, a scientific explanation of the cause of these harmful effects was lacking during the next 200 years. It is known nowadays that the harmful effects of high oxygen tensions on biological systems are due to the generation of reactive oxygen species (ROS) during cellular metabolism. The free radical theory of oxygen toxicity was first proposed by Rebeca Gerschman and colleagues in 1954 (40) by linking the effect of exposure to hyperbaric oxygen with alterations produced by ionizing radiation and with the concept of univalent oxygen reduction. Denham Harman was the first author who linked oxidative stress with aging. In his seminal article about the free radical theory of aging (46), Harman postulated that, even at normal oxygen tension, oxygen utilization causes tissue damage due to free radicals. The free radical theory of aging did not start to be widely considered until the discovery of the enzyme superoxide dismutase in 1969 by McCord and Fridovich (84). The presence in living things of an enzyme which decomposes an oxygen radical led to serious consideration of the role of free radicals in biology. Since then, the interest on free radical research has exponentially increased and today free radicals are considered by many as directly responsible or implicated in aging, cancer, diabetes, and many other degenerative diseases.

Univalent oxygen reduction generates three different ROS: superoxide radical, hydrogen peroxide, and hydroxyl radical (92). The hydroxyl radical is the most reactive ROS and can be generated due to the chemical interaction of superoxide radical with hydrogen peroxide in the presence of iron or copper during the Fenton-Haber-Weiss reaction. Hydrogen peroxide is not a free radical, and this allows it to diffuse away from the sites of ROS production to distant places in cells and tissues. Reaching those places, it generates there the hydroxyl and other reactive radicals thus propagating oxidative damage. Other ROS relevant for oxygen toxicity are the perhydroxyl radical, nitric oxide, and singlet oxygen. The possible role of these last radicals in aging is not so well understood. Nitric oxide plays a prominent role in controlling a variety of organ functions including the immune, cardiovascular, reproductive, and nervous systems. The inducible form of nitric oxide synthase is not normally present in the brain in youth but it can be detected in the brain after inflammatory, infectious or ischemic damage, as well as in the normal aging brain, supporting the idea of a possible role for this enzyme in aging (160). Various recent studies also suggest that a strong link between inflammation and aging exists (146). ROS are generated at various sites and situations in living cells and the exact mechanisms of production and regulation are still not fully known (reviewed in 32). However, in the absence of pathology and external oxidative stress, ROS are mainly produced in the mitochondrial electron transport chain both in complex I and complex III (11, 51, 93).

ROS can damage all kinds of cellular macromolecules including lipids, proteins, and DNA. Quantitatively, however, lipid peroxidation is a major oxidative process in tissues. This is mainly due to the strong chemical sensitivity to ROS-induced damage of polyunsaturated fatty acids (PUFA). This sensitivity exponentially increases as a function of the number of double bonds per fatty acid molecule. Moreover, lipid peroxidation not only damages the lipids, because its final products like malondialdehyde and others, as well as secondary radicals generated during the process, can also alter tissue proteins and DNA. Studies performed during the last years show that long-lived animal species have an intrinsically low degree of unsaturation of their tissue fatty acids (101), and this protects their membranes, the lipids, and other macromolecules against oxidative damage (see below).

ANTIOXIDANTS: THE INCOMPLETE PROTECTION OF CELLS

Cellular protection against oxidative damage includes both elimination of ROS and repair of damage. Antioxidants constitute a first line of defense contributing to maintenance of the cellular oxidative stress homeostasis. They can be classified in two categories: enzymatic and nonenzymatic antioxidants.

Direct ROS scavenging antioxidant enzymes are superoxide dismutase (SOD), glutathione peroxidase (Gpx), and catalase (CAT). SOD converts the superoxide radical to oxygen and hydrogen peroxide. There are different types of SOD in different cellular compartments: MnSOD in the mitochondrial matrix, Cu/Zn SOD in the cytosol and the intermembrane space, and a different Cu/Zn SOD in the extracellular compartment. Although SOD eliminates superoxide radical, it is not a complete antioxidant because it produces hydrogen peroxide. Two different but kinetically complementary enzymes eliminate H_2O_2, CAT and Gpx. CAT is most useful to eliminate high concentrations of hydrogen peroxide because it decomposes H_2O_2 at high rates and shows low affinity for the peroxide. Conversely, Gpxs (both the selenium- and nonselenium-dependent forms) are most functional at low hydrogen peroxide concentrations, since they have high affinity and low rates of catalysis.

Besides antioxidant enzymes, different kinds of endogenous nonenzymatic molecules cooperate to maintain cellular oxidative stress homeostasis. After reacting with ROS, these molecules are oxidized, and they must be reduced back by other molecules to regain antioxidant capacity. Their low molecular weight allows them to remove ROS at sites where the larger enzymes do not have access. Glutathione (GSH) and ascorbate are two main nonenzymatic antioxidants in the hydrophilic compartment. The antioxidant activity of GSH resides in the reduced thiol group of its cysteine residue. Glutathione can reacting directly with ROS or can act as a cosubstrate of Gpx enzymes. Oxidized glutathione (GSSG) can also play an important role in the regulation of protein func-

tion. Glutathionylation of enzymes has been described as a new and important way to control protein activity in cells (8). Furthermore, it has been recently described that glutathionylation of complex I increases its rate of ROS production (149). Moreover, the GSSG/GSH ratio positively correlates with the extent of mtDNA oxidative damage (30), also supporting a role for thiols in the regulation of ROS production in complex I. Another thiol-related redox-active substance is the protein thioredoxin (89). Thioredoxin has a redox-active disulfide/dithiol at the active site that regulates transcription factors like nuclear factor kappaB and AP-1. It is induced by various oxidative stresses and is translocated to the nucleus. Thioredoxin is cytoprotective against oxidative stress by scavenging ROS in cooperation with peroxiredoxin/thiredoxin-dependent peroxidase (89).

Following glutathione, ascorbate is the next most abundant reduced nonenzymatic antioxidant inside cells. In most vertebrates it is endogenously synthesized (although not in human beings), and it is maintained at levels as high as 1 m*M* in tissues. After reacting with ROS, the oxidized form of ascorbate must be reduced back again throughout NADPH-, GSH-, or NADH-dependent reductases. Tocopherols and carotenoids act inside lipophilic environments. The tocopherol vitamin E acts on lipid peroxyl groups inside membranes reducing them to hydroperoxides, thus inhibiting the propagation of lipid peroxidation. Vitamin E also reduces lipid alcoxyl radicals to lipid alcohols. Vitamin E can be recycled back to its reduced form by ascorbate or ubiquinone. Carotenoids quench singlet oxygen, and interact with other ROS at physiological tissue oxygen partial pressures. A most important carotenoid is coenzyme Q. It is a hydroquinone synthesized and present in all cellular membranes. Its antioxidant activity is exhibited through scavenging of lipids radicals or reduction of vitamin E radical. The regeneration of coenzyme Q is performed by reductase enzymes that use NADPH or NADH as cofactors.

ANTIOXIDANTS CAN NOT DETERMINE LONGEVITY IN VERTEBRATES

The level of oxidative stress can be controlled in principle by the rate of free radical generation or by the rate of free radical scavenging. But this does not necessarily mean that both factors act at the same level in the living organism. For example, different kinds of studies indicate that antioxidants do not determine maximum lifespan at least in vertebrates, and it seems that ROS production and not scavenging can be involved in the control of the rate of aging.

There are two general kinds of studies concerning antioxidants and longevity, and both indicate that antioxidants do not control maximum lifespan in vertebrates. Studies comparing animals of different longevities show that endogenous antioxidants, both enzymes and low molecular weight antioxidants, are negatively correlated with maximum longevity (reviewed in 113). These negative correlations constitute the strongest, although indirect, evidence available to date that oxygen radical generation in tissues *in vivo* under normal conditions must be lower in long-lived than in short-lived species.

Moreover, studies that increase antioxidants levels in tissue by dietary supplementation, injection, pharmacological reactive induction, or genetic manipulation, generally show that they do not increase maximum lifespan in vertebrates (Tables 1 and 2). Classic studies treating mammals with the spin trap phenyl-N-tert-butylnitrone indicate that it can decrease tissue oxidative stress (97) and extend lifespan (127). But in most dietary studies the antioxidants did not change maximum lifespan, while a few of them showed very small marginal increases in longevity (50, 88, Table 1). Moreover, these increases were only observed in investigations in which the MLSP of the control animals was under 3 years, rather small for the studied rodents, whereas in studies in which the maximum lifespan of controls was higher than 3 years there was no effect on MLSP. The most frequent result observed was an increase in mean lifespan. In most reports this effect was due to a decrease in early death provoked by a lower incidence of disease (47). Furthermore, replicates in different groups of animals showed that antioxidant supplementation increased mean lifespan when survival of the controls was suboptimal, whereas when it was optimal the antioxidants were without effect (68). Antioxidants never increased maximum lifespan beyond the values of controls reared under optimum conditions. Moreover, antioxidants never increased longevity beyond the maximum described for the species. These results suggest that antioxidant can protect against increased oxidative stress from pathological situations or exogenous damage without changing aging rate. This is confirmed in models in which transgenic mammals overexpressing different kinds of antioxidant enzymes show increased resistance to oxidative stress (Table 2), but no increase in maximum longevity has been reported. A recent report described 27% increases in the mean lifespan of mice after catalase overexpression in mitochondria, but the increase in maximum lifespan was not stated and the survival curves shown suggest that it was a 10% increase or less (130b). A 10% increase in maximum longevity seems very small for a 50-fold increase in mitochondrial catalase. Moreover, small increases in maximum longevity (4%–12%) were also observed in very few of the many old studies on life-long antioxidant feeding in rodents (Table 1), suggesting that perhaps random statistical effects on the small number of animals left at the very last period of the lifespan could be responsible for them. In the case of *Drosophila melanogaste*r, early studies found increases in maximum longevity after combined SOD plus catalase overexpression (not individually), but this was not found in later studies when longer-lived control strains were used (99). It is also possible that overexpression of antioxidant enzymes is more important in short-lived very active insects than in mammals. On the other hand, nutritional antioxidants are physiologically regulated at cell level and may also interfere with natural cell signaling. In animals with overexpressed SOD, for instance, other proteins and substrates may be affected. Without a gene array, one cannot be completely certain that potential confounding compensatory mechanisms are not involved. These compensations can affect mean lifespan and the incidence of chronic diseases. In summary, antioxidants may not have the capacity of caloric restriction or IGF-I/insulin disrupt signaling to decrease the aging rate in rodents (reviewed in 77). Antioxidant defenses seem to be

Table 1. Effect of Artificial Increases in Antioxidant Levels on the Mean and Maximum Longevity of Vertebrate Animals

Species	*Antioxidant increased*	*Survival curve*	*Effect on mean lifespan*	*Effect on maximum lifespan*	*MLSP of controls (yrs)*	*References*
BALB/c mice	BHT	Yes	Increase (22%)	NC	3	24
C3B1ORF1 mice	ETO+MEA	Yes	NC	NC	3.6	49
C57BL/6J mice	MEA	Yes	NC	NC	2.8	68
LAF1 mice	MEA	Yes	NC	NC	3.3	47
LAF1 mice	MEA	Yes	Increase (12%)	NC	2.2	47
LAF1 mice	BHT	Yes	Increase (31%)	NC	2.2	47
LAF1 mice	CYS, PG, DTBH or HNCL	No	NC	NC	2.2	47
C3H mice	ETO	Yes	Increase (19%)	NI	1.9	25
BC3F1 mice	MET	Yes	Increase (13%)	Increase (12%)	2.8	50
C3 mice	α-Tocopherol	Yes	NC	NC	3	73
CD1 mice	Mixture (a)	Yes	NC	NI	NS	148
Fisher 344 rats	Deprenyl	No	Increase (2%)	Increase (4%)	2.7	88
Wistar rats	α-Tocopherol	Yes	NC	NC	2.8	115
Frogs	GSH, GSH-Red	Yes	Increase	NC	6	78
C57BL/6 × C3H ($B6C3F_1$)	coenzyme Q α-lipoic acid	Yes	NC	NC	3.8	74

ASC, ascorbate; BHT, butylated hydroxytoluene; CYS, cysteine; DTBH, 2,6-di-*tert*-butyl hydroquinone; ETO, ethoxyquin; GSH, glutathione; GSH-Red, GSH reductase; HNCL, hydroxylamine hydrochloride; MEA, 2-mercaptoethylamine; MET, 2-mercaptoethanol; MLSP, maximum lifespan; NI, not investigated; NC, no change; Yes, shown; No, not shown; PG, propyl gallate; SOD, superoxide dismustae; TZC, thiazolidinecarboxylic acid; (a) mixture of antioxidants: α-tocopherol+BHT+ascorbic acid+DL-methionine+sodium selenite.

optimized *in vivo* in such a way that any additional expression is unable to increase protection against aging-related oxidative damage, whereas they are useful when oxidative damage is higher than normal.

Overexpression and knockout models of antioxidant enzymes in invertebrates are controversial and it is very difficult to reach a general conclusion. However, most studies discard a positive effect of antioxidants on aging rate (100). Experiments performed in knockout mouse models for different antioxidant enzymes have recently confirmed that antioxidants do not control directly maximum lifespan, although they have an important influence on resistance against oxidative stress (reviewed in Table 2). Some studies show that null mutations in the glutathione peroxidases or Cu/Zn SOD genes do not change significantly lifespan in mice (85, 123). Other studies in which the lifespan of controls is longer (indicating better rearing conditions) show that maximum lifespan is decreased in homozygous, but not in heterozygous mutants (35). Thus, null mutations in Gpx 1 and heterozygous for Sod2 ($Gpx1^{-/-}$, $Sod2^{+/-}$) are viable, although more sensitive to different kinds of oxidative stress (154), and this increase in sensitivity to oxidative damage is due to the decreased expression of antioxidants enzymes. The null mutation in the mitochondrial MnSOD (72) leads to death, probably due to increased oxidative stress and neural cell death (112). Knocking out Gpx 4 (162) also induces lethality in neonates. So these models do not inform about the role of these enzymes in longer-term aging. However, there is another interesting model to study the possible linking of MnSOD to aging. The knockout mice heterozygous for MnSOD show a 30%–80% reduction in SOD activity without any compensatory overexpression of other antioxidant enzymes (155, 156). In this study mitochondrial DNA showed increased levels of oxidative damage (estimated as the level of 8-oxo-7,8-dihydro-2′-deoxyguanosine: 8-oxodG). This increase was linked to increases in cancer rate in heterozygous mice, but the lifespan of the knockout mice was not different from that of controls, suggesting that oxidative damage to mtDNA could affect cancer but not the aging rate. The results obtained in this animal model do not agree with the mitochondrial free radical theory of aging, or at least with a direct involvement of mtDNA oxidation in the aging process. A possible explanation is that different types of oxidative damage are generated in cells leading to different effects. 8-oxodG, for instance, can be easily repaired, but free radicals also produce permanent alterations in mitochondrial DNA such as large deletions which increase during aging. It is not known if those mutations are increased in the heterozygous MnSOD knockout mice. In fact, a recent study further contributes to support a role for mtDNA in aging (151). In this study the rate of mtDNA mutations was increased in mice by inducing a homozygous mutation in the mitochondrial DNA polymerase PolgA, and the animals showed phenotypic signs of accelerated aging. Apart from antioxidants, other factors like stress proteins have been recently linked to aging. Thus, caloric restriction, a well-known antiaging intervention increases heat shock proteins in rat tissues (131). The link between oxidative stress, pathology, and aging is now well recognized clinically in the brain (114), while aging in muscle and heart could affect mean lifespan and chronic disease to a greater extent than maximum life-

Table 2. Effects of Increasing and Decreasing Tissue Antioxidant Enzymes *In Vivo* on MLSP: Transgenic/Knockout Mutants in Mice

Mutant	*Effect on maximum longevity*	*Other effects*	*Reference*
Overexpression			
CuZnSOD	None		61
Catalase (in nuclei)	Not reported	No increase in protection against oxidative damage	130a
Catalase or Catalase/ CuZnSOD on ApoE$^{-/-}$ mice	Not reported	Protection against oxidative stress	161
Catalase	Not reported	Hepatocytes and fibroblasts more resistant H_2O_2, but not to paraquat or TNF-α. Mice more sensitive to gamma radiation	22
Catalase (in mitochondria)	Increase of around 10% or less		130b
Gpx4	Not reported	More resistant to oxidative stress-induced apoptosis	124
Knockout			
Homozygous extracellular SOD	Viable at least 14 months	Increased sensitivity to oxidative stress	18
Homozygous CuZnSOD	Not reported	Increased sensitivity to axonal injury	122
Homozygous Gpx 1	Viable at least 20 months		56
Heterozygous MnSOD	No reported	No alteration in sensitivity to oxidative stress in heart	152
Homozygous CuZnSOD	No reported	Neuronal alterations	133
Homozygous MnSOD	Neonatal death	Antioxidant administration slightly increased longevity	87
Homozygous Gpx 4	Embryonic death		162
Heterozygous Gpx 4	No reported	More sensitive to oxidative stress	121
Heterozygous MnSOD	None	Increase in tumour incidence	156
Homozygous Gpx1/ Heterozygous SOD2	Viable at least 28 months	More sensitive to oxidative stress	154
Homozygous Catalase	No reported	Brain mitochondria more sensitive to oxidative damage	57
Heterozygous CuZnSOD	None		35
Homozygous CuZnSOD	Decrease in mean and maximum lifespan	Increase in liver tumor incidence	35

span. Diseases causing premature aging such as Werner's syndrome (98) and many mitochondrial diseases also have brain and neurons as important targets. In summary, studies comparing constitutive antioxidant levels between mammalian species, and experiments increasing or decreasing their tissue antioxidant concentrations in different ways, consistently indicate that antioxidants do not seem to control aging rate, although they can protect against different pathologies and early death. However, new mitochondrial antioxidant defenses have been recently described (20) and new strategies of antioxidant overexpression are being tested, for instance catalase overexpression targeted to mitochondria (22). The results of these and similar studies will be further informative and will allow more solid and definitive conclusions to be reached.

MITOCHONDRIAL FREE RADICAL PRODUCTION AS A MAIN DETERMINANT OF VERTEBRATE LONGEVITY

The negative correlation between antioxidants and MLSP indicates that free radical production, not scavenging, mechanistically connects oxidative stress to aging (3). A general compensation between free radical production and scavenging must occur in both short- and long-lived animals, allowing tissue homeostasis and thus survival in both cases; the cellular ROS turnover must be high in the first and low in the second case. But, if short-lived animals have high ROS production and high antioxidant levels, whereas long-lived animals have low ROS production and low antioxidant levels, why they do

not age at the same rate? This paradox can be explained if the high rate of ROS production of short-lived animals leads to a higher local concentration of ROS near the sites of ROS generation and near targets relevant for aging like mitochondrial DNA, when compared to long-lived animals. This is probable taking into account the proximity and even contact of mtDNA with the source of ROS generation at the inner mitochondrial membrane. Steady-state levels of 8-oxodG in mtDNA negatively correlate with longevity in the heart and brain of mammals (5), and in various comparisons between mammals and birds, in agreement with such hypothesis.

The relationship between mitochondrial free radical production and longevity has been studied many times. Most studies show a negative relationship between mitochondrial ROS production and MLSP. In different studies a negative exponential correlation has been found between ROS (superoxide and hydrogen peroxide) production and MLSP in different mammal tissues (reviewed in 137). However, these studies only included animal species following the rate of living theory, so the results obtained could also be interpreted as a correlate of that phenomenon. Species with short MLSP could show high mitochondrial ROS production simply because their rates of mitochondrial oxygen consumption are higher. Many biochemical reactions apart from oxygen consumption occur at an accelerated rate when the metabolic rate is high, and some of them, unrelated to ROS production, could also be in principle the ones responsible for the accelerated aging rate of the short-lived animals.

Fortunately, comparative physiology offers more possibilities to test the hypothesis that mitochondrial ROS production is involved in aging. Three groups of homoeothermic vertebrates have an extraordinarily high longevity in relation to their body size and metabolic rate: birds, bats, and primates. Birds can be a strong test to the mitochondrial theory of aging, because if a low rate of ROS production contributes to slow the aging rate, the mitochondria of birds should show a low rate of ROS generation in spite of the high rate of oxygen consumption of these animals. It was indeed found that the heart and brain mitochondrial production of hydrogen peroxide was lower in pigeons (MLSP = 35 years) that in rats (MLSP = 4 years), even though their metabolic rates and body sizes are of a similar magnitude (4). The difference in ROS production between these two species occurred at only one of the electron transport complexes: the NADH-quinone oxidoreductase or complex I (51). Analogous comparisons in other species of birds and mammals led to similar results (52). Furthermore, other interspecies comparisons between rodents with widely different longevities but similar body size (135), or between rodents and bats (17) also showed that the shortest-lived species had lowest mitochondrial ROS production. All the comparative investigations performed in mammals and birds show that mitochondrial ROS generation is lower in long- than in short-lived animals, in agreement with the mitochondrial theory of aging. If mitochondrial oxygen radical production controls aging rate, the rate of ROS production of animals with different metabolic rates and body sizes but similar longevity should be equal. This is exactly what happened when mitochondrial ROS production of mouse (MLSP = 3.5 years) and rat (MLSP = 4 years) were compared (51, 52).

On the other hand, in comparative studies in general the difference in mitochondrial ROS production between species is smaller than their difference in MLSP. This suggests that there are other causes of aging apart from ROS production. One of these alternative causes may be the control of aging by a highly conserved IGF-I/insulin-like signaling system. Mutations in this system can increase longevity in a wide array of animal species including yeast, nematodes, insects, or mammals (reviewed in 67). Although some studies indicate that insulin could directly affect ROS production (70), other results do not confirm this relationship (129). Furthermore, most studies indicate that the longevity-extending effects of IGF-I/insulin signaling disruption and caloric restriction in dwarf mice are independent of each other (7). Thus, present information suggests that ROS production and IGF I/insulin-like signaling can control aging through different mechanisms.

COMPLEX I ROS GENERATION AND AGING

It is widely accepted that both complex III (93) and complex I (51) produce ROS in mitochondria. However, the differences in ROS production between species of different longevities and between caloric restricted and *ad libitum*-fed animals seem to come exclusively from complex I (reviewed in 3). The respiratory complex responsible for the lower ROS generation of pigeon compared rat mitochondria is complex I, not complex III, because the difference in ROS production between both animal species with succinate as substrate disappears after the addition of rotenone. Further studies localized the ROS generator in the electron pathway inside complex I between the ferricyanide reduction site and the rotenone binding site. This discards the flavin, and suggests that the source of ROS may be one of the FeS clusters situated in the hydrophilic domain of complex I facing the matrix (54).

Studies comparing caloric restriction *versus ad libitum*-fed animals reinforce the conclusions obtained in comparative investigations. Caloric restriction is a well-known experimental manipulation that increases MLSP in many different animal species (138). It is most interesting that the oxidative stress-related parameter that varies between species also changes in the appropriate direction after subjecting rodents to caloric restriction. Caloric restriction significantly decreased the rate of mitochondrial ROS generation in the majority of the studies performed in rodents (9, 42). On the other hand, neither the expression (159) nor the activity (136) of the antioxidant enzymes SOD, catalase, or Gpx are modified in a consistent way by caloric restriction. These results show again that the parameter connecting oxidative stress to aging is the rate of mitochondrial ROS generation, not their rate of elimination.

In many studies, caloric restriction did not act by suppressing increases in ROS production with age (42, 79). Instead, mitochondrial ROS production was strongly decreased by caloric restriction below the basal levels of young animals fed *ad libitum* (42, 79). This decrease can be one the fundamental mechanisms responsible for the slow aging rate of caloric-restricted animals. Strikingly, it was found that the ROS gen-

erator responsible for the decrease in ROS production of caloric-restricted animals was situated in the same respiratory complex as in long-lived animals: at complex I. The mechanism allowing the decrease in ROS production during caloric restriction was not a simple decline in mitochondrial oxygen consumption since this parameter did not change. The results indicate that that mechanism is related to the degree of reduction of the complex I generator, because the lower ROS production of the restricted animals disappeared after fully reducing complex I. Strikingly again, the same was found in studies of animals with different longevities. Various potential mechanisms can be responsible for the decrease in ROS production of caloric restricted animals. One possibility is the differential expression of selected subunits leading to a more efficient complex I (with a lower ROS production). This hypothesis is supported by the large number of regulatory subunits of complex I. Another possibility is that the degree of phosphorylation or glutathionylation of the complex could be different in caloric restricted animals. These two protein regulatory mechanisms have been reported to control complex I ROS production (120, 149). It is interesting that complex I seems responsible for the differences in ROS production in the different models of aging studied, since it is also the mitochondrial respiratory complex with more subunits (seven) codified by mitochondrial DNA. Since mitochondrial DNA is specially sensitive to oxidative damage, complex I is specially sensitive too because the complex I subunits codified by mtDNA are essential to assemble a functional complex.

IS MITOCHONDRIAL DNA THE NEXUS BETWEEN ROS PRODUCTION AND AGING RATE?

Although free radicals attack all kind of macromolecules, DNA is specially important for aging. Carbohydrates, lipids, and proteins can be repaired or replaced when they are damaged, but when all cellular copies of a gene are seriously altered or deleted, the function codified in it is lost forever. ROS can attack DNA directly at the sugar-phosphate backbone or at the bases, producing many different oxidatively modified purines and pyrimidines and single and double strand breaks due to hydrogen abstraction or radical addition. Oxidatively modified bases are present in animal tissues even in healthy animals. Some of base adducts, like the most commonly used marker of DNA oxidative damage 8-oxodG, can cause DNA mutations during DNA replication or repair.

Mitochondrial DNA has two important characteristics in relation to the mitochondrial FRTA: (a) it is situated very close to the main site of ROS production, the inner mitochondrial membrane; and (b) homoplasmic mtDNA mutations lead to life-long detrimental consequences in postmitotic cells of aging organisms. Long-lived animals produce less ROS per unit time in their mitochondria than short-lived ones, and as consequence they should have lower steady-state levels of oxidative damage in their mtDNA. When 8-oxodG was measured in the mtDNA of heart and brain of eight different species of mammals differing by 13-fold in MLSP, a negative correlation between mtDNA oxidative damage and MLSP was found (5). In the same study, no correlation was found between 8-oxodG and MLSP in nuclear DNA (nDNA). The 8-oxodG concentration was also lower in the heart and brain mtDNA of long-lived birds compared to short-lived mammals, and again no differences were found in nDNA (53). Moreover, caloric restricted animals have lower levels of oxidative damage in their mitochondrial DNA than *ad libitum*-fed animals (42, 79). Recent data (39) show that the rate of urinary excretion of oxidized nucleotide bases also correlates negatively with MLSP (39), strongly agreeing with the similar kind of correlation previously described in tissues (5). All these results support the presence of a causal relationship between mitochondrial ROS production, oxidative DNA damage, and aging rate.

Available results indicate that oxygen radicals damage more intensively mitochondrial than nuclear DNA, and that the higher the rate of mitochondrial ROS generation, the higher is the steady- state level of oxidative damage in mtDNA. Low rates of ROS production are associated with low 8-oxodG steady-state levels in mtDNA, indicating that the flux of oxidative damage (attack and repair) through the mtDNA of caloric restricted or long-lived animals is lower than that of *ad libitum*-fed controls or short-lived animals (3). This idea is consistent with decrease in the activity of base excision repair in mtDNA induced by caloric restriction (145). Thus, the rate of ROS generation seems to be the most important determinant of mtDNA oxidative damage.

Although, 8-oxodG is present both in nDNA and mtDNA, its level is several fold higher in the last one (5, 45). A most probable reason for this is that mtDNA suffers higher levels of free radical attack than nuclear DNA because it is situated very close to a main site of free radical generation, the inner mitochondrial membrane. Experimental data discard differences in repair of 8-oxodG between nuclear and mitochondrial DNA as an alternative explanation (1). Other differences, such as the lack of introns in mtDNA, would amplify the detrimental consequences of the relatively higher level of oxidative damage to mtDNA. Since genetic information is tightly packed in the mtDNA of mammals, most mutational changes should have injurious effects.

Some investigations have found that the steady-state levels of 8-oxodG increase during aging both in nDNA and mtDNA in different tissues of rodents and humans (45), however this increase is not found in other studies except in very old animals (42). While oxidative damage seems to increase during aging, it is not clear if this damage is enough to account for the process of aging. Such increases in steady-state oxidative damage in old individuals are quantitatively limited due to presence of many repair systems. In addition to oxidized bases, ROS can generate many other kinds DNA modifications including DNA breaks leading to mutations like large deletions. Once formed, these modifications do not have any sign attesting their oxidative origin. However, they have important consequences for aging, for instance, mutations provoke loss of function or genome instability (157). DNA mutations in postmitotic cells can be of paramount long-term relevance, since, differing from damaged lipids and proteins, they cannot be repaired.

Many investigations have shown that mtDNA mutations increase with age in mammals. The rate of accumulation of mi-

tochondrial DNA mutations with age is higher in mice than in humans (158). This means that the rate of accumulation of those mtDNA mutations is much faster in short-lived than in long-lived animals, in agreement with their differences in rate of mitochondrial ROS rate production. Thus, the rate of mitochondrial ROS production of each animal species seems to determine its rate of ROS attack and oxidative damage to mtDNA, and then its rate of accumulation of mtDNA mutations and aging rate (3). Such a model fits well with available results of comparative, caloric restriction, and age-related studies. The accumulation of somatic mtDNA mutations during aging would be a main cause of detrimental changes observed in the aging organism, according to the mitochondrial FRTA. However, there are many open questions concerning this hypothesis. Somatic mtDNA mutations accumulate in aged tissues as mosaics of mutated and non-mutated cells (16). MtDNA mutated cells of old tissues usually show a predominant single kind of mutation, which is different in each mutated cell. Since ROS can cause many different kinds of DNA changes non-specifically, why only one kind of mutation finally accumulates? Clonal expansion of mutated mtDNA in individual cells seems to be responsible for this phenomenon, provided that the mutated mitochondria do not continue to produce ROS. On the other hand, the absolute level of accumulation of mtDNA mutations in post-mitotic tissues during aging seems too small to directly produce aging through deficits of mitochondrial energy generation (29), although decreases in maximum oxygen consumption in association with increases in mtDNA deletions haven been recently reported (44). But it is possible that a relatively small number of mutations can cause important deleterious effects by other mechanisms during aging in the cells harboring the mutations, in nearby cells of the same tissue, or even in cells situated far away in other organs (28). Furthermore, alterations in nuclear DNA should not be discarded as cause of aging. Can mutated mitochondria affect in some way the large amount of information coded in nuclear DNA? Further research can resolve these and other related issues.

PROTEIN RESTRICTION, METHIONINE, AND LONGEVITY

The studies described above show that caloric restriction decreases mitochondrial free radical production and oxidative DNA damage. These results connect caloric restriction with the mitochondrial free radical theory of aging. They offer a plausible mechanism by which caloric restriction could slow down the rate of aging, by decreasing oxidative damage and long-term accumulation of mutations in mtDNA (3). However, it has not been investigated until recently if the decreases in mitochondrial production of reactive oxygen species (ROS) and oxidative DNA damage during caloric restriction are due to the reduction in calories themselves or to decreases in the intake of specific dietary components. A study has shown that restricting by 40% the dietary intake of protein without strongly decreasing the caloric intake lowers mitochondrial ROS production at complex I and oxidative damage to mitochondrial DNA in the liver of a mammal (128). These decreases were of similar magnitude as the ones observed in caloric restriction, also occurred with an increase in the efficiency of the respiratory chain to avoid free radical leak, and the mechanism involved was also related to the degree of electronic reduction of the free radical generator. In other words, the protein restriction results perfectly mimicked those observed in caloric restriction.

It is generally believed that the antiaging effect of caloric restriction is due to the decreased intake of calories rather than to specific dietary components, although variations in the proportions of the main dietary constituents seem also to affect longevity (2, 82). Concerning the effects of protein restriction on aging, consideration of published studies performed in mammals shows that increases in longevity have been found much more commonly than no effects or decreases. Some authors found that decreasing the casein content of the diet from 42% to 18% decreased the survival of male rats (27). However, many other investigators found the contrary. Thus, low protein diets increased the lifespan of rats (6), increased the mean and maximum lifespan of Fisher 344 rats (59, 166), increased the lifespan of C57BL/6J and hybrid F1 mice (41,76), prolonged life expectancy in BALB/c mice (143), and increased the lifespan of DBA/2f mice (37). In some of these studies the diet was restricted only with respect to protein (37), as in the experiments measuring ROS production and mtDNA damage (128), whereas in other cases the decrease in protein was balanced by an increase in dietary carbohydrate (59). In some studies it was observed that the life extension effect (15%) of the low protein diets was smaller than that of caloric-restricted diets, and it was concluded that the retardation of aging was due to restriction of energy intake rather than to a specific nutrient (83). Other authors have found that protein restriction can increase the lifespan of Wistar rats by as much as 25% (6). Taken together, these data suggest that protein restriction can be responsible for part of the life prolonging effects of caloric restriction. Caloric and protein restriction share many common effects in addition to life prolongation, including delays in puberty, decreases in growth rate, changes in metabolic rate, boosting of cell-mediated immunity, lowering of cholesterol, or decreases in preneoplastic lesions and tumors (163, 164). Low protein diets also decrease IGF-1 levels and decelerate glomerulosclerosis in mice (31), delay the occurrence of chronic nephropathy and cardiomyopathy in rats (80), and protect rat liver against exposure to toxic chemicals (125). A lower but significant life extension effect in protein than in caloric restriction would agree with the widely accepted notion that aging has multiple causes. Restriction of protein intake can be responsible for part of the aging-delaying effect of caloric restriction by decreasing mitochondrial ROS production and oxidative DNA damage (128). The remaining effects of caloric restriction on aging rate could be related to decreases in other dietary components or in the calories themselves through different additional mechanisms. In any case, it is most interesting that protein restriction can decrease mitochondrial oxidative stress and aging rate, because this dietary manipulation does not imply the strong behavioral stress of caloric restriction and thus seems a more feasible option for adult humans.

On the other hand, it is well known that methionine dietary restriction increases MLSP in mammals (124, 167). A plausible mechanism by which caloric and protein restriction extends lifespan is by decreasing mitochondrial ROS production (3, 128). Thus, it is possible that methionine restriction can be responsible for the decrease in ROS production observed both in protein and in caloric restriction and for the ensuing decrease in aging rate. This possibility warrants further investigation. Moreover, we have recently found that the methionine content of cardiac proteins is negatively correlated with mammalian MLSP in a strong way, and this does not occur for any other amino acid (unpublished results). In agreement with this, methionine content of skeletal muscle proteins is also lower pigeons than in rats (119). These are striking findings, since many recent investigations point to a relationship between methionine and aging (91, 126, 142, 143). There are various possible mechanisms by which a high methionine content could induce damage. Methionine residues of proteins are the most susceptible to oxidation by ROS (91, 134) and sensitivity of proteins to oxidative stress increases as a function of the number of methionine residues in the protein (141).

Methionine dietary supplementation also increases iron and lipid peroxidation in rat liver (90). Oxidation of methionine residues generates methionine sulfoxide in proteins which deprives proteins of their function as methyl donors and may lead to loss of their biological activity (19, 23). However, this modification can be repaired by methionine sulfoxide reductase in a thioredoxin-dependent reaction. In this context it is most interesting that knocking out methionine sulfoxide reductase-A lowers MLSP and increases protein carbonyls and sensitivity to hyperoxia in mice (91). The opposite manipulation, overexpression of methionine sulfoxide reductase A has been reported to increase lifespan and to delay the start of the aging process in *Drosophila* (126). On the other hand, the oxidized form of thioredoxin produced in the reduction of methionine sulfoxide can be converted back to reduced thioredoxin by the enzyme thioredoxin reductase. In agreement with the methionine oxidation hypothesis above, overexpression of thioredoxin reductase seems to increase longevity in mice (89, 94).

A high methionine content could also be detrimental because it is sequentially converted to and increases the levels of *S*-adenosyl-methionine and homocysteine (90). Homocysteine levels increase with age in humans and they are a risk factor for aging and free radical-associated pathologies including atherosclerosis, thrombosis, cancer, stoke, wasting, chronic kidney disease, and Parkinson's disease (32, 33, 38, 96). Homocysteine has a free thiol group that can be readily oxidized leading to protein mixed disulfides or thiol bridges between proteins. On the other hand, *S*-adenosyl-methionine is a potent alkylating agent by virtue of its destabilizing sulphonium ion. The methyl group of *S*-adenosyl-methionine is subject to attack by nucleophiles and is thus strongly reactive (75). Interestingly, long-lived Ames dwarf mice have altered methionine metabolism including lowered tissue *S*-adenosyl-methionine levels (153). All these data point to a possible relationship between protein and caloric restriction, protein methionine levels, oxidative stress, and longevity that should be further investigated.

BIOLOGICAL MEMBRANES AND UNSATURATED FATTY ACIDS

Biological membranes generally consist of bilayers of amphipathic molecules held together by noncovalent bonds. In eukaryotic cells, phospholipids are the predominant membrane lipids and consist of a hydrophilic head group with attached hydrophobic acyl chains. Phospholipids play multiple roles. They constitute a permeability barrier, provide a matrix for the assembly and function of a wide variety of catalytic processes, act as donors during the synthesis of macromolecules, and modulate the functional properties of membrane-associated activities. The wide range of processes in which phospholipids are specifically involved explains the need for diversity in phospholipid structures and fatty acid composition.

The acyl chains are either saturated, monounsaturated, or polyunsaturated hydrocarbon chains that normally vary from 14 to 22 carbons in length. Polyunsaturated fatty acids (PUFAs) are essential components of cellular membranes in higher eukaryotes that strongly affect their fluidity, flexibility, and selective permeability. PUFAs affect many cellular and physiological processes in animals, including cold adaptation and survival, modulation of ion channels and carriers, regulation of gene expression, endocytosis/exocytosis, pathogen defense, and activities of membrane-associated enzymes. In vertebrates, C20 PUFAs are metabolized by oxygenases and other enzymes to produce short-lived prostaglandins, leukotrienes, and thromboxanes that bind to specific G-protein-coupled receptors and signal cellular responses that mediate fever, inflammation, vasodilation, blood pressure, and pain, among others. Polyunsaturated fatty acids are generally synthesized by the modification of saturated fatty acid precursors that are products of fatty acid synthase. The desaturase enzymes insert double bonds at specific carbon atoms in the fatty acid chain and the fatty acid elongation system elongates the precursors in two-carbon increments. The pathways for the synthesis of arachidonic acid (20:4n-6) and eicosapentaenoic acid (20:5n-3) involve alternating fatty acid desaturation and elongation reactions that have been characterized biochemically and are supported by the recent cloning and characterization of desaturase and elongase genes. The pathway to 22:6n-3 involves synthesis and desaturation of 24:5n-3 followed by one cycle of β-oxidation in the peroxisome (95).

MEMBRANE UNSATURATION AND LONGEVITY

Studies summarized in the previous sections indicate that MLSP is inversely related to mitochondrial free radical generation and mtDNA oxidative damage. Nevertheless, additional factors that may operate in a parallel fashion can also lead to a low level of oxidative damage in long- lived *versus* short-lived animal species. Oxygen radicals attack all cellular macromolecules. In addition to DNA modification, damage to other cellular components can be also relevant for aging. The PUFA residues of phospholipids are extremely sensitive to oxidation. Every membrane phospholipid contains an un-

saturated fatty acid residue esterified to the 2-hydroxyl group of its glycerol moiety. Many of these are polyunsaturated and the presence of a methylene group between two double bonds renders the fatty acid sensitive to ROS-induced damage, their sensitivity to oxidation exponentially increasing as a function of the number of double bonds per fatty acid molecule (10). Consequently, the high concentration of PUFAs in phospholipids not only makes them prime targets for reaction with oxidizing agents but also enables them to participate in long free radical chain reactions.

With these premises, and maintaining other physiological properties, a low degree of fatty acid unsaturation in cellular membranes, and particularly in the inner mitochondrial membrane, may be advantageous by decreasing their sensitivity to lipid peroxidation. This would also protect other molecules against lipoxidation-derived damage. In agreement with this, it has been found that long-lived animals have a lower degree of total tissue and mitochondrial fatty acid unsaturation (low Double Bond Index, DBI) than short-lived ones. The findings summarized in Table 3 indicate that a low degree of unsaturation of cellular membranes in postmitotic tissues seems to be a general characteristic of long-lived vertebrate homeotherms, both birds and mammals.

The membrane acyl composition of the mammals and birds studied indicates that their biological membranes maintain an identical fatty acid average chain length (18 carbon atoms), and a similar ratio of saturated *versus* unsaturated fatty acids irrespective of animal longevity. The low DBI observed in long-lived species is due to changes in the type of unsaturated fatty acid that participates in membrane composition. So, there is a systematic redistribution between the types of PUFAs present from the highly unsaturated docosahexaenoic acid (22:6n-3, DHA) and sometimes arachidonic (20:4n-6, AA) acid in short-lived animals to the less unsaturated linoleic acid (18:2n-6, LA), and, in some cases linolenic acid (18:3n-3, LNA) in the long-lived ones, at mitochondrial and tissue level. Furthermore, the DBI of the respective diets did not correlate with MLSP. This indicates that the contribution of the variations in the degree of unsaturation of dietary fats to the inter-species differences is, if any, very modest.

What are the mechanisms responsible for the maintenance of this singular fatty acid profile? In relation to the mechanisms responsible for the particular fatty acid composition of homeothermic vertebrates with different longevities, the following may be involved: the fatty acid desaturation pathway and the deacylation-reacylation cycle. The estimation of delta-5 and delta-6 desaturase activities indicates that they are various fold lower in long-lived species than in short-lived ones (104, 106, 107, 110). This can explain why DHA and AA decrease, and LA and LNA increase, from short- to long-lived animals, since desaturases are the rate-limiting enzymes of the n-3 and n-6 pathways synthesizing the highly unsaturated PUFAs AA and DHA from their dietary precursors, LA and LNA respectively. Thus, desaturation pathways would make available *in situ* the n-6 and n-3 fatty acids to phospholipid acyltransferases in order to remodel the phospholipid acyl groups, postulating the presence of constitutively low species-specific desaturase activities in long-lived animals. The fact that acyltransferase/n-6 desaturase activity ratio is about 10:1 in tissues (66) reinforces the idea that regulation of desaturases can be the main limiting factor responsible for the observed DBI-longevity relationship. In addition, a relevant role for a phospholipid-specific deacylation-reacylation system cannot be discarded since it has been observed that this longevity-related redistribution particularly affects the phosphatidylcholine and phosphatidylethanolamine fractions in liver mitochondria, and does not modify cardiolipin (118).

The presence of constitutively low desaturase activities in long-lived animals can explain why feeding corn oil (rich in LA) to primates increases mainly LA (to 30% of total fatty acids) instead of AA (only to 10% of total) in their tissues (21), whereas in short-lived rodents dietary LA leads to strong increases in AA. In a similar fashion, human monastic

Table 3. Comparative Studies of Membrane Unsaturation in Vertebrates with Different Maximum Longevities

Species compared	*MLSP (yrs)*	*Organ*	*Correlation with MLSP*	*References*
Rat-Pigeon-Human	4–120	mtLiver	Negative	110
Rat vs. Pigeon	4, 35	mtLiver	Negative	43
8 Mammals	3.5–46	mtLiver	Negative	108
Rat vs. Pigeon	4, 35	mtHeart	Negative	104
Mouse vs. Canary	3.5, 24	Heart	Negative	105
Mouse vs. Parakeet	3.5, 21	Heart	Negative	105
Rat vs. Pigeon	4, 35	mtHeart and microsomes	Negative	43
7 Mammals	3.5–46	Liver	Negative	106
8 Mammals	3.5–46	Heart	Negative	107
8 Mammals	3.5, 46	Liver	Negative	118
11 Mammals + 9 Birds	3.5–120	Skeletal muscle	Negative	64, 65
9 Mammals + 8 Birds	3.5–120	mtLiver	Negative	14, 116
Rat vs. Pigeon	4, 35	Skeletal muscle	Negative	119

Mt, mitochondria.

communities who chronically consumed only corn oil as the main dietary fat source (67% rich in LA) had lipid profiles with around 30% LA but only 9% AA in their lipoproteins (139).

Animals with high MLSP have a low degree of membrane fatty acid desaturation based in the redistribution between types of PUFAs without any alteration in the total (%) PUFA content or in the average chain length. This may be viewed as an elegant evolutionary strategy, since it decreases the sensitivity to lipid peroxidation and lipoxidation-derived damage to cellular macromolecules without strongly altering fluidity/microviscosity, a fundamental property of cellular membranes for the proper function of receptors, ion pumps, and transport of metabolites. This occurs because membrane fluidity increases acutely with the formation of the first and less with the second double bond due to their introduction of "kinks" in the fatty acid molecule, whereas additional (the third and following) double bonds cause few further variations in fluidity (15). This is so because the kink has a larger impact on fluidity when the double bond is situated near the center of the fatty acid chain (first double bond) than when it is situated progressively nearer to its extremes (next double bond additions). In the case of sensitivity to lipid peroxidation, however, double bonds increase it irrespective of being situated at the center or laterally on the fatty acids. Thus, by substituting fatty acids with four or six double bonds by those having only two (or sometimes three) double bonds, the sensitivity to lipid peroxidation is strongly decreased in long-lived animals, whereas the fluidity of the membrane would be essentially maintained. This hypothesis, reminiscent of membrane acclimation to temperature at PUFA level in poikilotherms, has been denominated *homeoviscous longevity adaptation* (101). The adjustment of the DBI of each organ and species independently of the diet also indicates that it is an endogenous trait under genotypic control. The diet-independency occurs through PUFA-induced repression of the expression of genes controlling PUFA synthesis, and through increases in the expression of those genes induced by PUFA-deficient diets (132). These genome-based mechanisms are responsible for the decreases in PUFAn-6 induced by diets rich in PUFAn-3 as well as for the reverse, which operate mainly trough variations in delta-5/-6 activities (95).

AGING RATE VERSUS MEMBRANE UNSATURATION: PHYSIOLOGICAL SIGNIFICANCE

Membrane unsaturation versus sensitivity to lipid peroxidation

What could be the physiological significance of the allometry in double bond content? Various possibilities have been proposed. Some authors (for reviews see 62, 63) have suggested that mammals of large body size have a low DBI in order to decrease their metabolic rates, because the lower the DBI of a membrane, the lower would be its permeability to ions (maintenance of various trans-membrane gradients is one of the main determinants of metabolic rate), suggesting that membranes can act as pacemakers for overall metabolic activity.

While this holds true among mammals or birds of different sizes, it cannot explain the low DBI of birds because they have a metabolic rate similar or even higher than that of mammals of similar body size. Therefore, the high specific metabolic activity of birds must be due to other factors different from the low degree of unsaturation of their cellular membranes. However, both the studied birds (pigeons, canaries, and parakeets) and mammals of large body size share two traits: a high maximum longevity and a low degree of fatty acid unsaturation. Thus, it could be hypothesized that the low DBI of long-lived homeotherms (either mammals or birds) could have evolved to decrease membrane lipid peroxidation and its lipoxidative consequences to other cellular macromolecules including proteins and DNA, to make possible their extension of longevity.

Lipid peroxidation generates hydroperoxides and endoperoxides, which undergo fragmentation to produce a broad range of reactive intermediates, like alkanals, alkenals, hydroxyalkenals, glyoxal, and malondialdehyde (MDA; 36). These carbonyl compounds, and possibly their peroxide precursors, react with nucleophilic groups in proteins, resulting in their modification. The modification of amino acids in proteins by products of lipid peroxidation results in the chemical nonenzymatic formation of a variety of adducts collectively named Advanced Maillard Products. These products include, among others, malondialdehyde-lysine adducts (150), which can be useful indicators of lipoxidative protein stress *in vivo*. In this context, it has been demonstrated that in long-lived animal species a low degree of total tissue and mitochondrial fatty acid unsaturation (low DBI) is accompanied by a low sensitivity to *in vivo* and *in vitro* lipid peroxidation (104–106, 110) and a low concentration of lipoxidation-derived adducts in several tissue and mitochondrial proteins (102, 104, 106, 119). A negative correlation between MLSP and the sensitivity to lipid autoxidation of homogenates from mammalian kidney and brain has been also described by independent researchers (26).

However, the occurrence of correlation does not necessarily mean that a cause-effect relationship is operative. In order to clarify whether the low DBI of long-lived animals protects their mitochondria from lipid oxidation and lipoxidation-derived protein modification, studies of experimental dietary modification of *in vivo* membrane fatty acid unsaturation have been performed. These studies were specially designed to partially circumvent the homeostatic system of compensation of dietary-induced changes in DBI which operates at tissue level (55, 109, 117). The obtained findings suggest that lowering the DBI of cellular membranes protects postmitotic tissues against lipid peroxidation and lipoxidation-derived macromolecular damage.

Lipid peroxidation, free radical production, and uncoupling proteins

As mentioned above, peroxidation of the polyunsaturated fatty acyl chains of phospholipids generates a complex mixture of short-chain aldehydes. Initially, these aldehydes were believed to produce only cytotoxic effects associated with oxidative stress, but evidence is increasing that these compounds can also have specific signaling roles.

Available studies support the notion that superoxide radical produced by the electron transport chain can cause mild uncoupling of mitochondria by activating the membrane proton conductance by uncoupling proteins (UCPs; for review see 12). Insight into the mechanism by which superoxide radical activates UCPs comes from the finding that the lipid peroxidation product 4-hydroxy-*trans*-2-nonenal and its homologs induce uncoupling of mitochondria through UCP1, UCP2, and UCP3 and also through the adenine nucleotide translocase (34). This and other observations support a model in which endogenous superoxide production generates carbon-centered radicals that initiate lipid peroxidation, producing alkenals like 4-hydroxy-*trans*-2-nonenal that may activate UCPs and adenine nucleotide translocase.

Whilst the thermogenic function of UCP1 has been well characterized, a function for its homologs (UCP2, UCP3, avian UCP, and plant UCP) has yet to be unambiguously defined. A possible physiological function for UCPs has been proposed (34). In this model, UCPs respond to overproduction of matrix superoxide by catalyzing mild uncoupling, which lowers proton motive force and would decrease superoxide production by the electron transport chain. This will attenuate superoxide-mediated molecular damage (13), at the cost of a slightly lowered efficiency of oxidative phosphorylation. It was hypothesized by others that this negative feedback loop will protect cells from ROS-induced damage and might represent the ancestral function of all UCPs (12).

AGING RATE VERSUS MEMBRANE UNSATURATION: EFFECT OF AGING AND CALORIC RESTRICTION

The singular importance of membrane unsaturation in the aging process is highlighted by studies showing: (a) Increases in DBI and peroxidizability indexes during aging in an organ-dependent way (69, 71, 165). This is mainly due to decreases in the less unsaturated LA and LNA and to increases in the highly unsaturated AA, 22:4n-3, 22:5n-3, and DHA. Indirect evidences such as the amount ethane and pentane in exhaled air, or biophysical studies on membrane order also support these findings; (b) The senescent accelerated prone mouse (SAM-P) has higher levels of the very unsaturated AA, DHA and peroxidizability index and lower levels of LA than SAM-resistant controls (111); and (c) Caloric restriction attenuates age-related changes in lipid peroxidation (69, 71, 103, 165). The effects of CR on membrane unsaturation can be divided in three stages depending of CR duration in rats. During short-term CR periods (weeks), decreases in the rate of mitochondrial ROS production and lipoxidation-derived protein damage are observed in some tissues together with minor changes in membrane fatty acid composition. The decreases in lipoxidation probably reflect a decrease in free radical attack to the membrane. If CR is applied for several months, changes in particular fatty acids with slight or no changes in DBI occur, although the magnitude of the changes depends on the organ and the intensity of the restriction. Finally, in long-term CR (longer than 1 year), the beneficial effects on ROS production, DBI-fatty acid composition, and lipoxidation-derived protein damage are evident. In fact, CR diminishes the slope of the relationship between age and age-related lipid peroxidation. Thus, the CR manipulation, as pointed out by Yu and co-workers (165) seems to trigger an adaptive response protecting the most basic requirements of membrane integrity.

Additional observations suggesting that membrane unsaturation is related to aging include data showing that strongly unsaturated fatty acids like AA and DHA can have detrimental effects *in vivo*. Examples of this include decreases in respiratory control and increases in proton leak in mitochondria, increased mitochondrial breakage and dysfunction, peroxisome proliferation, fatal ventricular fibrillation in rats, neurological damage, increased lipid peroxidation in association with various diseases, increased incidence of death from apoplexy, or sudden cardiac death in humans induced by AA or DHA. Increases of more than one order of magnitude in AA (to 500 μ*M*) occur in the brain during ischemia and even concentrations of AA and eicosapentaenoic acid (20:5n-3) in the much lower 20–40 μ*M* range uncouple mitochondria and cause tissue edema (147). Hypermetabolic uncoupling effects of thyroid hormones on rat liver mitochondria are due to a great extent to increased AA/LA ratios caused by increases in desaturase activities induced by the hormone, whereas LA is considered a "proton plug" or coupler (see 58 for review). Furthermore, the largest amounts of unsaturated fats in the healthy human diet must be present as fatty acids with low degrees of unsaturation like oleic acid and LA, whereas beneficial levels of dietary n-3 PUFAs (the n-3 "paradox") occur only at the low (1%) optimum dietary levels recommended by the World Health Organization. These beneficial effects are probably observed because the conversion of dietary LNA to highly unsaturated fatty acids like DHA is strongly limited thanks to the constitutively low delta-5/-6 desaturase activities of humans. In this context, two studies deserve special attention: (a) Inuits are human populations showing unusually low incidence of coronary heart disease, psoriasis, rheumatoid arthritis, and asthma, and have very low levels of AA in plasma phospholipids due to a genetic abnormality in essential fatty acid desaturation which persists even after changing them to a LA-rich diet (60); and (b) In a recent prospective study on old healthy subjects, it was found that a higher monounsaturated fatty acid intake (Mediterranean diet) increased survival, whereas a higher unsaturated/saturated fatty acid ratio increased total mortality (140).

ABBREVIATIONS

8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; AA, arachidonic acid (20:4n-6); CAT, catalase; DBI, double bond index; DHA, docosaexahenoic acid (22:6n-3); FRTA, free radical theory of aging; GSH, glutathione; Gpx, glutathione peroxidase; LA, linoleic acid (18:2n-6); LNA, linolenic acid (18:3n-3); ROS, reactive oxygen species; nDNA, nuclear DNA; mtDNA, mitochondrial DNA; MLSP, maximum lifespan potential; PUFA, polyunsaturated fatty acids; SOD, superoxide dismutase; UCP, uncoupling proteins.

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