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The biological basis of the aging process

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Resum

El procés biològic bàsic subjacent de l'envelliment va ésser avançat per la teoria de l'envelliment basada en els radicals lliures l'any 1954: la reacció dels radicals lliures actius, produïts fisiològicament en l'organisme, amb els constituents cel·lulars inicia els canvis associats a l'envelliment. La implicació dels radicals lliures en l'envelliment està relacionada amb el seu paper clau en l'origen i l'evolució de la vida. La informació disponible avui en dia ens mostra que la composició específica de les macromolècules cel·lulars (proteïnes, àcids nucleics, lípids i carbohidrats) en les espècies animals longeves tenen intrínsicament una resistència elevada a la modificació oxidativa, la qual cosa probablement contribueix a la longevitat superior d'aquestes espècies. Les espècies longeves també mostren unes taxes reduïdes de producció de radicals lliures i de lesió oxidativa. D'altra banda, la restricció dietària disminueix la producció de radicals lliures i la lesió molecular oxidativa. Aquests canvis estan directament associats a la reducció de la ingesta de proteïnes dels animals sotmesos a restricció, que alhora sembla que són deguts específicament a la reducció de la ingesta de metionina. En aquesta revisió s'emfatitza que una taxa baixa de generació de lesió endògena i una resistència intrínsecament elevada a la modificació de les macromolècules cel·lulars són trets clau de la longevitat de les espècies animals.

Paraules clau: envelliment; grau d'insaturació de membrana; lesió molecular; longevitat; mitocondris; oxidació d'àcids nucleics, carbohidrats, lípids i proteïnes; radicals lliures; restricció calòrica, de proteïnes i de metionina

Abstract

The basic chemical process underlying aging was first put forward by the free radical theory of aging in 1956; the reaction of active free radicals (normally produced within an organism itself) with cellular constituents initiates the changes associated with aging. The involvement of free radicals in aging is related to their key role in the origin and evolution of life. The specific composition of tissue macromolecules (proteins, nucleic acids, lipids and carbohydrates) in long-lived animal species gives them an intrinsically high resistance to modification that probably contributes to the superior longevity of these species. Long-lived species also show low rates of reactive oxygen species (ROS) generation and oxidative damage to their mitochondria. Dietary restriction further decreases mitochondrial ROS production and oxidative molecular damage due to the decreased intake of dietary proteins. These effects of protein restriction seem to be specifically due to the lowered methionine intake of protein and dietary restricted animals. Both a low rate of generation of endogenous damage and an intrinsically high resistance to the modification of tissue macromolecules are key traits of animal longevity.

Keywords: aging; caloric, protein and methionine restriction; carbohydrate oxidation; DNA oxidation; free radicals; lipid oxidation; longevity; membrane unsaturation; mitochondria; molecular damage; protein oxidation; ROS production

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Abbreviations: DR, dietary restriction; 8-oxodG, 8-oxo-7,8-dihydro-2'deoxyguanosine (DNA specific oxidation marker); MetR, L-methionine restriction; MitROS, mitochondrial ROS; mtDNA, mitochondrial DNA; PR, protein restriction; ROS, reactive oxygen species.

When Jeanne Calment died in a nursing home in southern France in 1997, she was 122 years old; the longest-living human ever documented. But Calment's status will change in forthcoming decades if the predictions of biogerontologists and demographers are borne out. Lifespan extension in species from yeast to mice and extrapolation from life expectancy trends in humans have convinced scientists that humans will routinely live beyond 100 or 110 years of age. Today, 1 in 10,000 people in industrialized countries holds centenarian status. By 2025 the United Nations anticipates that there will be 822 million people in the world aged 65 and over. The elderly population will have grown by a factor of 2.5 between 1990 and 2025. This is faster than total population growth, resulting in the world's elderly population increasing from 6.2 to 9.7 percent.

The rise in life expectancy related to improvements in health is among the most remarkable demographic changes of the past century. For the world as a whole, life expectancy more than doubled from around 30 years in 1900, to 65 years by 2000, and it is projected to rise to 81 by the end of this century. Most of the historical rise reflects declines in infant and child mortality due to public health interventions related to drinking water and sanitation, and to medical interventions such as vaccination and the use of antibiotics. By contrast, the life expectancy gains observed over the past few decades (especially in high-income countries) and which are projected into the future are predominantly associated with reductions in age-specific death rates at the middle and older ages. These reductions are typically associated with improvements in medical technology, life-style changes, and income growth.

What structural components and physiological mechanisms determine the aging process? Why can human beings, for instance, reach 122 years whereas rats only live at most 4 years? "How much can human lifespan be extended?" was one of the questions featured by the journal *Science* recently on the occasion of its 125th anniversary. It addresses what the journal regards as one of the frontiers of science for the next 25 years. An answer to the question, if available, will probably emerge from many different investigative avenues, with possibly the most important being an examination of how nature determines the diverse and distinctive maximum lifespans throughout the animal kingdom.

The post-reproductive phase of life of virtually all cellular species is characterized by the progressive decline in the efficiency of maximum physiological functions. Consequently, the ability to maintain homeostasis is correspondingly attenuated – leading eventually to an increased risk of developing cancer, and to neurodegenerative and cardiovascular diseases– increasing the chances of death. Any theory that explains aging must fit in with four main characteristics of this natural process: aging is progressive, endogenous, irreversible and deleterious (in the sense that it damages the soma) for the individual [1]. First, the progressive character of aging means that the causes of aging must be present during the whole lifespan: in both young and old. Second, since aging is an endogenous process, exogenous factors (such as UV rays and dietary antioxidants) are not causes of aging though they may interact with endogenous causes enhancing or mitigating their effects. The endogenous character of aging means that the rate of aging of different animal species, and thus their maximum lifespan potentials (MLSPs), is genotipically determined, not dependent upon the environment. Hence, different animal species age at widely different rates in similar environments. In contrast, the mean lifespan (frequently and wrongly termed "longevity") which is calculated from the amount of time that each individual lives, is mainly determined by the environment and to a lesser extent by the genotype. (Genetic determination of mean lifespan in humans is commonly agreed to be around 30%). This is the reason why many environmental factors such as smoking, the amount of saturated fat consumed, an unbalanced diet, a sedentary life, and possibly the action of antioxidants are so important in determining the age of death. Conversely, no matter what an elephant eats or does, it will never age in two years like a healthy rat does, and no diet will make a mouse survive for 85 years. Thus, mortality should not be confused with aging, even though advancing age increases the probability of death. In relation to this, the inter-individual variation in the time lived for a given species (mainly environmentally determined) should not be confused with interspecies variation in longevity (which is genetically determined).

Although aging seems to be a multi-causal process, it is perhaps mainly due to a small number of principal causes with major effects. In this review we update the available evidence concerning the mitochondrial oxidative stress theory of aging [2-4] and focus on comparative and dietary restriction models and the underlying mechanisms involved. We highlight two main characteristics that link slow animal aging to oxidative stress: i) slow generation of endogenous damage, and ii) a macromolecular composition that is highly resistant to oxidative modification.

The rate of generation of damage induced by mitochondrial free radicals and longevity

About 85 to 90 per cent of oxygen is used by the mitochondria; these organelles are the major source of energy (as adenosine triphosphate [ATP] molecule) in aerobic organisms. Electrons from reduced substrates move from complexes I and II of the electron transport chain through complexes III and IV to oxygen, forming water and causing protons to be pumped across the mitochondrial inner membrane. The proton motive force set up by proton pumping drives protons back through the ATP synthase in the inner membrane, forming ATP from their precursors ADP (adenosine diphosphate) and phosphate. There are two major side reactions that are relevant here: electrons leak from the respiratory chain and react with oxygen to form free radicals; and pumped protons leak back across the inner membrane, diverting the conserved energy away from ATP biosynthesis and into heat production. Apart from the nucleus, mitochondria are the only cellular organelles that have their own DNA, and they have a significant capacity for continuous free radical (reactive oxygen species, ROS) production. – (A free radical is any molecule capable of independent existence that contains one or more unpaired electrons. They are

extremely reactive and have damaging effects.) The fact that they are responsible for free radical production suggests that mitochondria play a causal role in the progressive phenomenon that is aging. Oxidative stress could be related to aging through ROS generation, ROS elimination, or both.

Aerobic life demands antioxidant defences. An antioxidant is: "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate." An antioxidant either reacts with an oxidant and neutralizes it, or it regenerates other molecules capable of reacting with the oxidant. Oxidative damage is a broad term used to cover the attack upon biological molecules by free radicals. Cellular protection against oxidative damage includes both the elimination of ROS and repairing damage, with antioxidants constituting a fundamental line of this defence. Although antioxidants may protect against various age-related diseases, they do not seem to control the rate of aging [reviewed in 4] since: i) long-lived species, including both invertebrates and vertebrates, constitutively have lower (not higher) tissue levels of antioxidant enzymes and of low molecular weight endogenous antioxidants than short-lived ones; ii) experimentally increasing tissue antioxidants through dietary supplementation, pharmacological induction, or transgenic techniques sometimes moderately increases mean lifespan but does not change maximum lifespan; and iii) animals in which genes coding for particular antioxidant enzymes are knocked out may show different pathologies, but their rates of aging do not seem to be affected.

The strong negative correlation between endogenous tissue antioxidants and maximum longevity suggests that the rate of endogenous free radical production in vivo must be much lower in long-lived than in short-lived animals. If long-lived animals had high rates of ROS production together with their very low levels of endogenous antioxidants, their tissue cells would not be able to maintain their oxidative stress balance. Decreasing mitochondrial reactive oxygen species (MitROS) production instead of increasing antioxidants or repair systems makes sense when considered from the point of view of the evolution of longevity among species. It would be very inefficient to generate large amounts of ROS and, afterwards, try to intercept them before they reach biomolecules, or even worse, try to repair biomolecules after they have been heavily damaged. This makes even more sense if we take into account: a) the high energetic cost of continuously maintaining high levels of antioxidant and repair molecules in tissues, and b) the capacity of all kinds of animals (short-lived and long-lived) to temporarily induce these protective molecules when needed in larger amounts. All the research published in this field has found that the rate of MitROS production is lower in the tissues of long-lived than in those of short-lived animal species [4,5]**.** This occurs in all kinds of long-lived homeothermic vertebrates independently of their mass-adjusted rates of O_2 consumption, which is low in animals of large body size like cows and high in animals of small size like the birds studied. This characteristic thus explains why endogenous tissue antioxidants correlate negatively with maximum longevity across species: long-lived

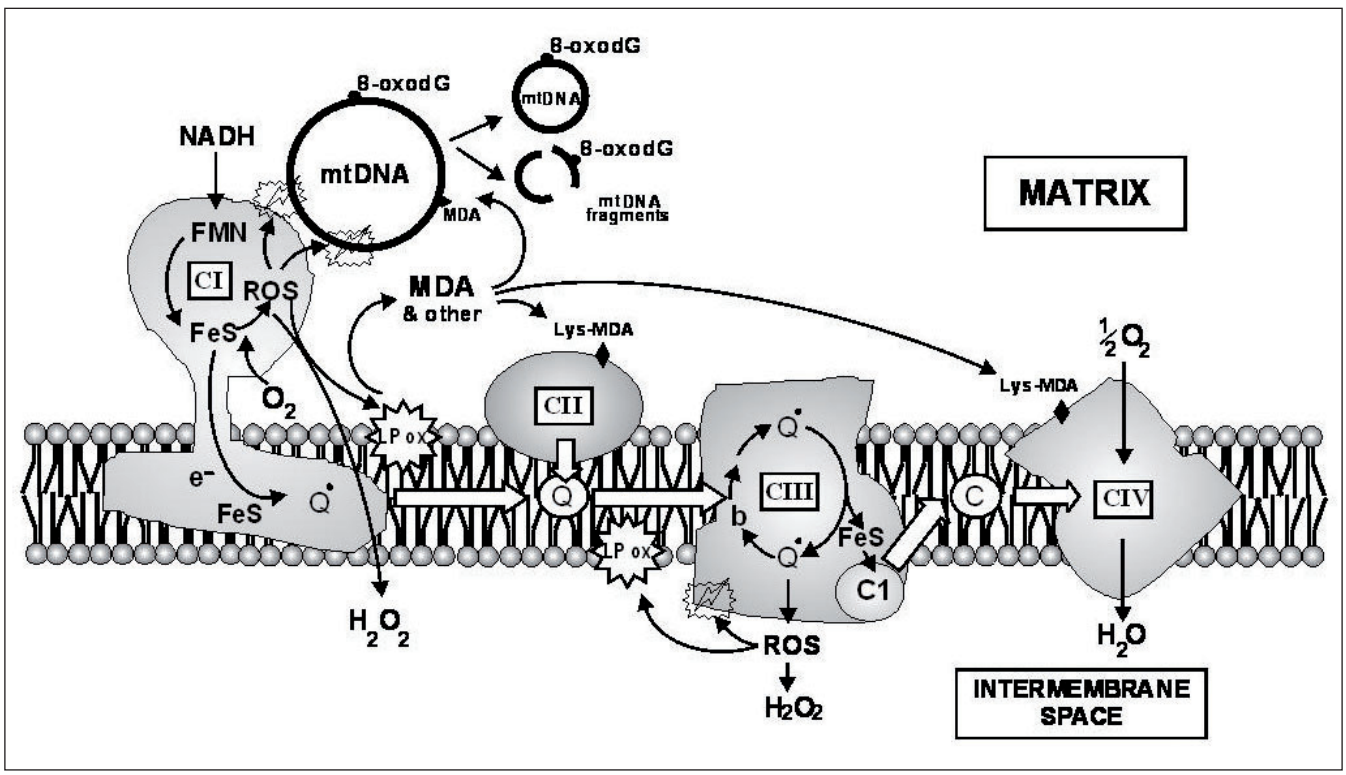

Figure 1. Mitochondrial complex I and III are the main free radical generators. Under normal physiological conditions, in healthy tissues most reactive oxygen species are generated by the mitochondrial respiratory chain at complex I and III. Several physiological mechanisms influence the rate of mitochondrial ROS generation including: i) the relative concentration of the respiratory complexes; ii) the degree of electronic reduction of the generators; iii) the uncoupling proteins; iv) the cardiolipin content, and v) specific chemical modifications. Oxygen radicals attack lipids, carbohydrates, proteins and DNA inducing non-enzymatic and irreversible chemical modifications and leading to structural and functional changes (modified for ref. [20]).

animals have constitutively lower levels of antioxidants because they produce ROS at a low rate. This is possible because the percentage of total electron flow in the respiratory chain directed to MitROS production (the percentage of free radical leak, %FRL) is lower in long-lived animals. This means that their respiratory chains transport electrons more efficiently avoiding univalent electron leaks to oxygen upstream of cytochrome oxidase. Recent studies have also found much lower rates of ROS generation in human than in rat brain mitochondria [6]. Primates–and especially humans–also live much longer than their body size and metabolic rate would suggest.

MitROS generation occurs at complex I and III (Figure 1) [4,5]. Studies indicate that the respiratory complex responsible for the lower MitROS generation of long-lived species is complex I. Flavin mononucleotide, ubisemiquinone species, or iron-sulphur clusters have been proposed as the electron transport component responsible for MitROS generation within complex I. We wish to know whether there are physiological mechanisms regulating the rate of mitochondrial free radical generation. The evidence seems to suggest that this is the case [7,8]. The following mechanisms or factors are believed to regulate the rate of free radical production: a) A decrease in the concentration of the respiratory complex(es) responsible for ROS generation. Thus, long-lived birds have a lower content of complex I protein than short-lived mammals. This decrease in the amount of complex I protein can lead to a decreased rate of ROS generation in slowly aging animals. b) The degree of electronic reduction of these generators: the higher their degree of reduction, the higher their rate of ROS production. c) Uncoupling proteins: mitochondrial free radical production is very sensitive to the proton motive force, so it can be strongly decreased by mild uncoupling. An ancestral function has been proposed for uncoupling proteins; to cause mild uncoupling and so diminish mitochondrial free radical production, hence protecting against oxidative damage at the expense of a small loss of energy. d) Glutathionylation of complex I increases free radical production by the complex, and when mixed disulfides are reduced, free radical production returns to basal levels. Within intact mitochondria, oxidation of the glutathione pool to glutathione disulfide also leads to glutathionylation of complex I, which correlates with increased free radical formation. This mechanism of reversible mitochondrial ROS production suggests how mitochondria might regulate redox signalling and shows how oxidation of the mitochondrial glutathione pool could contribute to the pathological changes that mitochondria undergo during oxidative stress, and finally, e) the cardiolipin content, and other modifications such as S-nitrosylation.

Structural components that are highly resistant to oxidative stress and longevity

Molecular damage caused by oxidation is one of the natural consequences of aerobic life. Classically, cellular protection against oxidative damage includes free radical elimination and repair/turnover systems. These are considered as the first and the second lines of defence, respectively. Recent studies, however, support the notion of another line of defence based on the inherent susceptibility of macromolecules to oxidative damage. This susceptibility (defined as the ease with which a macromolecule suffers an oxidative injury) is intrinsically associated with the specific chemical composition of proteins, nucleic acids, lipids and carbohydrates. In the context of oxidative stress, the following are especially relevant: a) Methionine residues from proteins are among the amino acids most susceptible to oxidation by free radicals [9]. b) Of the four nucleobases, guanine has the lowest oxidation potential and is thus generally most easily oxidized [10]. c) Highly unsaturated fatty acids in cell membranes are the macromolecules most susceptible to oxidative damage in cells, and this sensitivity increases as a function of the number of double bonds they contain. This means that saturated and monounsaturated fatty acyl chains (SFA and MUFA) are essentially resistant to peroxidation whereas polyunsaturates (PUFA) are easily damaged [8]. Finally, d) carbohydrate reactivity, referring very particularly to monosaccharides of biological interest, is dependent on the extent to which it exists in the open (carbonyl) structure rather than in the ring (hemiacetal or hemiketal) structure. Glucose is the most stable and least reactive monosaccharide [11].

Based on these premises, the available evidence verifies that:

a) Methionine is the amino acid that on average has the smallest percentage presence in cellular proteins [12-14]. In addition, the longer the lifespan of a species, the lower its tissue protein methionine content (Figure 2) and the less the protein damage derived from glycoxidation and lipoxidation (Figure 3), because the longer the longevity of a species, the higher the content of proteins resistant to oxidative damage [8,15].

b) Guanine is the least abundant nucleotide in mitochondrial DNA [16]. In addition, the longer the maximum lifespan

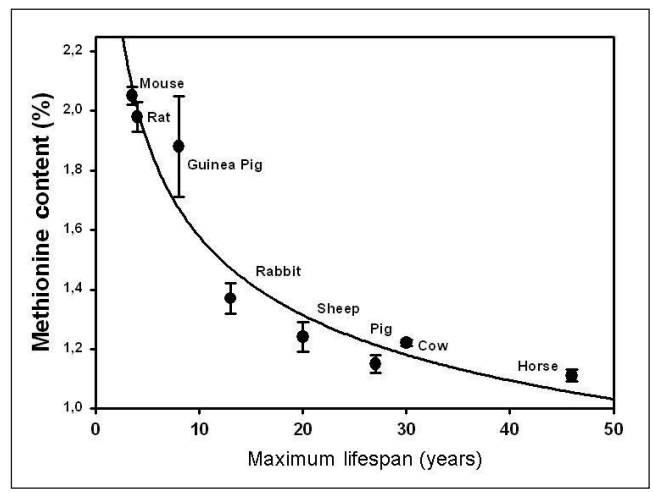

Figure 2. Correlation between methionine content of heart proteins and maximum longevity in mammals. Methionine protein content negatively correlates with maximum longevity in the heart of eight mammalian species with widely varying MLSPs ($r = -0.96$; $p<0.001$). Methionine values are plotted as a function of maximum longevity and data are fitted to the power function $y = a.x^b$, where y is the methionine content and x is maximum longevity. Values are given as mean±SE (data from ref. [13]).

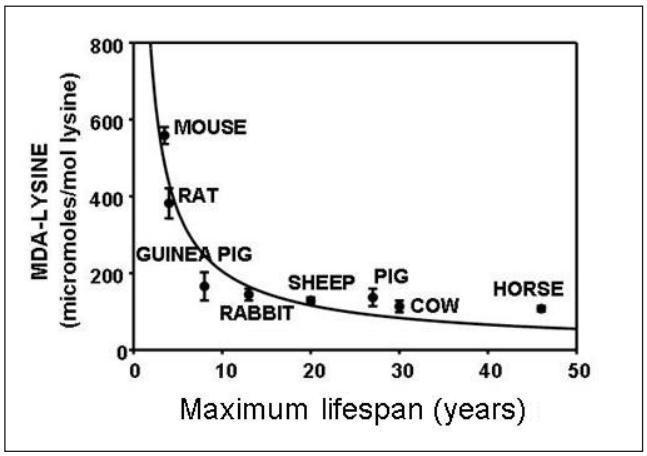

Figure 3. Correlation between the level of MDA-lysine (MDAL) protein adducts–a specific biomarker of lipoxidation-derived protein damage–in the heart of mammals and maximum longevity. MDAL adducts in heart proteins negatively correlate with MLSP in mammals (r = –0.92; p<0.001). MDAL values are plotted as a function of maximum longevity and data are fitted to the power function $y = a.x^b$, where y is the level of MDAL and x is maximum longevity. Values are given as mean±SE (data from ref. [13]).

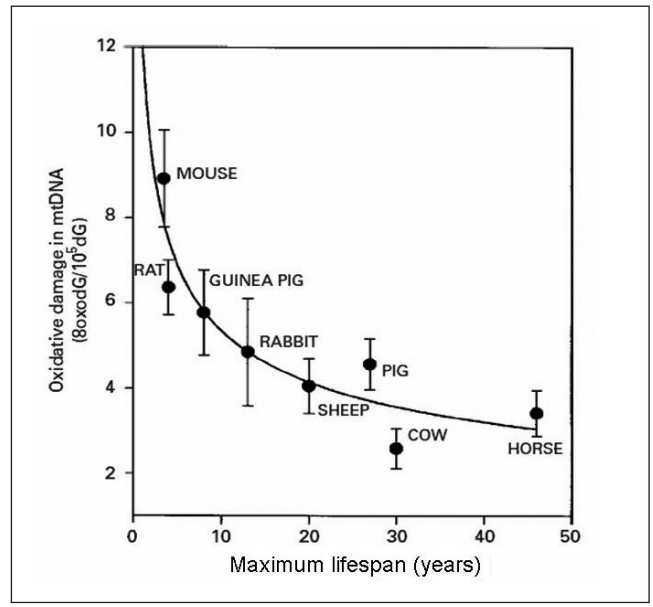

Figure 4. Inverse correlation between the level of heart 8-oxodG/105dG in mtDNA and maximum longevity in eight mammalian species. 8-oxodG/105dG values are plotted as a function of maximum longevity and data are fitted to the power function $y = a.x^b$, where y is the level of 8-oxodG in mtDNA and x is maximum longevity (r=-0.92, P<0.001). Values are given as mean±SE (data from ref. [5]).

of a species (including invertebrates, birds and mammals) the lower the mtDNA free energy (which is a physical property of the double-stranded DNA molecule that measures the binding energy between the two DNA strands, and can be interpreted as a measure of the susceptibility of mtDNA to mutation) [16]. Consequently, the mtDNA sequence composition expresses a highly resistant longevity adaptation. In agreement with this, long-lived mammals and birds have lower steady-state levels of 8-oxodG (a specific marker of DNA oxidation) in their mtDNA [4,5] (Figure 4) and lower rates of urinary excretion of 8-oxodG than short-lived ones [17]. It has also been observed that the rate of accumulation of mtDNA oxidation-derived mutations with age is much slower in humans than in mice [18].

c) Highly unsaturated fatty acids (more than 2 double bonds) are on average the least abundant fatty acids in cell membranes [8,19]. In addition, a low degree of unsaturation (double bond index and peroxidizability index) of cell membranes is a general characteristic of long-lived

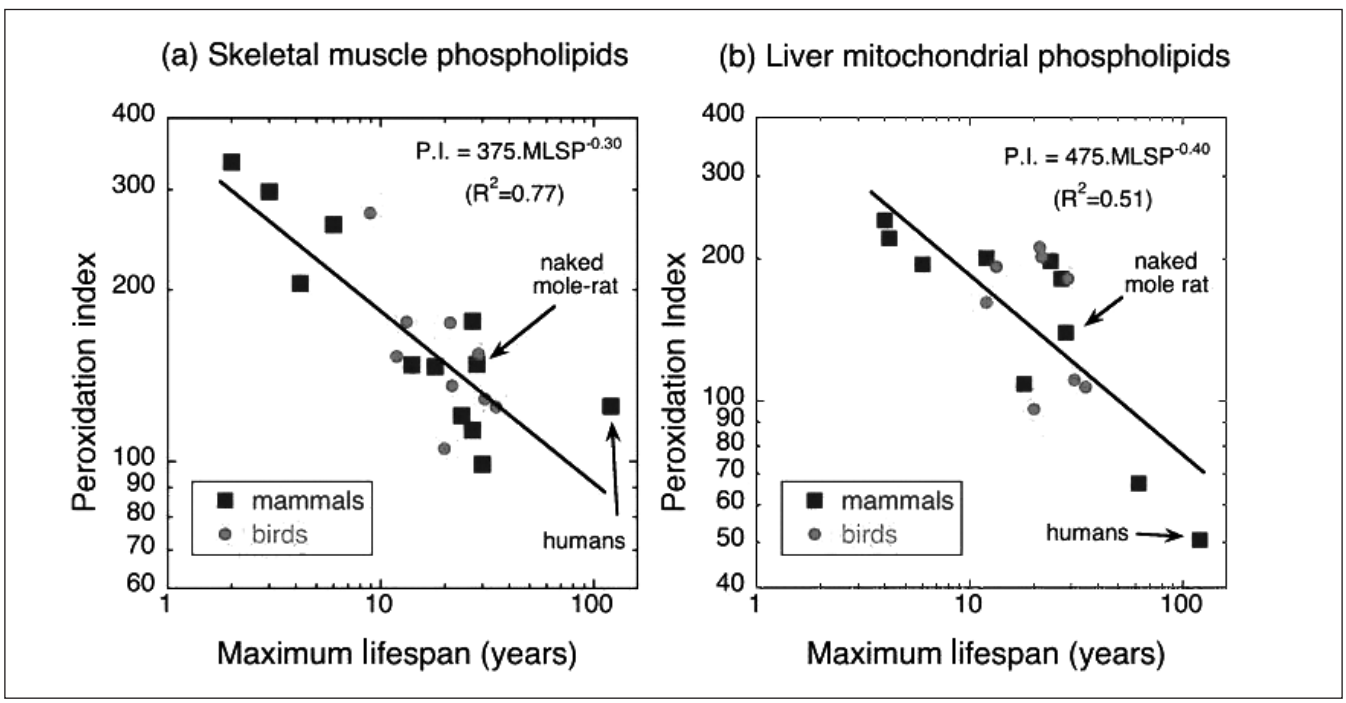

Figure 5. Correlation between peroxidizability index and maximum longevity. The relationship between maximum lifespan of mammals and birds and the peroxidation index of (a) skeletal muscle phospholipids and (b) liver mitochondrial phospholipids (data from ref [8]).

species [4,8,19,20] (Figure 5). Species (including humans, and species with an exceptional longevity such as the naked mole-rat) with a high maximum longevity have a low degree of membrane fatty acid unsaturation based on the redistribution between types of PUFA without any alteration in the total (percentage) PUFA content and 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 greatly altering fluidity/microviscosity (a fundamental property of cell membranes for the proper function of receptors, ion pumps, and transport of metabolites). This occurs because membrane fluidity increases sharply with the formation of the first double bond but less so with the second, due to the introduction of "kinks" in the fatty acid molecule. Additional (third and subsequent) double bonds cause little further variation in fluidity [21], since the kink has a larger impact on fluidity when the double bond is situated near the centre of the fatty acid chain (first double bond) than when it is situated progressively nearer to its extremes (subsequent double bond additions). However, double bonds increase sensitivity to lipid peroxidation irrespective of where they are situated on the fatty acids. Thus, by replacing 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 is essentially maintained. This effect, reminiscent of membrane acclimation to temperature at PUFA level in poikilotherms, has been denominated homeoviscous longevity adaptation [20].

Finally, d) glucose has emerged as the most important carrier of energy from cell to cell in animal species, precisely because it is the slowest reacting carbohydrate. From experimental data obtained by Bunn and Higgins [11], it is apparent that other sugars (such as ribose) are of the order of 100 times more reactive in the browning reaction (also named Maillard reaction; it leads to the formation of advanced glycation/glycoxidation endproducts [15,22]) than glucose. Thus, the lower the plasma and cellular concentration of these highly reactive sugars, the less the biological stress [23]. For instance, a look at the plasma concentrations of reducing sugars and related compounds offers an insight into the type of reactants that are expected to modify plasma protein in vivo. As expected, the most abundant sugar is glucose (concentration in the range of mM). Methylglyoxal, free pentoses and other sugars are present in much lower concentrations (μM). In a similar way, data on the intracellular level of glycolytic intermediates show that concentrations are also in the micromolar range, and are overall tightly controlled.

As a rule, chemical reactions in living cells are under strict enzyme control and conform to a tightly regulated metabolic program. One of the attractors involved in biomolecular evolution is the minimizing of unwanted side reactions. Nevertheless, uncontrolled and potentially deleterious reactions occur, even under physiological conditions. Consequently, we can infer from the available evidence that aerobic life, and long-lived species, evolved by reducing the relative abundance of those structural components that are highly susceptible to oxidative damage, but without renouncing them, thus conferring to the macromolecules a higher structural stability and lower susceptibility to oxidative stress.

Oxidative stress, aging and dietary restriction

Caloric restriction

The comparative studies described above strongly suggest a causal relationship between MitROS production, oxidative molecular damage and the rate of aging. However, correlation does not necessarily indicate a cause-effect relationship; experimental studies are needed for confirmation. Dietary restriction (DR) is the experimental intervention described in most detail that slows down aging and increases maximum longevity [24]. It is therefore very interesting to study whether DR can change the rate of MitROS generation. Much research has shown that changes in antioxidant levels during DR are inconsistent [25] and cannot explain the increase in lifespan due to this dietary manipulation. The effect of DR on MitROS production has been studied intensively in rodents and especially in rats [5]. These studies, usually applying 40% DR, consistently demonstrate that long-term dietary restriction (between 1 year of DR and life-long DR) significantly decreases the rate of MitROS generation in rat tissues including heart, brain, skeletal muscle, liver and kidney [4,5,7]. Furthermore, 6-7 weeks of dietary restriction is enough to decrease MitROS production and 8-oxodG in mtDNA and nDNA in rat liver. It was also found that the decrease in MitROS generation in DR rats occurs specifically at complex I in all the organs studied (heart, liver and brain) and that it is not accompanied by changes in mitochondrial O_2 consumption but does correspond to a decrease in %FRL. This indicates that the mitochondria of DR animals are more efficient at avoiding ROS production per unit electron flow. These characteristics were also observed in long-lived animal species when compared to those of short-lived ones (see above). The lack of changes in mitochondrial O_2 consumption also agreed with the lack of variations in basal metabolic rates in DR [26,27]. In addition, the decrease in MitROS generation observed in DR rats was accompanied by significant decreases in 8-oxodG levels either in mtDNA alone, or in mtDNA and nDNA, depending on the tissue studied. It was also accompanied by decreases in membrane unsaturation, and damage to tissue and mitochondrial proteins resulting from oxidation, glycoxidation, and lipoxidation [4, 8,15,19,20] (see Table 1).

In contrast, the repair of 8-oxodG in mtDNA via the mitochondrial base excision repair pathway does not increase, and even decreases, during DR [28]. Thus, the decrease in 8-oxodG steady-state levels in mtDNA during DR must be due to a decrease in 8-oxodG formation. This is further supported by the fact that the three parameters involved (level of MitROS generation, steady state 8-oxodG levels in mtDNA, and degree of 8-oxodG repair via mitochondrial base excision repair) all decrease to a similar extent during 40% DR; around 30-40%.

Table 1. Summary of changes in mitochondrial ROS generation, oxidative damage, and maximum lifespan in dietary, protein and methionine restriction in rats or mice

	Dietary Restriction	*Protein Restriction*	*Methionine Restriction*
Mitochondrial ROS production	↓	↓	↓
Free radical leak (%FRL)	↓	↓	↓
Membrane unsaturation (1)	↓	↓	↓
mtDNA Oxidative damage (2)	↓	↓	↓
Oxidation-derived protein damage (3)	↓	↓	↓
Glycoxidation-derived protein damage (4)	↓	↓	↓
Lipoxidation-derived protein damage (5)	↓	↓	↓
Maximum lifespan	↑	↑	↑

↑, increase; ↓, decrease. The variations shown in the table for the biochemical and mitochondrial parameters correspond to rat liver during short-term (7-week) restrictions. Similar changes in most parameters have been found during long-term (1-year, or whole lifespan) caloric restriction in rat heart and brain, and during short-term MetR in rat heart and brain. The decrease in MitROS production and %FRL has been found both in 80% and in 40% MetR. (1) Measured as Double Bond Index and Peroxidizability Index; (2) measured as 8-oxo-7,8-dihydro-2'deoxyguanosine; (3) measured as the specific protein carbonyls glutamic semialdehyde and aminoadipic semialdehyde; (4) measured as the advanced glycoxidation endproduct nepsilon-(Carboxyethyl)lysine; (5) measured as the advanced lipoxidation endproducts nepsilon-(carboxymethyl)lysine and nepsilon-(malondialdehyde)lysine.

This agrees with the idea that there is a decrease in the flow of oxidative damage through the mtDNA in DR, which is similar to what we observe in long-lived compared to short-lived species. In other words, mitochondrial 8-oxodG repair, like tissue antioxidants in long-lived animals, is lower (instead of higher) in tissues of DR rodents because their rate of MitROS production is lower than it is in those fed ad libitum. Neither 8-oxodG repair nor endogenous antioxidants should be continuously maintained at levels higher than those required, as that would be energetically costly. Both the antioxidants and the mtDNA repair systems are inducible. Thus, they can be temporarily increased at moments of higher than normal oxidative damage and then quickly return to their basal levels when the situation is normalized. For instance, the repair of 8-oxodG is maximally induced in the rat kidney after only 6 hours of exposure to potassium bromate [29]. The lower steady-state oxidative damage in mtDNA is most efficiently and simply achieved in long-lived and DR animals by decreasing the rate of generation of damage (MitROS generation).

In contrast to the repair of 8-oxodG in mtDNA in the postmitotic tissues of long-lived animals caused by endogenous oxidative damage, the rate of repair of UV-induced (exogenous) damage to genomic DNA ("unscheduled" DNA synthesis) in dermal cultured fibroblasts (cells with mitotic capacity) is higher in long-lived than in short-lived mammalian species [30,31]. This is logical since it does not make sense to design a slowly aging organism if it will die soon due to externally induced damage (radiation in this case). The same kind of reasoning is also applicable to the observation that long-lived animals are usually more resistant to external sources of stress. This was found, for instance, for skin fibroblasts from different donor species exposed in culture to diverse external stresses including H_2O_2, paraquat, butyl-OOH, sodium arsenite or alkaline conditions [32]. Long-lived animal models have a low rate of endogenous aging which must go hand-in-hand with high levels of protection (or with rapidly inducible protective systems) against externally induced damage such as UV radiation or exogenous chemicals. Otherwise their superior longevity would not have the opportunity to be expressed.

Finally, a series of effects have been found in DR models, including mammals, nematodes, yeast and cell cultures [27, 33-36]. These effects include: increases in mitochondrial biogenesis and mitochondrial bioenergetic efficiency; up-regulation of multiple plasma membrane redox system (PMRS) enzymes and reduced levels of markers of oxidative stress. Together with: up-regulation of uncoupling proteins associated with a reduction in ROS production and increased resistance to oxidative and mitochondrial stress; decreases in cellular steady-state ROS levels as measured with dichlorodihydroflorescein diacetate; and a lack of changes, or even increases, in O_2 consumption. This is interesting for various reasons, including the finding that the concentration of complex I decreases considerably during protein [37], methionine [7,38,39] and dietary restriction [unpublished results].

In summary, lowering the rate of MitROS production seems to be a very conservative mechanism mechanism which has evolved both within (DR) and between species. It allows both long-lived species and DR animals to decrease steady-state oxidative damage to macromolecules, especially that to mtDNA, and probably the rate of accumulation of mtDNA mutations and the rate of aging too.

Protein restriction

As described in the previous section, much research has consistently found that DR decreases MitROS production and oxidative damage to macromolecules. However, the specific dietary factor that causes these beneficial changes remained unknown. A systematic series of studies was recently performed on rats to answer this question [37-39]. It is commonly believed that all the anti-aging effect of DR is due to the decreased caloric intake not to decreases in specific dietary components. The few studies that we are aware of on the subject do not support the possibility of life-long carbohydrate or lipid restriction increasing rodent longevity. According to the two published studies in which dietary lipid restriction was applied to Fischer 344 rats, their longevity remained unchanged [40,41]. The two available studies of carbohydrate restriction led to minor and contradictory changes in rat longevity [42,43]. In contrast, reconsideration of classic studies of protein restriction (PR) performed on rats and mice shows that PR increases maximum longevity in the large majority of cases [reviewed in 39]. Ten of the eleven published studies concerning rats or mice (and 16 of the 18 different life-long survival experiments contained in them) found that protein restriction increases maximum lifespan. The magnitude of this increase (a mean 19.2% increase in the 16 positive studies) was lower than that

typically found in 40% DR (about a 40% increase). The mean degree of PR applied in the 16 positive studies was 66.7%. Thus, assuming proportionality between the life extension and the degree of restriction (as is known to occur in DR) the increase in maximum longevity expected at 40% PR would be 11.5%. The decrease in protein intake therefore seems to be responsible for around one third of the total life extension effect of DR in rodents. A significant PR life extension effect which is lower than the total DR effect agrees with the widely accepted notion that aging has more than one single main cause.

It seems that, even though caloric restriction is one important mechanism underlying the effects of different dietary restriction regimes on lifespan and disease susceptibility, at least some beneficial effects of dietary restriction regimens may result from a mechanism other than an overall reduction in caloric intake. One such possible mechanism is the stimulation of cellular stress resistance pathways, which are strongly induced by intermittent (alternate-day) fasting–DR regimes that maintain overall food intake and body weight [44]. A recent study in *Drosophila* also suggests that the life extension effect of caloric restriction is not simply due to the number of calories [45]. The study did not use chemically defined diets, but the results were compatible with a specific role of the decreased protein intake in life extension. This was further supported by the observation that decreasing the amount of dietary casein from 4% to 2% and from 2% to 1% or 0.5% increases *Drosophila* maximum longevity. Furthermore, DR and protein restriction share many common effects in mammals, in addition to life prolongation. These include: transitory decreases in metabolic rate; boosting of cell-mediated immunity; decreases in IGF-1; increased hepatic protection against xenobiotics; and decreases in the incidence of tumours, glomerulosclerosis, or chronic nephropathy and cardiomyopathy [reviewed in 39].

But what is responsible for the decrease in MitROS generation in DR? Is the decrease in MitROS production due to PR, or is it due to restriction of some other dietary components or simply the overall reduction in calories? Recent research suggests answers to these questions. The effect of PR on MitROS generation and oxidative stress was studied in rats, without changing the amount eaten per day of the other dietary components. It was found that 40% PR decreases MitROS production, specifically at complex I, lowers the %FRL, and decreases 8-oxodG in mtDNA [46]. It also decreases specific markers of protein oxidative modification, membrane unsaturation and complex I content in rat liver mitochondria and tissue [37] (Table 1). Interestingly, the magnitude, direction of change, mechanisms and site of action of many of these changes are very similar to those found in DR. In the 40% PR studies an 8.5% caloric restriction in the PR group was unavoidable, since that is the caloric content of the proteins themselves that were withheld from the experimental animals. However, we recently found that a total food restriction that decreased the caloric intake by precisely 8.5% does not decrease MitROS generation, %FRL or 8-oxodG in mtDNA in rat liver (unpublished results). Thus, the decrease in MitROS generation and oxidative mtDNA damage observed during 40% PR was specifically due to the lower protein consumption of the PR group, not to its 8.5% lower caloric intake.

The possible effects of lipid or carbohydrate restriction without changing the intake of the other dietary components were also studied. It was found that neither lipid nor carbohydrate restriction changes MitROS production or oxidative damage to mtDNA in rat liver [39]. In agreement with a role for MitROS generation in aging, neither lipid nor carbohydrate restriction seems to increase maximum longevity in rats either. All this, together with the decrease in MitROS generation and increase in longevity caused by PR, demonstrates that proteins are the dietary component responsible for the decreases in MitROS generation and oxidative stress that take place in DR and possibly for part (about 30%) of the increase in maximum longevity during DR. The rest of the effect of DR on longevity (the other two thirds) must be due to other mechanisms which may depend on oxidative stress or not.

Methionine restriction

Since dietary protein is responsible for the decrease in MitROS generation during DR, the next step was to establish if some specific protein component(s) are responsible. Since it was known that L-methionine restriction (MetR), like PR, increases maximum longevity, it was logical to suspect that dietary methionine could be involved. It is known that MetR increases maximum longevity in rats and mice independently of energy restriction, although until recently there have been no clues as to what molecular mechanisms mediate this effect. Decreased methionine ingestion could be responsible for the PR-induced increase in longevity and for part of the life-extension effect of DR. In the mouse study, 65% MetR led to significant increases in maximum longevity (at least 10%) when the unfinished lifespan experiment was published [47]. This would agree in general terms with the mean increase in longevity at 40% PR calculated above from the different PR studies (a 11.5% increase) [39]. The rat MetR studies found a 44% increase [48] and an 11% increase [49] in maximum longevity at 80% MetR, which would be equivalent to a mean 14% increase at 40% MetR. This value is also within the range reported for MetR mice and for PR in rats and mice. The MetR studies available suggest that the reduced dietary intake of this amino acid could be responsible for all the life extension effect of PR. The decrease in methionine intake seems to be responsible for around one third (11-14% increase in longevity) of the life extension effect of DR in rodents (approximately a 40% increase in longevity).

Regarding oxidative stress, only changes in liver and blood glutathione (GSH), the major cellular thiol or redox buffer, had been studied in MetR models [48]. Thus, we decided to study the effect of MetR on MitROS generation and oxidative stress. We found that the protocol of 80% MetR (without caloric restriction), as well as that of 40% MetR (unpublished results) also decrease MitROS generation (mainly at complex I), %FRL, 8-oxodG in mtDNA, complex I content, specific markers of protein oxidative modification, and membrane unsaturation, in rat heart and liver mitochondria [38] These decreases were dose dependent and stronger at 80% than 40% MetR [unpublished results] (see Table 1). Strikingly, the pattern of many of these changes was very similar to those previously found in DR and PR. Both the occurrence and the intensity of the changes

at 40% MetR (without changing other dietary components) strongly suggest that MetR may be responsible for 100% of the decrease in MitROS generation and oxidative stress that occur at 40% PR and 40% DR, and possibly for all (PR) or part (DR) of the life extension effect.

Any treatment that increases maximum longevity must also decrease the incidence of age-related degenerative diseases [reviewed in 39]. It is known that excessive methionine dietary supplementation damages many vital organ systems and increases tissue oxidative stress. Thus, methionine supplementation increases plasma hydroperoxides and LDL-cholesterol, raises liver iron, lipid peroxidation, conjugated dienes, is hepatotoxic, alters liver antioxidant enzymes and glutathione, and decreases vitamin E levels in liver and heart. It also raises plasma, heart and aortic homocysteine levels leading to angiotoxicity, mitochondrial degeneration in arterial smooth muscle cells and accelerated aging of the rat vascular system, induces hypertension and coronary disease, and seems to accelerate brain aging [39]. Interestingly, the negative effects observed in rats fed high protein (50%) or high methionine (2%) diets for *2* years are similar. Thus, the high methionine and protein content of the Western diet could predispose people in the West to cardiovascular and other degenerative diseases. Most interestingly, the influence of dietary methionine on age-related changes is not limited to excess methionine dietary supplementation. Recent studies have found that the same MetR protocol that increased rodent longevity slows cataract development, minimizes age-related changes in T cells, increases macrophage migration inhibition factor, and lowers serum glucose, IGF-I and insulin levels in mice [47]. It also decreases visceral fat mass (by around 70%), plasma insulin, IGF-1, and insulin response to glucose, and avoids the age-related increases in blood cholesterol and triglycerides in rats [50]. The marked decrease in visceral fat mass described for MetR is striking and suggests that such a change, typical in DR animals, is not necessarily linked to a decreased caloric dietary intake, contrary to common belief. In summary, the association between methionine dietary intake and deleterious changes appears through a wide range of dietary concentrations covering both methionine restriction and supplementation below and above optimum dietary levels.

The capacity of MetR and PR to decrease MitROS generation, oxidative molecular damage and many diseases, and to increase longevity, is most interesting since these interventions are more easily practicable for humans than DR is. DR is a difficult manipulation for human populations due to: a) the marked difficulty in modifying acquired nutritional habits in human adults; b) the high risk of malnutrition; and c) possible decreases in acute resistance to the normally stressful human living conditions. The last two reasons present even bigger problems in the case of children and the elderly. At present, Western human populations consume levels of dietary protein that are 3-4 fold higher than the recommended values (0.5-0.75 g/kg body weight per day). Therefore, there is ample room for safely decreasing the amount of protein ingested. A lack of negative effects of diets containing as little as 0.5 g of protein per kg per day for periods of up to one year in human adult males was described as early as 1909 [51]. Decreasing only the ingestion of protein, or even of a single molecule (methionine) through emphasizing the intake of foods that are particularly low in methionine, without having to decrease the total ingestion of calories and the overall food intake, is much easier than DR for most people.

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