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On the importance of fatty acid composition of membranes for aging

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Abstract

The membrane pacemaker theory of aging is an extension of the oxidative stress theory of aging. It emphasises variation in the fatty acid composition of membranes as an important influence on lipid peroxidation and consequently on the rate of aging and determination of lifespan. The products of lipid peroxidation are reactive molecules and thus potent damagers of other cellular molecules. It is suggested that the feedback effects of these peroxidation products on the oxidative stress experienced by cells is an important part of the aging process. The large variation in the chemical susceptibility of individual fatty acids to peroxidation coupled with the known differences in membrane composition between species can explain the different lifespans of species, especially the difference between mammals and birds as well as the body-size-related variation in lifespan within mammals and birds. Lifespan extension by calorie-restriction can also be explained by changes in membrane fatty acid composition which result in membranes more resistant to peroxidation. It is suggested that lifespan extension by reduced insulin/IGF signalling may also be mediated by changes in membrane fatty acid composition.

Keywords: Membrane fatty acids; Lipid peroxidation; Maximum lifespan; Calorie-restriction; Insulin/IGF signalling; Docosahexaenoic acid

1. Introduction

Despite being a very long-living species, most of us desire more. Humankind's first literary achievement, the 4000 year old "Epic of Gilgamesh", tells the story of a search for immortality (George, 1999). The maximum lifespan of mammal species increases allometrically with body mass (Sacher, 1959), with the maximum lifespan of mice being 3–4 years and for elephants ~80 years. Although elephants are much larger than humans, they are shorter-living than *Homo sapiens* with has a maximum lifespan of ~115 years (Carey and Judge, 2000).

Aging is measured demographically as an increase in the "age-dependent mortality". This is a reflection that death results from a variety of causes and for many diseases the biggest risk factor is age. Undoubtedly, there is both a genetic and an environmental contribution basis to aging. In humans, studies of Danish twins suggest that the heritability of longevity is 0.26 for males and 0.23 for females (Herskind et al., 1996). Theories of aging are of two types; those that seek to explain "why" aging occurs (evolutionary theories) and those that seek to explain "how" aging occurs (mechanistic theories). These two types of theories are not independent of each other, in that evolutionary theories must operate within the constraints of the mechanisms that cause aging. Most multicellular animals have a finite maximum lifespan yet we do not know the cause of this fundamental difference between species.

This contribution will describe a mechanistic theory of aging that for convenience I have called the *membrane pacemaker* theory of aging. It is not a completely new theory and can be regarded as an extension of the *oxidative stress* theories of aging. It is strongly

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influenced by the work of Pamplona and colleagues (for reviews see Pamplona et al., 2002a; Pamplona and Barja, 2003) and extends their perspective beyond the lifespan comparison between species. Mechanistic theories of aging should give insight to four paradigms. These are; (a) the changes that occur in individuals during aging, (b) the physiological treatments that alter lifespan and thus aging, (c) the different lifespans of strains and specific mutants within species, and (d) the different maximum lifespans that are characteristic of species. After describing the evolution of the *membrane pacemaker* theory, I will discuss its contribution to understanding these four paradigms.

2. The *membrane pacemaker* theory and a link to lifespan?

Within mammals and birds, about 60% of the statistical variation in maximum lifespan between species within the group can be explained by body mass (Sacher, 1959; Lindstedt and Calder, 1976). Although birds and mammals differ in lifespans, in both groups of homeotherms the allometric exponent relating lifespan to body mass is ~0.20. Indeed, at this broad comparative level of very different-sized animals, the "rate of living" theory of aging holds within (but not between) these two groups of homeotherms, in that maximum lifespan is associated with a species metabolic intensity and this maximum lifespan loosely approximates a fixed number of shorter events. These shorter events include muscle twitch time, circulation and filtration of blood plasma, respiratory and metabolic cycles, embryonic development, growth, sexual maturation, and even minimum population doubling time (see Lindstedt and Calder, 1981). All these events have similar allometric exponents and are linked by the concept of "physiological time" to the allometric relationship between body mass and metabolic rate of mammals and birds, where small birds and mammals have higher mass-specific metabolic rates than large birds and mammals.

The *membrane pacemaker* theory of metabolism has been reviewed/described in detail elsewhere (Hulbert and Else, 1999, 2000). The *membrane pacemaker* theory of metabolism is currently the only mechanistic explanation for the very different and distinctive metabolic rates of different-sized vertebrates. In summary, it proposes that:

(i) membrane-associated activities (e.g. maintenance of the plasmalemmal Na^+ gradient and mitochondrial H^+ gradient) are very significant and dominant components of the BMR,
(ii) that when BMR varies between species, all the activities that constitute BMR vary in unison,
(iii) species with high mass-specific BMR have highly polyunsaturated membranes and species with low BMR have membranes that are less polyunsaturated,
(iv) highly polyunsaturated membranes have distinctive physical properties that cause the proteins in the membranes to have a high molecular activity and thus result in higher rates of metabolic activities of cells, tissues and consequently the whole animal.

The long chain n-3 PUFA, docosahexaenoic acid (DHA or 22:6n-3) is particularly important and its abundance in tissue phospholipids is strongly correlated with body size in mammals (Couture and Hulbert, 1995; Hulbert et al., 2002b) and birds (Hulbert et al., 2002a; Brand et al., 2003). Fig. 1 shows the allometric relationships between body mass and DHA and OA content (oleic acid or 18:1n-9) in muscle phospholipids of both mammals and birds. Although much of the data

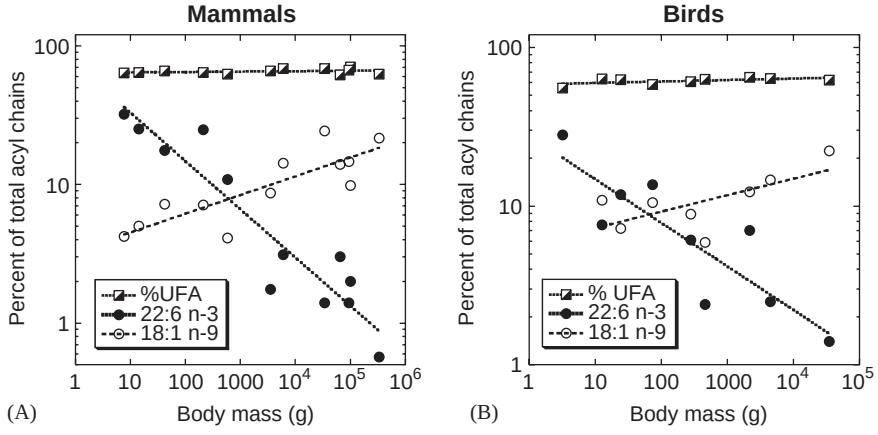

Fig. 1. A comparison of the fatty acid composition of skeletal muscle phospholipids from mammal species (A) and bird species (B) that vary in body size. The three parameters shown are: (i) content of total unsaturates (% UFA), (ii) content of the monounsaturate oleic acid (18:1 n-9), and (iii) content of the omega-3 polyunsaturate docosahexaenoic acid (22:6 n-3). The mammal data are from Hulbert et al. (2002b) and the bird data are from Hulbert et al. (2002a).

supporting the theory is correlative, there is also experimental evidence where membrane lipid composition has been altered and predicted changes in membrane function observed (e.g. Stillwell et al., 1997; Else and Wu 1999; Wu et al., 2004). The finding of similar body-size trends previously observed in mammals, in bird species (Hulbert et al., 2002a,b; Brand et al., 2003; Turner et al., 2003; Else et al., 2004), is strong evidence supporting the theory.

On a simplistic level it is suggested that it is predominantly the physical properties that acyl chains impart to membranes are important for their effects on metabolic rate (e.g. Wu et al., 2001), while it is their chemical properties that are important for their influence on aging. Individual acyl chains differ greatly in their chemical propensity for oxidative damage (Holman, 1954). Fig. 2 presents the relative peroxidisability of acyl chains found in membrane phospholipids. The n-3 PUFA are more peroxidation-prone than n-6 PUFA and within each PUFA class there is 4-fold increase in peroxidisability between the short- and long-chain fats. DHA (22:6) is 320-fold more susceptible to peroxidation than OA (18:1).

A link between MR and lifespan was suggested about a century ago (Rubner, 1908) and later elaborated into the *rate of living* theory (Pearl, 1928). This theory has a number of problems that still have to be resolved. There is considerable variation in lifespan of animals that cannot be explained by variation in metabolic rate. For example, birds live much longer than mammals although they have similar metabolic rates (Holmes and Austad, 1995). While rats and mice have maximum lifespans of 3–4 years, the similar-sized naked mole rat lives for ~28 years but this is not due to a dramatically reduced MR (O'Connor et al., 2002).

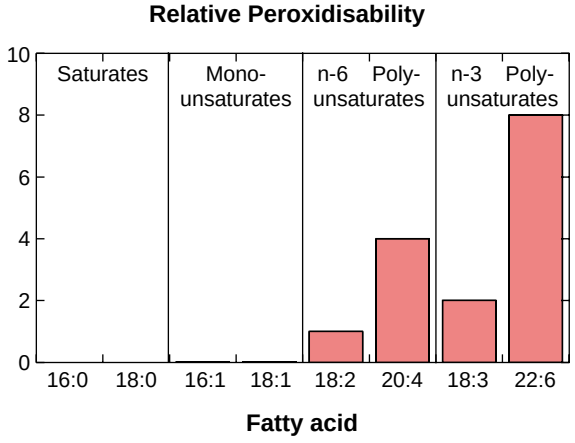

Fig. 2. The relative susceptibilty of different common membrane fatty acids to peroxidation. The individual fatty acids are identified by a numbering system. The number before the semi-colon indicates the number of carbons and the number after the colon represents the number of double bonds in the acyl chain. The relative values were empirically determined and are taken from Holman (1954).

Within species there is no evidence of a direct connection between MR and lifespan (Speakman et al., 2000; Hulbert et al., 2004a) and no relationship between daily food consumption and individual lifespan (Hulbert et al., 2004c). Similarly, voluntary exercise does not shorten the lifespan of either rats (Holloszy et al., 1985) or humans (Lee et al., 1995).

The *membrane pacemaker* theory of aging proposes that a missing piece of the jigsaw is the acyl composition of membrane bilayers. Membranes that have different fatty acid composition will differ dramatically in their susceptibility to oxidative damage and this can account for much of the to-date unexplained variation in lifespan.

In the 1950s, the *free radical* theory gave a molecular basis to the *rate-of-living* theory of aging (Harman, 1956). This proposed that normal oxygen consumption by mitochondria inevitably results in the production of oxygen free radicals, which in turn damage important biological molecules and the accumulation of this damage is manifest as aging. This has further evolved into the *oxidative stress* theory of aging following the observation that not all "reactive oxygen species" (ROS) are free radicals. The *oxidative stress* theory is currently the most popular theory of aging and there is much evidence, mainly indirect, to support it (Sohal and Weindruch, 1996). The *rate-of-living* and *oxidative stress* theories of aging are sometimes theoretically reconciled by supposing that higher levels of ROS are generated by a higher MR (Sohal and Weindruch, 1996; Beckman and Ames, 1998).

Free radicals are molecules capable of independent existence that contain one or more unpaired electrons (Halliwell and Gutteridge, 1999). An important free radical in biological systems is the superoxide radical, $O_2^{\bullet -}$, which is produced as a by-product of normal mitochondrial respiration. ROS is the collective term used to describe both radical and non-radical derivatives of oxygen. Antioxidant defence system (ADS) consists of a number of mechanisms to minimise the effects of ROS on biological molecules. Scavenger antioxidants include enzymes and non-enzymic small molecules. Some important ROS and important parts of the ADS are listed in Table 1. A detailed discussion of both ROS and ADS is beyond the current contribution and is excellently covered in the encyclopaedic "Free Radicals in Biology and Medicine" (Halliwell and Gutteridge, 1999). There are also enzyme systems to remove and repair oxidatively damaged molecules not discussed here.

Normal mitochondrial respiration produces small amounts of ROS which is often measured as mitochondrial H_2O_2 production. Superoxide is likely produced in the protonated state at complex III within the inner mitochondrial membrane (Muller, 2000). Initial measurements suggested 1–3% of normal oxygen consumption might be

Table 1
Some examples of Reactive Oxygen Species (ROS) and Lipid Aldehydes and Antioxidant Defence Systems. "Scavenger" antioxidants react with and remove ROS while "Prevention" antioxidants are proteins that bind ROS and thus hinder formation of additional ROS

Reactive oxygen species (ROS) and aldehydes	
Radicals	Non-Radicals
Hydroxyl ($HO^{\bullet}$)	Hydrogen peroxide H_2O_2
Superoxide ($O_2^{\bullet -}$)	Singlet oxygen $^1O_2$
Lipid peroxyl ($LOO^{\bullet}$)	Lipid hydroperoxide LOOH
Alkoxyl ($RO^{\bullet}$)	4-Hydroxy-2-hexenal HHE
Peroxyl ($ROO^{\bullet}$)	4-Hydroxy-2-nonenal HNE
Antioxidant defence systems	
Small molecules	Large enzymes
"*Scavenger*" *antioxidants*	
Ascorbic acid (vitamin C)	Superoxide dismutases (SOD)
Glutathione (GSH)	Glutathione peroxidases (GPx)
α-Tocopherol (vitamin E)	Catalases (CAT)
Carotenoids, coenzyme Q	Other peroxidases
"*Prevention*" *antioxidants*	
Albumin, metallothionein, transferrin, ceruloplasmin, myoglobin and ferritin	

diverted to ROS production (Chance et al., 1979) however recent measurements suggest that it is much less and is estimated to be a maximal 0.15% in rat mitochondria with palmitoyl carnitine as subtrate (St-Pierre et al., 2002). ROS production is also strongly influenced by mitochondrial membrane potential. In one study (Miwa et al., 2003) when membrane potential dropped by only 10 mV, ROS production decreased by 70%. This is likely important in muscle in minimising mitochondrial ROS production during animal activity (Herrero and Barja, 1997). ROS production cannot be simply calculated as an invariant proportion of mitochondrial oxygen consumption.

Once produced, ROS will oxidatively damage proteins, nucleic acids and lipids. The *membrane pacemaker* theory of aging emphasises the damage done to membrane fatty acids for a number of reasons. The first is that membrane fatty acids are located at the primary site of ROS production (the mitochondrial membrane) and are in such close proximity that no antioxidant defence system will be able to prevent their peroxidation. The second is that lipid peroxidation is an autocatalytic (and thus self-propagating) chain reaction that once initiated will continue unless stopped by antioxidant mechanisms. The third reason is that many of the products of lipid peroxidation are very reactive molecules themselves (see Table 1) and are thus very potent damagers of other molecules.

The protonated and uncharged form of superoxide ($HO_2^{\bullet}$) is reactive enough to initiate peroxidation of lipids as are hydroxyl ($HO^{\bullet}$), alkoxyl ($RO^{\bullet}$) and peroxyl ($ROO^{\bullet}$) radicals. Lipid peroxidation is propagated due to the fact that the initial lipid radical produced generally reacts with a membrane-located oxygen molecule to produce a lipid peroxyl radical ($LOO^{\bullet}$) that is capable of initiating another cycle of lipid peroxidation and in the process itself becomes a lipid hydroperoxide (LOOH) molecule, which is also a ROS molecule (Halliwell and Gutteridge, 1999).

The products of membrane lipid peroxidation are many and include the hydroxyl radical, lipid peroxyl radicals, lipid hydroperoxides as well as hydrocarbons and aldehydes. Two hydrocarbons produced, ethane and pentane, are volatile and exhaled. Measurement of their exhalation rate enables assessment of the rate of lipid peroxidation in vivo (see Kneepkens et al., 1994). Ethane is from n-3 PUFA whilst pentane is the result of peroxidation of n-6 PUFA. In human exhaled breath CO_2 concentration is ~2 mmol/l, while ethane is ~100 pmol/l (i.e. 1/20 millionth the CO_2 concentration).

The aldehydes produced by lipid peroxidation include hydroxynonenal (HNE, from n-6 PUFA) and hydroxyhexenal (HHE, from n-3 PUFA) and it has been proposed that much of the cellular and subcellular damage associated with oxidative stress is attributable to the deleterious actions of these peroxidation products. HNE decreases mitochondrial membrane fluidity (Chen and Yu, 1994) and HNE and HHE inhibit the mitochondrial adenine nucleotide translocase (Chen et al., 1995). HHE is a potent inducer of the mitochondrial permeability transition (Kristal et al., 1996) and HNE at high concentrations is cytotoxic, while at intermediate concentrations it inhibits DNA and protein synthesis and stops cell growth (Esterbauer et al., 1991). HNE stimulates mild uncoupling of mitochondria through the uncoupling proteins (UCP1, UCP2 & UCP3) and the adenine nucleotide translocase (Echtay et al., 2003). This role is interesting because it likely is part of a negative-feedback system keeping mitochondrial ROS production under control. Increased mitochondrial ROS production will result in increased HNE, which in turn will increase mitochondrial proton leak, consequently decrease mitochondrial membrane potential which in turn will decrease ROS production. The consequence is that the whole loop might act as a negative feedback homeostatic system limiting mitochondrial ROS production.

The fundamental aspects of the *membrane pacemaker* theory of aging are conceptually described in Fig. 3. The fact that membrane fatty acid composition differs in various situations (such as between species, during calorie-restriction, etc.) coupled with the different peroxidation susceptibilities of individual fatty acids, and the potency of lipid peroxidation products is the key addition of the *membrane pacemaker* theory to the *oxidative stress* theory of aging. Most of the interactions

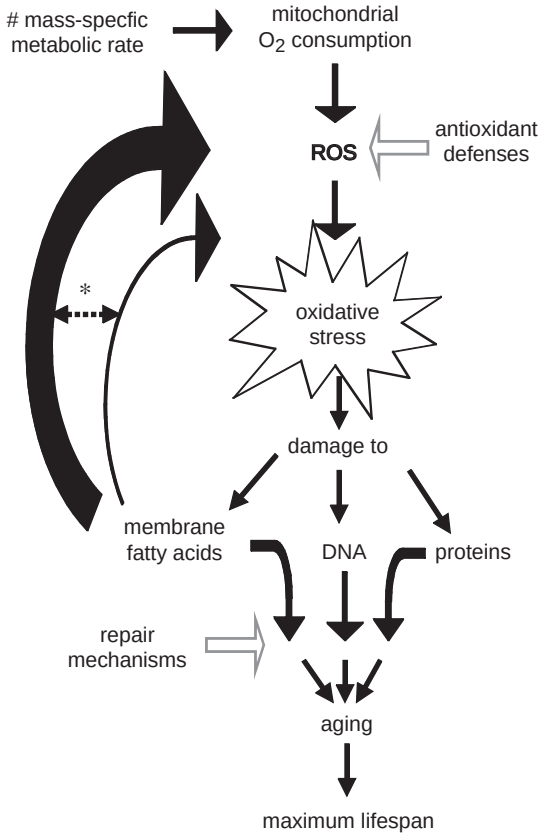

Fig. 3. Schematic representation of the membrane pacemaker theory of aging. Solid black arrows represent stimulatory influences while white arrows represent inhibitory influences. The fatty acid composition of membranes influences mass-specific metabolic rate largely via physical effects (indicated by #). Membrane fatty acid composition also influences the degree of positive feedback on oxidative stress by the chemical properties, specifically the different susceptibilities of individual fatty acids to peroxidation (indicated by *).

shown in this figure describe the oxidative stress theory of aging as generally perceived, however the feedback influence from membrane lipid peroxidation to ROS production is the additional feature suggested by the membrane pacemaker theory. This feedback is variable and depends on the fatty acid composition of the membrane. Membranes with large amounts of highly polyunsaturated fatty acids will have a large positive feedback influence, while membranes with a low PUFA content will have only a very small feedback influence.

3. Changes during the lifetime of individuals: the process of aging

Aging can be defined as the accumulation of diverse deleterious changes in cells with time that increase the risk of disease, the breakdown of homeostatic control and death (Harman, 2001). Ironically, the acquisition of cell immortality can be a manifestation of the aging process. However the process of aging is also manifest as senescence, which involves loss of functional ability at the cellular, tissue and whole organism level. While there is no doubt that damage to genetic material and proteins represents important deleterious change, oxidative damage to membrane lipids is also likely an important part of senescence, especially in view of the role of membranes in normal metabolism and in cell proliferation. Membranes become more rigid during senescence in animals and the processes of lipid peroxidation are responsible for this change (Choe et al., 1995). Interestingly, senescence in plants is also associated with membrane deterioration due to lipid peroxidation (Thompson et al., 1997).

As animals age, lipid peroxidation products accumulate. Old rats have greater endogenous levels of HNE and enhanced HNE formation upon stimulation of in vitro lipid peroxidation than young rats (Chen and Yu, 1994). The accumulation of "age pigment" (lipofuscin) in post-mitotic cells is the most consistent and phylogenetically constant morphological change of aging. In vitro studies suggest that granules of lipofuscin contain the end products of lipid peroxidation and lipoxidatively damaged protein (Porta, 2002).

Lipid peroxidation during aging does not necessarily mean that membranes will become less polyunsaturated with age. Phospholipids are more peroxidation-prone than triglycerides (e.g. Catala and Cerruti, 1997). However, when lipid peroxidation is induced in isolated liver cells there is no change in the fatty acid composition of phospholipids but a selective decrease in PUFA contents of triglycerides. This is because continual membrane remodelling eliminates peroxidised fatty acids in phospholipids replacing them with fresh PUFA from the triglyceride pool (GironCalle et al., 1997). Some studies have shown membrane fatty acid composition becomes more polyunsaturated with age and thus more prone to peroxidation (e.g. Laganiere and Yu, 1989) however other comparisons show the opposite trend (e.g. Andersson et al., 2000, 1998; Vessby et al., 1994).

The rate of lipid peroxidation is not constant and appears to increase with age. Exhalation of both ethane and pentane both increase with chronological age in rats (Sagai and Ichinose, 1980; Matsuo et al., 1993) and pentane exhalation increases with age in humans (Zarling et al., 1993) and flies (Sohal et al., 1985).

We know remarkably little of the biology that precedes natural death because most biochemical measurement requires the death of the individual being investigated and thus it is not possible to know the time before natural death. I speculate that towards the end of an individual's natural life, the capacity of antioxidant defences are exceeded and that when this occurs there is a rapid positive-feedback increase in lipid peroxidation with the consequent breakdown of cellular and organismal homeostatic systems and death from old age ensues.

4. Physiological treatments that alter the rate of aging

The only treatment that extends lifespan in a wide range of species is dietary calorie-restriction. It is one of the most examined aspects of aging and there are several excellent reviews (e.g. Merry and Holehan, 1994; Yu, 1994, 1996; Ramsey et al., 2000; Merry, 2002; Masoro, 2002). It has been most studied in rats and mice where it extends both mean and maximum lifespan. Within limits, the degree of life extension is linearly related to the degree of dietary calorie restriction and is not dependent on the age at which it is imposed (Merry, 2002). Calorie-restriction exerts its effects by both slowing the intrinsic rate of aging and suppressing pathogenesis (Yu, 1996). It can exert its effects very rapidly. In a series of nutritional-shift experiments where groups of *Drosophila melanogaster* were shifted between full-diets and restricted-diets at various times during their adult life, mortality rates changed within two days of the dietary shift (Mair et al., 2003).

The precise mechanisms of calorie-restriction are not known. A detailed discussion of the potential mechanisms is covered in detail elsewhere (e.g. Masoro, 2002). It does not decrease mass-specific MR of rodents (McCarter et al., 1985; Masoro, 2000) or *Drosophila melanogaster* (Hulbert et al., 2004a). Calorie-restriction decreases oxidative damage to many cellular macromolecules, including lipids, proteins and nucleic acids. Measurement of in vitro ROS production, such as the rate of mitochondrial H_2O_2 production (Sohal et al., 1994; Gredilla et al., 2001) is reduced. That the rate of in vivo lipid peroxidation is also decreased by calorie-restriction is demonstrated from its effects on ethane and pentane exhalation (Habib et al., 1990; Matsuo et al., 1993) as well as the urinary excretion of aldehydes (De Tata et al., 2001). The effect of calorie-restriction on antioxidant defences is more equivocal.

Of particular interest for the *membrane pacemaker* theory of aging is the finding that calorie-restriction is associated with substantial changes in the fatty acid composition of membranes resulting in a decreased susceptibility to lipid peroxidation (Laganiere and Yu, 1987). Since this seminal observation, similar fatty acid changes have been reported for phospholipids from other tissues (Laganiere and Yu, 1991; Tacconi et al., 1991; Laganiere and Fernandes, 1994; Lee et al., 1999; Cefalu et al., 2000), as well as for different classes of liver phospholipids (Cha and Jones, 2000; Jeon et al., 2001). However, some studies have either not found such changes (e.g. Pamplona et al., 2002b,c; Ramsey et al., 2004) or have only observed changes following long-term calorie restriction (Lambert et al., 2004). In our laboratory we have found in mice that such membrane changes occur early, are responsive to the degree of calorie restriction, and precede other changes. Within one month of calorie-restriction there was a significant 25% decrease in the peroxidation index of membranes lipids, but no statistical change in mitochondrial ROS production or tissue antioxidant status (S.C. Faulks, P.L. Else and A.J. Hulbert, unpublished observations). A reduced oxidative stress would be expected to result in greater membrane PUFA content and thus these membrane changes are not the consequence of a reduced oxidative stress (as suggested by some authors) as they are in the opposite direction. They are more likely a causal factor in the reduction in oxidative damage associated with calorie-restriction.

How such changes in membrane composition come about following calorie-restriction is unknown. Merry (2002) suggests there are two possible hormonal candidates; insulin and thyroid hormones. Blood concentrations of insulin and triiodothyronine are significantly lowered by calorie-restriction (see Masoro, 2002). Many studies have shown that low thyroid hormone levels affect membrane fatty acid composition with the most consistent finding being an decrease in the ratio of 20:4/18:2 (see Hulbert, 2000). The lowering of this ratio, with it's consequent lowering of the peroxidation-susceptibility of membranes, is a consistent finding of calorie-restriction. Similarly, desaturase enzyme activities are decreased when insulin levels fall which will also influence membrane fatty acid composition (Brenner, 1981). In diabetes, phospholipids containing 20:4 decrease while those containing 18:2 increase (McHowat et al., 2000; Head et al., 2000; Han et al., 2000). The potential role of insulin is highlighted by the finding that mitochondrial changes following calorie-restriction in rats are reversed by insulin treatment (Lambert and Merry, 2004).

Calorie-restriction also influences the secretion and levels of growth hormones (GH) and insulin-growth-factor-1 (IGF-1) in rodents (see Masoro, 2002). To my knowledge nothing is known as to whether these influence the fatty acid composition of membranes. An interesting perspective is that IGF-1 and insulin-signalling pathways involve membrane-bound receptors and changes in membrane fatty acid composition have been shown to influence both IGF-1 signalling (Lanson et al., 1997) and insulin-signalling (see Hulbert et al., 2004b).

5. Insight from long-living strains and mutants within a species

Within many species there are strains that differ in lifespan. The nature of scientific method suggests the most important information will come from those strains/mutants that slow aging and extend lifespan. This is because many treatments that are capable of shortening lifespan have little to do with the normal aging process. Artificial selection has produced strains of *Drosophila melanogaster* that differ in longevity but

not in their mass-specific MR (Arking et al., 1988), nor their antioxidant defences (Mockett et al., 2001), but mitochondrial H_2O_2 production is low in the long-living *Drosophila* strain (Ross, 2000).

Genetic manipulation of various antioxidant enzymes and repair enzymes have been used to investigate the mechanisms of aging (see Muller et al., 2003). These studies have shown that some antioxidant systems have a greater influence than others. For example, ablation of the extracellular and cytosolic SOD (CuZnSOD) in mice increases sensitivity to exogenous oxidative stress but has only a small influence on lifespan. However, ablation of the SOD in mitochondria (MnSOD) is neonatally lethal when homozygous but although heterozygotes show diminished antioxidant defence they have no change in lifespan. Over-expression of the cytosolic SOD antioxidant enzyme does not extend lifespan of mice and may even shorten life expectancy by interfering with immune function (Muller et al., 2003). The product of the *Gpx4* gene is a phospholipid hydroperoxidase which limits the production of the highly reactive hydroxyl radical and thus inhibits lipid peroxidation. Mice homozygous for null mutations of the *Gpx4* gene die early during embryonic development (Muller et al., 2003). It may be that, as metabolic control theory studies suggest is the situation for most enzymes (see Fell, 1997), antioxidant enzymes are normally present in excess and increases in their abundance does not thus result in greater effect. Similarly, although complete loss of the enzyme (e.g. from homozygous null mutations) may have a consequence, partial loss (e.g. in heterozygotes) may not exhibit any significant effect.

Interference with oxidative damage repair mechanisms can also influence aging and shorten lifespan in mice. For example, null mutation of the enzyme responsible for the repair of oxidised methionine results in a shortened lifespan (Moskovitz et al., 2001). Manipulation of the mitochondrial DNA polymerase to remove it's capacity to repair mitochondrial DNA mutations, results in both premature aging and a shortened lifespan in mice (Trifunovic et al., 2004). An intriguing finding is that null mutation of the $p66^{shc}$ gene results in mice with enhanced resistance to oxidative stress and an increased lifespan (Migliaccio et al., 1999). The $p66^{shc}$ protein is an important signalling protein in cellular apoptosis following oxidative stress.

In *Drosophila melanogaster*, the long-living CHICO-mutant with reduced insulin/IGF-signalling, does not have a reduced mass-specific MR (Hulbert et al., 2004a) but lives longer than wild-type *Drosophila,* when both are provided with food of the same energy density (Clancy et al., 2002). It maintains this difference, with both strains living longer as the energy density of the food is decreased. The CHICO strain achieves a peak lifespan at a higher food concentration than the wild-type flies but this peak lifespan is the same as that of wild-type flies at a lower food concentration. This interesting finding suggests that a diminished insulin/IGF signalling may extend lifespan by the same mechanism as calorie-restriction in these flies (see Gems et al., 2002).

Little is known of the biochemical mechanisms whereby reduced insulin/IGF-signalling extend lifespan. To my knowledge there are no measurements of membrane fatty acid composition of these mutants compared to wild-type strains. However, if the reduction in insulin/IGF-signalling is analogous to the reduced insulin-signalling associated with type-1 diabetes then the long lifespan of these strains may be mediated by changes in membrane fatty acid composition and its consequent diminution of lipid peroxidation. This is because insulin stimulates the desaturase enzymes responsible for increased polyunsaturation of fatty acids (Brenner, 1981). For example, when diabetes is induced in rats, there are marked changes in the fatty acid composition of heart phospholipids characterised by decreases in the content of the highly polyunsaturated 20:4 and 22:6 acyl chains, and increases in the less polyunsaturated 18:2 acyl chain (McHowat et al., 2000). Such changes in membrane composition will result in decreased susceptibility to membrane lipid peroxidation and if also manifest in mutant strains with lower insulin/IGF-signalling pathways may be the biochemical mechanism responsible for their extended lifespan.

An common observation concerning the lifespan of different strains within a species is that the relationship between body size and lifespan is the opposite of the relationship between species. Commonly, the long-living strains within a species are small and this seems to be associated with low IGF levels in these small individuals. In a study of body weight and longevity of 598 genetically heterogeneous mice, low body mass early in life was found to be strong correlate (and thus predictor) of lifespan (Miller et al., 2002). Between breeds of dogs, small body size is also associated with a long lifespan (Li et al., 1996). In the case of mice, low body mass early in life was significantly correlated with low IGF-1 levels in females (Miller et al., 2002) and in dogs, body size parallels IGF-1 levels but not growth hormone secretory capacity (Eigenmann et al., 1984).

6. Differences in maximum lifespan between species

Maximum lifespan is a species-characteristic and was used in the earliest attempts to understand the mechanisms of aging (e.g. Rubner, 1908). Maximum lifespan is allometrically related to body mass in both mammals (Sacher, 1959) and birds (Lindstedt and Calder, 1976), and has been used as evidence that the "rate of living" theory of aging operates on a very broad scale in both mammals and birds. However, this is not supported by

some specific comparisons within each group and also notably between mammals and birds, where birds are much longer-living than similar-sized mammals (Holmes and Austad, 1995).

The large differences in lifespan between species have been used to examine the importance of various mechanisms involved in aging. It was shown that long-living species had, surprisingly, low anti-oxidant defences (Perez-Campo et al., 1994, 1998). This has been observed for a number of anti-oxidant defence systems and suggests that antioxidant defences are not limiting for the aging process. It is also compatible with the finding that experimental addition of anti-oxidant defences to animals (either by dietary manipulation or genetically enhanced expression) does not increase maximum lifespan. This does not negate the fact that ablation of anti-oxidant defences can significantly decrease lifespan. From an evolutionary perspective, it would be surprising if an easy modification (an increased level of antioxidant defences) had not already occurred so that antioxidant defences were already optimal in animal species.

Following the initial observations that membrane fatty acid composition in mammals varied with body mass (Gudbjarnason et al., 1978; Couture and Hulbert, 1995), it was pointed out that the different membrane composition of mammals were related to their maximum lifespans (Pamplona et al., 1998, 1999a, 2000; Portero-Otin et al., 2001). Short-living small mammal species have membrane lipids high in PUFA and are thus very prone to lipid peroxidation (Pamplona et al., 1998, 1999a) and this is associated with elevated lipoxidative damage to proteins in small mammals (Pamplona et al., 2000).

Several investigators have used the 9-fold difference between the lifespans of pigeons and rats to investigate the mechanisms of aging. The antioxidant defences of tissues from pigeons and rats do not differ (Ku and Sohal, 1993; Barja et al., 1994). Mitochondrial H_2O_2 production by rat tissues is slightly greater than that of pigeon tissues but not 9-fold different (Ku and Sohal, 1993; Barja et al., 1994). While Barja et al. (1994) suggested rat mitochondria produce more ROS relative to oxygen consumption than pigeon mitochondria, Ku and Sohal (1993) suggested there were very small differences between the two species. A more recent rat–pigeon comparison found no difference in H_2O_2 production by heart mitochondria (St-Pierre et al., 2002). A similar bird-mammal comparison used the 7-fold difference in lifespan between canaries and parakeets compared to mice and showed the ratio of H_2O_2 production to respiration in heart mitochondria was the same in the mouse and parakeet but reduced in the canary (Herrero and Barja, 1998). A low mitochondrial ROS production might explain some of the relative longevity of birds compared to mammals, but it does not seem to be a complete explanation.

The mitochondrial membranes of pigeon liver and heart are less unsaturated than rat liver and heart mitochondria (Pamplona et al., 1996, 1999b) and the same difference has been demonstrated for the heart mitochondria of canaries and parakeets compared to mice (Pamplona et al., 1999c). Membrane fatty acid composition of bird tissues is related to body mass in a similar manner to that previously observed in mammals, namely small species have membrane lipids that are more polyunsaturated than large bird species (Hulbert et al., 2002a,b; Brand et al., 2003). Interestingly, bird membranes have a lower ratio of n-3/n-6 PUFA than do mammal membranes (Hulbert, 2003). This means that the membranes of birds are more resistant to lipid peroxidation than the membranes of similar-sized mammals. Thus not only does membrane peroxidisability correlate with body-size-related differences in maximum lifespan within mammals and birds but it also correlates with the lifespan difference between mammals and birds. This is illustrated in Fig. 4 where the data for skeletal muscle phospholipids and liver mitochondrial phospholipids of a variety of different-sized species of bird and mammals are plotted against the maximum lifespan of the species. For both membranes, birds follow the same relationship as mammals. The allometric equations describing these relationships show that a 24% decrease in the peroxidation index of liver mitochondrial phospholipids and a 19% decrease in the peroxidation index of skeletal muscle phospholipids is associated with a doubling of lifespan.

As noted at the beginning of this contribution, *Homo sapiens* is a very long living species of mammal, yet we are not sure why. It is thus of interest that membranes from humans have a very low peroxidation index compared to other mammal species (see Fig. 4) and this may explain our relatively long lifespan.

7. Conclusions

The *membrane pacemaker* theory of aging includes all the mechanistic details that are part of the *oxidative stress* theory of aging. Namely, that mitochondria are important participants in the aging process; that mitochondrial oxygen consumption inevitably results in the production of reactive oxygen species; and that this ROS production also damages other biological molecules, such as nucleic acids, proteins and lipids. Any additional production of reactive molecules apart from normal mitochondrial oxygen consumption will add to this damage and exacerbate aging. The *membrane pacemaker* theory emphasises the role of membrane fatty acid composition in determining the susceptibility

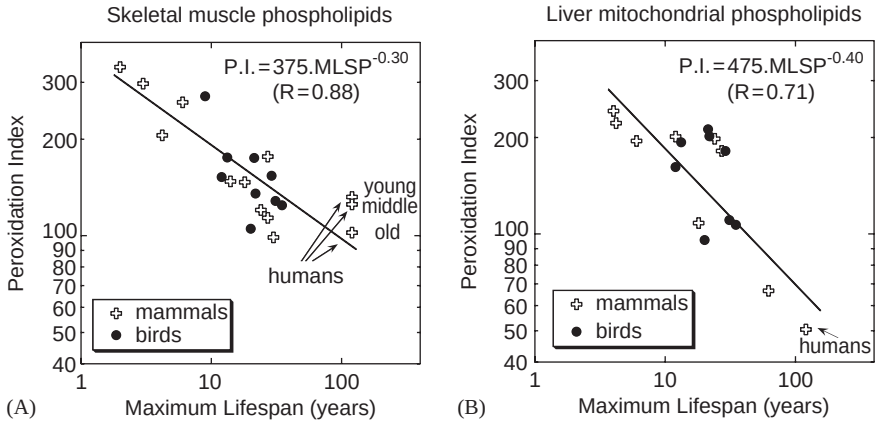

Fig. 4. The relationship between maximum lifespan of mammal and bird species and the peroxidizability index of their skeletal muscle phospholipids (A) and liver mitochondrial phospholipids (B). Peroxidation index was calculated from the fatty acid composition of (a) skeletal muscle phospholipids from mammals (data from Hulbert, Rana and Couture, 2002) and birds (data from Hulbert et al., 2002), and (b) liver mitochondrial phospholipids from mammals (data from Porter et al., 1996) and birds (data from Brand et al., 2003). The data for human skeletal muscle are the control groups from Andersson et al. (2000) for 23 year old men, Andersson et al. (1998) for 44 year old men and from Vessby et al. (1994) for the 70 year old men. The data for the human liver mitochondria are from Benga et al. (1978). An earlier version of Fig. 4A has previously appeared in Hulbert (2003).

of different biological systems to oxidative damage and highlights the role of PUFA together with the products of lipid peroxidation in the aging process. It suggests that death from old age likely involves an explosive and uncontrollable increase in lipid peroxidation. It proposes that membrane fatty acid composition is an important determinant of the maximum lifespan of different species. It proposes that the life-extending effect of caloric restriction is primarily due to its influence on membrane fatty acid composition resulting in peroxidation-resistant membranes and consequently less oxidative damage to cellular components. It suggests that although little is known about the effects of various lifespan-influencing mutations on membrane fatty acid composition, it is an area worthy of future investigation. It suggests that reduced insulin/IGF signalling might extend lifespan via modification of membrane fatty acid composition.

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