Experimental Gerontology 48 (2013) 1362–1368

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Age-induced perturbation in cell membrane phospholipid fatty acid profile of longevity-selected *Drosophila melanogaster* and corresponding control lines

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ARTICLE INFO

Article history:
Received 7 May 2013
Received in revised form 9 August 2013
Accepted 29 August 2013
Available online 5 September 2013

Section Editor: T.E. Johnson

Keywords:
Peroxidation
Membrane fluidity
Lifespan
Aging
Membrane pacemaker theory
Reactive oxygen species

ABSTRACT

Various compositions of fatty acids can produce cell membranes with disparate fluidity and propensity for oxidation. The latter characteristic, which can be evaluated via the peroxidation index (PI), has a fundamental role in the development of the "membrane-pacemaker theory" of aging. This study tried to evaluate differences between the membrane phospholipid fatty acid (PLFA) profile of longevity-selected (L) and corresponding control (C) lines of *Drosophila melanogaster* with age (3, 9, 14 and 19 days) and its consequences on phase transition temperature as a function of membrane fluidity. Despite an equal proportion of polyunsaturated fatty acids, PI and double bond index over all ages in both experimental groups, monounsaturated fatty acids showed significant variation with advancement of age in both L and C lines. A significant age-associated elevation of the unsaturation vs. saturation index in parallel with a gradual reduction of the mean melting point was observed in longevous flies. PLFA composition of the L vs. C lines revealed a dissimilarity in 3-day old samples, which was based on the positive loading of $C_{14:0}$ and $C_{18:3}$ as well as negative loading of $C_{18:0}$. The findings of this study are not in agreement with the principle of the "membrane pacemaker theory" linking PI and longevity. However, the physiochemical properties of PLFAs in longevity lines may retard the cells' senescence by maintaining optimal membrane functionality over time. Identical susceptibility to peroxidation of both types of lines underlines the involvement of other mechanisms in protecting the bio-membrane against oxidation, such as the reduced production of mitochondrial reactive oxygen species or improvement of the antioxidant defense system in longer-lived phenotypes. Concurrent assessments of these mechanisms in relation to cell membrane PLFA composition may clarify the cellular basis of lifespan in this species.

1. Introduction

Accumulation of endogenous deleterious effects on biological macromolecules such as nucleic acids, proteins and lipids can cause irreversible disorders in cell function (Harman, 1988), enhance the risk of age-specific diseases and consequently death of organisms (Buffenstein et al., 2008). Discrepancy in the onset and pace of this natural phenomenon has brought about a wide variety in maximum lifespan of diverse species and given rise to numerous assays and theories.

Most of the major mechanistic theories on aging arise from a seminal idea by Pearl (1928), in which an inverse correlation was postulated between the basal metabolic rate and the maximum lifespan of organisms (Pearl, 1928). This notion introduced the "rate of living theory" and substantiated that the low metabolic rate of cold-adapted strains of *Drosophila melanogaster* is one of the prerequisite physiological factors to their long lifespan compared to warm-adapted equivalents (Loeb and Northrop, 1916; Pearl, 1928). However, subsequent investigations on longevity-selected phenotypes of this experimental model were in conflict with this classical model, thus refuting Pearl's hypothesis on the restrictive role of metabolic rate in longevity (Khazaeli et al., 2005; Van Voorhies et al., 2004).

Following the demonstration of oxidative damage by reactive oxygen species (ROS) with age, these genetically-regulated factors (Barja, 2013) were selected as a link between metabolic rate and lifespan (Harman, 1956). However, subsequent studies did not support the "oxidative stress theory" and the debate on how to explain the various lifespans of different species is ongoing (Andziak and Buffenstein, 2006; Buffenstein et al., 2008).

Mitochondria, as massive oxygen consumers in aerobic species, play a prominent role in the generation of reactive molecule species (Camougrand and Rigoulet, 2001; Ross, 2000). According to Chance et al. (1979), around 2% of mitochondrial oxygen expenditure in rat liver and heart can be converted to hydrogen peroxide (H_2O_2), a potent

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http://dx.doi.org/10.1016/j.exger.2013.08.018

ROS, under the presence of NAD-linked substrates or succinate (Chance et al., 1979). However, in another experiment on rat skeletal muscle with palmitoyl carnitine as substrate, the estimation of mitochondrial H_2O_2 production was 0.15% (St-Pierre et al., 2002).

Under normal physical conditions, the antioxidant defense system (ADS) regulates the balance between generation and elimination of ROS and ameliorates this physiological redox procedure. Age-related perturbation of this equilibrium through degradation of mitochondrial respiratory chain reactions might increase the emission of reactive molecules and their detrimental consequences on nucleic acids, proteins and lipids (Hulbert, 2003; Sanz et al., 2006). Among these biological macromolecules, the "self-propagating autocatalytic" feature of lipid peroxidation, makes it one of the influential intrinsic factors of aging (Halliwell and Chirico, 1993; Hulbert et al., 2007). The reactive nature of most lipoxidation end-products predisposes them to commence an infinite oxidative chain reaction (Hulbert, 2008, 2010). Moreover, the extraordinary hydrophilic feature of lipid hydroperoxides, as non-radical intermediates of lipid peroxidation, can cause fundamental disorders in cell membrane integrity and functionality (Girotti, 1998; Hulbert et al., 2007; Spiteller, 2003).

Lipid peroxidation tendency relies on the presence of bis-allylic hydrogen (i.e. the hydrogen atom attached to a single-bonded C atom between double-bonded C atoms) in the acyl chain structure, which is the initial site of oxidative damage (Bielski et al., 1983). The absence of this atom in saturated (SFAs) and monounsaturated fatty acids (MUFAs) implies their peroxidation resistance, whereas polyunsaturated fatty acids (PUFAs) show an exponential trend to oxidize in proportion to their bis-allylic hydrogen content (Hulbert et al., 2007). According to the constructive role of the phospholipids in cell bilayers, various compositions of fatty acids (FAs) can create membranes with disparate propensities for oxidation. This characteristic, which can be estimated via the "peroxidation index" (PI) (Hulbert et al., 2007), led to the "membrane-pacemaker theory" of aging, in which a part of the age-associated cell membrane deteriorations in short-lived species, had been related to chemical properties that constitutive FAs, especially PUFAs impart to the cell membrane (Hulbert, 2005; Pamplona et al., 2002). Comparative studies across a wide range of birds and mammals showed the low proportion of vulnerable phospholipid fatty acids (PLFAs) or their substitution with more oxidative resistant groups in long-lived species and verified the impact of PUFAs as crucial aging-modulator factors and their linkage with the rate of living and oxidative stress theory of aging (Hulbert et al., 2006; Pamplona et al., 2002; Portero-Otin et al., 2001). The role of PUFAs in the determination of lifespan has also been reported for invertebrates such as *D. melanogaster* where a negative correlation was observed between the mean lifespan and cell membrane PI when manipulating the flight activity (Magwere et al., 2006).

Perturbation in the cell membrane structure of organisms, due to peroxidation or FAs' redistribution to enhance resistance to oxidation, may have consequences on lateral and rotational movements of hydrocarbon chains, and subsequently on membrane fluidity (Hulbert, 2003). Preserving the optimal membrane fluidity is essential for proper operation of membrane-bound proteins regulating activities such as ion permeability, enzyme activity or receptor responsiveness (Choe et al., 1995).

Among cell membrane components, hydrophobic chains in combination with other substances such as cholesterol and proteins play a pivotal role in adjusting the membrane viscosity (Stubbs and Smith, 1984). This property stems from the coherence between the rotation and mobility of FAs and acyl chain length as well as the degree of unsaturation. Besides a reduction in chain length (Lewis et al., 1989), the presence and position of double bonds in unsaturated fatty acids (USFAs), increase their motional capabilities and reduce phase transition temperature. It is noteworthy that the correlation between the number of double bonds and phase transition temperature is not straightforward. Assessment of various FAs indicates that the presence of the first double bond can cause a profound reduction in the melting point, however the second and subsequent double bonds cannot bring about such significant changes (Coolbear et al., 1983; Stubbs et al., 1981). Thus, the mean melting point of the constitutive PLFAs is suggested as an estimate of membrane fluidity (Stubbs and Smith, 1984).

Reduction of membrane fluidity in senescent cells is reported in rat hepatic mitochondrial (Chen and Yu, 1994), microsomal (Yu et al., 1992), intestinal microvillus (Wahnon et al., 1989) and erythrocyte membranes (Levin et al., 1992). In these experiments, degeneration of membrane viscosity is ascribed to the detrimental effects of peroxidation and its products on membrane PLFAs. By incorporating peroxidized and cholesterol loaded liposomes into rat liver microsomal membranes, Choe et al. (1995) showed that the age-induced membrane rigidity mainly depends on the peroxidation intensity in comparison to the cholesterol content. Hence, the structure of PLFAs, by modifying the cell membrane fluidity and peroxidizability, might have a dual effect on species-specific lifespan.

From a biological standpoint, studying the interaction between cell membrane fluidity and peroxidizability and their alteration with age in long- vs. normal-lived strains might shed light on the fundamental molecular mechanisms behind longevity. The main aims of this study were: 1) comparing the PLFA profile of *D. melanogaster* longevity selected and non-selected counterparts, 2) evaluating the age-related reconstruction in PLFA composition of long- and normal-lived strains and 3) assessing the fluctuation in mean melting point of constitutive PLFAs, as an indicator of membrane fluidity, in response to age-derived alteration in the cell membrane composition.

2. Materials and methods

2.1. Sample collection

The experiment was based on three replicate longevity-selected (L) and corresponding control lines (C) of *D. melanogaster*, in which the mean lifespan of mated females after 31 generations of selection was $\geq$1.5 times higher than that of non-selected flies (for details see: Sarup et al., 2011). These lines were derived from a mass population established in September 2002 via combination of four pre-existing laboratory stocks; ca. 2400–2800 flies, originating from different geographical regions (Bubliy and Loeschcke, 2005). For the purpose of this assay, eggs were collected at controlled densities (40 eggs per vial), transferred to 35 mm plastic vials (60 vials per replicate) containing a 7 ml standard *Drosophila* oatmeal-sugar-yeast-agar medium and kept at 25 $\pm$ 1 °C with a 12:12 light:dark cycle. The age of the emerged flies was controlled by collecting them over a period of 24 h after the first eclosion. The offspring's rearing conditions were similar to the previous stage. The flies were transferred to fresh food vials every other day and their density was kept close to 40 individuals (male and female) per vial during the maintenance period. Samples were snap frozen in liquid N_2 at four different ages: 3-, 9-, 14- and 19-days old, at the same time of the day (2 p.m.). These age groups were selected in accordance to a pilot study on daily mortality rates of selected and non-selected female flies. Because of the high mortality rate of flies of the C lines at higher age, the age of 19 days was chosen as the last age group to reduce the effect of selection due to mortality. The female samples were separated in a cooling room (4 °C), on a piece of glass over a thick layer of dry ice to diminish the effect of ambient temperature on FA composition, and finally preserved at −80 °C for subsequent analysis.

2.2. Lipid extraction

The modified Bligh–Dyer single-phase extraction method using chloroform:methanol:phosphate buffer (2:1:1 v/v/v) was used (Bligh and Dyer, 1959) to extract ester-linked membrane PLFAs of intact flies in two steps as described by Bayley et al. (2001). After phase separation, the chloroform layer was transferred to another vial and evaporated by