Exceptionally old mice are highly resistant to lipoxidation-derived molecular damage

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Received: 31 October 2011 / Accepted: 8 February 2012 / Published online: 25 February 2012
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Abstract Membrane unsaturation plays an important role in the aging process and the determination of inter-species animal longevity. Furthermore, the accumulation of oxidation-derived molecular damage to cellular components particularly in the nervous and immune systems over time leads to homeostasis loss, which highly influences age-related morbidity and mortality. In this context, it is of great interest to know and discern the degree of membrane unsaturation and the steady-state levels of oxidative damage in both physiological systems from long-lived subjects. In the present work, adult (28 ± 4 weeks), old (76 ± 4 weeks) and exceptionally old (128 ± 4 weeks) BALB/c female mice were used. Brain and spleen were analysed for membrane fatty acid composition and specific markers of protein oxidation, glycoxidation and lipoxidation damage, i.e. glutamic semialdehyde, amino adipic semialdehyde, carboxyethyl-lysine, carboxymethyl-lysine and malondialdehyde-lysine, by

gas chromatography–mass spectrometry. The results showed significantly lower peroxidizability index in brain and spleen from exceptionally old animals when compared to old specimens. The higher membrane resistance to lipid peroxidation and lower lipoxidation-derived molecular damage found in exceptionally old animals was associated with a significantly lower desaturase activity and peroxisomal β -oxidation. Protein oxidation markers in brain and spleen from adult and exceptionally old animals showed similar levels, which were higher in old mice. In addition, the higher levels of the glycoxidation-derived marker observed in exceptionally old animals, as well as in adult mice, could be considered as a good indicator of a better bioenergetic state of these animals when compared to the old group. In conclusion, low lipid oxidation susceptibility and maintenance of adult-like protein lipoxidative damage could be key mechanisms for longevity achievement.

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Keywords Aging · Brain · Longevity ·
Peroxidizability index · Protein oxidation · Spleen

Abbreviations

ACL	Average chain length
AASA	Amino adipic semialdehyde
ALEs	Advanced lipoxidation end-products
CEL	Carboxyethyl-lysine
CML	Carboxymethyl-lysine
DBI	Double bond index
ELOVL	Elongation of very long chain fatty acids

GC/MS	Gas chromatography/mass spectrometry
GSA	Glutamic semialdehyde
MDA	Malondialdehyde
MDAL	Malondialdehyde-lysine
MUFAs	Monounsaturated fatty acids
PI	Peroxidizability index
PUFAs	Polyunsaturated fatty acids
RCS	Reactive carbonyl species
SFAs	Saturated fatty acids
UFAs	Unsaturated fatty acids

Introduction

Understanding the mechanisms underlying healthy aging and longevity is highly relevant in an expanding aged population. However, the reason(s) why some individuals survive over the mortality peak of the general population and achieve longevity close to the maximum of their species is poorly understood to date. Available evidences suggest that long-lived species evolved by reducing the relative abundance of those structural components that are highly susceptible to oxidative damage, thus conferring to the cellular constituents a higher structural stability and lower susceptibility to oxidative damage, along with low rate of mitochondrial free radical generation and oxidative molecular damage (Pamplona et al. 2002; Pamplona and Barja 2011; Pamplona and Costantini 2011). This intrinsically high resistance to modification that likely contributes to their superior longevity is obtained in the case of lipids by decreasing the degree of fatty acid unsaturation (Hulbert et al. 2007; Pamplona 2008). The susceptibility of membrane lipids to oxidative alterations is directly related to the chemical reactivity of the fatty acids composing the own membrane. Different fatty acids vary severely in their susceptibility to free radicals attack; those highly polyunsaturated are much more vulnerable than less polyunsaturated fatty acids, with monounsaturates and saturates being essentially unable of peroxidation (Holman 1954; Bielski et al. 1983; Hulbert et al. 2007; Xu et al. 2009). This fact, along with the findings that fatty acid composition of cellular membranes varies systematically with species longevity, both in mammals and birds, has highlighted the important link between membrane unsaturation and longevity (Pamplona et al. 2002; Hulbert et al. 2007; Pamplona 2008; Pamplona and Barja 2011).

The high concentration of polyunsaturated fatty acids (PUFAs) in membrane phospholipids not only makes them prime targets for reaction with oxidizing agents but also enables them to participate in long free radical chain reactions (Spiteller 2010). Lipid peroxidation generates hydro- and endoperoxides which undergo fragmentation to produce a broad range of reactive intermediates called reactive carbonyl species (RCS) with three to nine carbons in length, the most reactive being α,β -unsaturated aldehydes, dialdehydes such as malondialdehyde (MDA) and glyoxal, and keto-aldehydes (Esterbauer et al. 1991). These carbonyl compounds, ubiquitously generated in biological systems, have unique properties contrasted with free radicals. Thus, compared with free radicals, reactive aldehydes have a much longer half-life (i.e. minutes to hours instead of microseconds to nanoseconds for most free radicals). Further, the non-charged structure of aldehydes allows them to migrate with relative ease through hydrophobic membranes and hydrophilic cytosolic media, thereby extending the migration distance far from the production site. Based on these features alone, these carbonyl compounds can be more destructive than free radicals and may have far-reaching damaging effects on target sites within or outside membranes (Pamplona 2011). Carbonyl compounds react with nucleophilic groups in macromolecules like proteins, DNA and aminophospholipids, among others, resulting in their chemical, nonenzymatic and irreversible modification and formation of a variety of adducts and crosslinks collectively named advanced lipoxidation end-products (ALEs) (Thorpe and Baynes 2003; Pamplona 2011). Thus, by reacting with nucleophilic sites in proteins, carbonyl compounds generate ALE adducts such as malondialdehyde-lysine (MDAL) and carboxymethyl-lysine (CML), among others. The consequent loss of function and structural integrity of modified biomolecules by RCS can have a wide range of downstream functional consequences and may be the cause of subsequent cellular dysfunctions and tissue damage determining the rate of aging and, consequently, the longevity of the animal species (Pamplona 2011).

Thus, the aim of the present work was to study the degree of membrane unsaturation and specific markers of protein damage in exceptionally old mice and compare them to adult and old individuals. Importantly, these mice are genetically identical, but some die before the mean lifespan of the

group, some die at the mean lifespan and some have non-genetic factors that help them to live longer than would be expected. With those purposes and in order to discern what makes mice in the last group—exceptionally old—different from those that die earlier, we used highly sensitive gas chromatography/mass spectrometry methods. We chose two major organs from the nervous and immune regulatory systems, namely brain and spleen, since their age-related impairment is highly associated with morbidity and mortality throughout the aging process (De la Fuente and Miquel 2009). Despite age-related oxidative stress-derived molecular damage accumulation has been associated with the rate of aging (Sanz et al. 2006), it remains undetermined in individuals who have achieved extreme longevity. In this scenario, this is the first study in which lipoxidation-derived molecular damage from exceptionally old mice is analysed.

Experimental procedures

Animals

We used 26 ex-reproductive females of inbred BALB/c mice (*Mus musculus*) strain, purchased from Harlan Ibérica (Barcelona, Spain) at the young adult age (28 ± 4 weeks). The mice were specifically pathogen free as tested by Harlan and according to the Federation of European Laboratory Science Associations recommendations. In our Animal Facility, placed at the Faculty of Biology (Complutense University of Madrid, Madrid, Spain), they were housed at 6 ± 1 per cage and maintained at a constant temperature ($22 \pm 2^\circ\text{C}$) in sterile conditions inside an aseptic air negative-pressure environmental cabinet (Flufrance, Cachan, France), on a 12:12-h reversed light/dark cycle (lights on at 2000 hours). Mice had access to tap water and standard Sander Mus pellets (A04 diet from Panlab L.S., Barcelona, Spain) ad libitum. Diet was in accordance with the recommendations of the American Institute of Nutrition for laboratory animals. This cross-sectional study was performed simultaneously on mice of different ages, namely adult (28 ± 4 weeks, $n=6$), old (76 ± 4 weeks, $n=11$) and exceptionally old (128 ± 4 weeks, $n=9$), which had aged in our Animal Unit under the above-specified conditions from the adult age. Each age group category was formed by animals that

had been purchased in the same set. The extreme long-lived mice had naturally achieved healthy and successful aging since the average longevity for females of BALB/c mice strain in our Animal House is 99 ± 5 weeks (unpublished observation). Mice were treated according to the guidelines of the European Community Council Directives (86/6091 EEC). They were sacrificed by rapid cervical dislocation during the dark phase of the cycle (0800–1000 hours). Brain and spleen were removed aseptically and immediately frozen down at -80°C until further proceeding.

Fatty acid analysis

Fatty acyl groups of total brain and spleen lipids were analysed as methyl esters derivatives by gas chromatography (GC) as previously described (Pamplona et al. 2005a, 2005b). Total lipids were extracted with chloroform/methanol (2:1, v/v) in the presence of 0.01% butylated hydroxytoluene. The chloroform phase was evaporated under nitrogen, and the fatty acids were transesterified by incubation in 2.5 ml of 5% methanolic HCl for 90 min at 75°C . The resulting fatty acid methyl esters were extracted by adding 2.5 ml of *n*-pentane and 1 ml of saturated NaCl solution. The *n*-pentane phase was separated, evaporated under nitrogen and redissolved and 4 μl was used for GC analysis. Separation was performed by a DB-WAX capillary column (30 m $\times$ 0.25 mm $\times$ 0.20 μm) in a GC System 7890A with a Series Injector 7683B and a FID detector (Agilent Technologies, Barcelona, Spain). The injection port was maintained at 220°C and the detector at 250°C ; the temperature program was 2 min at 100°C , then $10^\circ\text{C}/\text{min}$ to 200°C , then $5^\circ\text{C}/\text{min}$ to 240°C and finally hold at 240°C for 10 min. Identification of fatty acid methyl esters was made by comparison with authentic standards (Larodan Fine Chemicals, Malmö, Sweden). Results are expressed as mole percent.

The following fatty acyl indexes (Pamplona et al. 2005a, 2005b; Hulbert et al. 2007) were also calculated: saturated fatty acids, unsaturated fatty acids, monounsaturated fatty acids, PUFA, PUFA from *n*-3 and *n*-6 series and average chain length = $[(\Sigma\% \text{Total}_{14} \times 14) + (\Sigma\% \text{Total}_{16} \times 16) + (\Sigma\% \text{Total}_{18} \times 18) + (\Sigma\% \text{Total}_{20} \times 20) + (\Sigma\% \text{Total}_{22} \times 22) + (\Sigma\% \text{Total}_{24} \times 24)]/100$. The density of double bonds in the membrane was calculated by the double bond index (DBI) = $[(1 \times \Sigma \text{mol}\% \text{ monoenoic}) + (2 \times \Sigma \text{mol}\% \text{ dienoic}) + (3 \times \Sigma \text{mol}\% \text{ trienoic}) + (4 \times \Sigma \text{mol}\%$

tetraenoic)+(5×Σmol% pentaenoic)+(6×Σmol% hexaenoic)]. Finally, the membrane susceptibility to peroxidation was calculated by the peroxidizability index $PI=[(0.025\times\Sigma\text{mol\% monoenoic})+(1\times\Sigma\text{mol\% dienoic})+(2\times\Sigma\text{mol\% trienoic})+(4\times\Sigma\text{mol\% tetraenoic})+(6\times\Sigma\text{mol\% pentaenoic})+(8\times\Sigma\text{mol\% hexaenoic})]$.

Desaturase activities were estimated from specific product/substrate ratios (Guillou et al. 2010): for Δ9 (*n*-7) desaturase, 16:1*n*-7/16:0; for Δ9(*n*-9) desaturase, 18:1*n*-9/18:0; for Δ8(*n*-6) desaturase, 20:3*n*-6/20:2*n*-6; for Δ5(*n*-6) desaturase, 20:4*n*-6/20:3*n*-6; and for Δ6(*n*-3) desaturase, 24:6*n*-3/24:5*n*-3. Elongase activities were estimated from specific product/substrate ratios (Guillou et al. 2010): for ELOVL3 (*n*-9) elongase, 20:1*n*-9/18:1*n*-9; for ELOVL6 elongase, 18:0/16:0; for ELOVL1, 3 and 7 elongases, 20:0/18:0, 22:0/20:0 and 24:0/22:0; for ELOVL5 elongase, 20:2*n*-6/18:2*n*-6; for ELOVL2 and 5 (*n*-6) elongases, 22:4*n*-6/20:4*n*-6; for ELOVL2 and 5 (*n*-3) elongases, 22:5*n*-3/20:5*n*-3; and for ELOVL2 (*n*-3) elongase, 24:5*n*-3/22:5*n*-3. Finally, peroxisomal β-oxidation was estimated according to the ratio 22:6*n*-3/24:6*n*-3.

Oxidation-derived protein damage markers

Five oxidative damage markers of protein oxidation (the specific protein carbonyls glutamic and amino-adipic semialdehydes, GSA and AASA), glycoxidation (carboxyethyl-lysine CEL) and lipoxidation (CML and MDAL) were measured by GC/mass spectrometry (MS) as previously described (Pamplona et al. 2005a, 2005b). Samples (brain and spleen homogenates) containing 500 μg of protein were delipidated using chloroform/methanol (2:1, v/v), and proteins were precipitated by adding 10% trichloroacetic acid (final concentration) and subsequent centrifugation. Protein samples were reduced overnight with 500 mM NaBH₄ (final concentration) in 0.2 M borate buffer, pH 9.2, containing one drop of hexanol as an anti-foam reagent. Proteins were then reprecipitated by adding 1 ml of 20% trichloroacetic acid and subsequent centrifugation. The following isotopically labelled internal standards were then added: [²H₈]Lysine (d₈-Lys; CDN isotopes); [²H₅]glutamic semialdehyde and [²H₄]amino-adipic semialdehyde, prepared as described Requena et al. (2001) and [²H₄]CML, [²H₄]CEL and [²H₈]MDAL prepared as described Fu et al. (1996) and Requena et al. (1997). The samples were hydrolysed at 155°C for 30 min in 1 ml of 6 N HCl

and then dried in vacuo. The *N,O*-trifluoroacetyl methyl ester derivatives of the protein hydrolysate were prepared as previously described Knecht et al. (1991). GC/MS analyses were carried out on a Hewlett-Packard model 6890 gas chromatograph equipped with a 30-m HP-5MS capillary column (30 m×0.25 mm×0.25 μm) coupled to a Hewlett-Packard model 5973A mass selective detector (Hewlett-Packard Española, S.A., Barcelona, Spain). The injection port was maintained at 275°C; the temperature program was 5 min at 110°C, then 2°C/min to 150°C, then 5°C/min to 240°C, then 25°C/min to 300°C and finally hold at 300°C for 5 min. Quantification was performed by external standardisation using standard curves constructed from mixtures of deuterated and non-deuterated standards. Analyses were carried out by selected ion monitoring GC/MS. Analytes were detected by selected ion-monitoring GC/MS. The ions used were lysine and [²H₈]lysine, *m/z* 180 and 187, respectively; 5-hydroxy-2-aminovaleric acid and [²H₅]5-hydroxy-2-aminovaleric acid (stable derivatives of GSA), *m/z* 280 and 285, respectively; 6-hydroxy-2-aminocaproic acid and [²H₄]6-hydroxy-2-aminocaproic acid (stable derivatives of AASA), *m/z* 294 and 298, respectively; CML and [²H₄]CML, *m/z* 392 and 396, respectively; CEL and [²H₄]CEL, *m/z* 379 and 383, respectively; and MDAL and [²H₈]MDAL, *m/z* 474 and 482, respectively. The amounts of products were expressed as the ratio micromoles glutamic semialdehyde, amino-adipic semialdehyde, CML, CEL or MDAL/mol lysine.

Immunodetection of malondialdehyde-modified proteins by western blot

The nonenzymatic modification of proteins by malondialdehyde was also estimated using western blot analyses. The protein concentration from both brain and spleen samples was measured using the Bradford method (Bio-Rad Protein Assay 500-0006), and proteins were separated by one-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Samples were in the sample buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 20% 2-β-mercaptoethanol and 0.02% bromophenol blue) and heated at 95°C for 5 min. Proteins (10 μg) were subjected to electrophoresis on 10% SDS–polyacrylamide minigels. For immunodetection, proteins were transferred using a Mini Trans-Blot Transfer Cell

(Bio-Rad) in a buffer containing 25 mM Tris, 192 mM glycine and 20% methanol, to polyvinylidene difluoride membranes (Immobilon-P Millipore, Bedford, MA, US). The membranes were immersed in blocking solution (0.2% I-Block Tropix AI300, 0.1% Tween in PBS) at room temperature for 1 h. After blocking, the membrane was washed two times using 0.05% TBS-T buffer. Afterwards, the membrane was incubated in primary solution using specific antibody for MDA (HRP-goat anti-MDA, MD20H-G1b, Academy Bio-Medical Company, Inc., Houston, TX, USA; 1:1,000) and for beta-actin (HRP-mouse anti-beta-actin, Ab20272, Abcam, Cambridge, MA, USA; 1:1,000) in order to determine the proportion of protein levels referred to total mass. Primaries antibodies were incubated 1 h at room temperature. After five washes with 0.05% TBS-T buffer, bands were visualized by using an enhanced chemiluminescence HRP substrate (Millipore, MA, USA). Signal quantification and recording was performed with a ChemiDoc equipment (Bio-Rad Laboratories, Inc., Barcelona, Spain).

Statistical analyses

All statistics calculations were performed using the SPSS software (SPSS Inc., Chicago, IL, USA). Data

were analysed by one-way ANOVA. After the ANOVA, a post hoc analysis with the least significant difference (Tukey) test was performed for multiple comparisons between pairs of groups. The minimum level of statistical significance was set at $P < 0.05$ in all the analyses.

Results

Available evidence supports the hypothesis that membrane fatty acid composition is regulated in a longevity-related species-specific way (Naudi et al. 2011). As a whole, the membrane acyl composition of the studied groups (adult, old and exceptionally old) verifies that their biological membranes maintain, for both brain and spleen, a similar fatty acid average chain length (18 carbon atoms), as well as ratio saturated versus unsaturated fatty acids (ratio ~40:60) (Table 1). The old group, however, shows some significant changes in membrane fatty acid composition for general indexes when compared to the adult group. Thus, the brain from old group shows an increase in unsaturated fatty acid content basically due to an increase in the content of mono-unsaturated fatty acids (Table 1); in contrast, in spleen from the old group, it was observed a

Table 1 General indexes from fatty acid composition of both brain and spleen from adult, old and exceptionally old mice

	Brain			Spleen		
	Adult	Old	Exceptionally old	Adult	Old	Exceptionally old
ACL	18.82±0.02	18.88±0.03	18.70±0.05	18.49±0.02	18.61±0.02	18.48±0.08
SFA	41.89±0.49	39.12±0.56 ^{a*}	41.77±1.16 ^{b*}	36.64±0.29	38.73±0.55	40.16±0.91 ^{a*}
UFA	58.10±0.49	60.87±0.56 ^{a*}	58.22±1.16 ^{b*}	63.35±0.29	61.26±0.55	59.83±0.91 ^{a*}
MUFA	24.63±0.36	27.54±0.57 ^{a*}	27.93±0.81 ^{a**}	21.82±0.24	15.80±0.78 ^{a**}	16.35±1.56 ^{a*}
PUFA	33.47±0.54	33.32±0.50	30.28±1.51 ^{a*,b*}	41.53±0.05	45.46±0.64	43.47±2.35
PUFAn-6	12.38±0.35	12.78±0.33	12.43±0.69	25.53±0.01	32.29±0.96 ^{a*}	26.64±2.41 ^{b*}
PUFAn-3	21.08±0.31	20.54±0.30	17.85±0.93 ^{a**}	15.99±0.04	13.17±1.13	16.82±1.59 ^{b*}
DBI	185.57±1.89	192.65±2.90	173.03±6.54 ^{a*,b**}	185.78±1.18	202.04±2.82 ^{a*}	190.46±3.81 ^{b*}
PI	190.52±2.29	199.25±4.09	171.91±8.23 ^{a*,b**}	169.67±1.49	197.05±4.00 ^{a*}	181.14±5.42 ^{b*}

Values are means±SEM from six to 11 different animals

ACL average chain length, SFA saturated fatty acids, UFA unsaturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids, PUFAn-6 PUFA *n*-6 series, PUFAn-3 PUFA *n*-3 series, DBI double bond index, PI peroxidizability index

* $P < 0.05$; ** $P < 0.01$

^aRepresents significant differences compared to adults

^bRepresents significant differences between old and long-lived groups

significant increase in polyunsaturated fatty acids of the *n*-6 series along with a decrease in the mono-unsaturated fatty acid content. These changes in global fatty acid indexes lead to an increase in the double bond index (5% for brain and 8% for spleen) and peroxidizability index (5% for brain and 13% for spleen) in the old group when compared to the adult group (Table 1). In terms of specific fatty acids, there is an increase, in both brain and spleen, in 20:4 and 22:6 contents in the old group (Table 2). The verified changes in the old group contrast with the membrane fatty acid composition and derived degree of membrane unsaturation of the exceptionally old group. Thus, exceptionally old animals show in brain and spleen

a membrane unsaturation similar or even lower than the adult group for double bond index and peroxidizability index resulting in a biological membrane more resistant to lipid peroxidation (Table 1). The maintenance of the double bond index and peroxidizability index at levels of the adult group in exceptionally old animals can be appointed to the preservation of the content of specific fatty acids, particularly 20:4 and 22:6 (Table 2).

Mechanisms responsible for the longevity-related differences in fatty acid profile can be related, in principle, to the fatty acid desaturation–elongation pathway (Naudi et al. 2011). For this reason, different desaturase and elongase activities, as well as the peroxisomal β -oxidation activity, were estimated from

Table 2 Fatty acid composition of both brain and spleen from adult, old and exceptionally old mice

	Brain			Spleen		
	Adult	Old	Exceptionally old	Adult	Old	Exceptionally old
14:0	0.49±0.04	0.26±0.02***	0.34±0.03***,b*	0.74±0.09	0.51±0.03**	0.62±0.06
16:0	18.71±0.44	18.18±0.44	19.02±0.37	19.98±0.31	22.03±0.37***	21.38±0.44 ^a *
16:1 <i>n</i> -7	0.67±0.02	0.61±0.04	0.72±0.06	1.58±0.17	1.48±0.14	1.38±0.19
18:0	19.19±0.42	17.98±0.11	19.60±0.95	12.69±0.04	14.54±0.56	15.64±0.96 ^a *
18:1 <i>n</i> -9	20.04±0.49	23.03±0.40***	23.49±0.59***	19.23±0.33	13.69±0.65***	14.08±1.40 ^a *
18:2 <i>n</i> -6	1.09±0.08	0.78±0.10	1.18±0.33	5.89±0.28	5.25±0.19	5.42±0.25
18:3 <i>n</i> -3	0.40±0.07	0.26±0.04	0.39±0.07	0.78±0.04	0.38±0.09**	0.55±0.12
18:4 <i>n</i> -3	4.28±0.23	2.26±0.32***	2.72±0.39 ^a *	7.23±0.39	4.00±1.06	8.43±1.86 ^b *
20:0	0.85±0.07	0.70±0.02**	0.80±0.04	0.79±0.06	0.41±0.07***	0.64±0.06 ^b *
20:1 <i>n</i> -9	3.91±0.15	3.90±0.19	3.71±0.25	1.00±0.08	0.62±0.06***	0.87±0.06 ^b *
20:2 <i>n</i> -6	0.81±0.09	0.85±0.15	0.98±0.16	1.44±0.11	0.78±0.12***	0.99±0.08 ^a *
20:3 <i>n</i> -6	0.54±0.05	0.31±0.04***	0.47±0.03 ^b *	1.02±0.13	0.38±0.05***	0.93±0.06 ^b **
20:4 <i>n</i> -6	6.40±0.06	7.30±0.24 ^a *	6.68±0.31 ^b *	13.41±0.22	19.47±0.87 ^a *	14.78±1.78 ^b *
20:5 <i>n</i> -3	0.49±0.10	0.30±0.07	0.28±0.03	0.92±0.10	0.30±0.06***	0.58±0.06 ^a , ^b *
22:0	0.93±0.05	0.75±0.03	0.86±0.07	1.14±0.30	0.54±0.08**	0.76±0.13
22:4 <i>n</i> -6	2.89±0.03	3.02±0.08	2.60±0.09 ^a , ^b **	2.79±0.09	5.04±0.29***	3.54±0.47 ^b *
22:5 <i>n</i> -6	0.62±0.11	0.49±0.08	0.49±0.18	0.96±0.07	1.35±0.13 ^a *	0.96±0.07 ^b *
22:5 <i>n</i> -3	0.57±0.11	0.37±0.04 ^a *	0.24±0.01***	0.95±0.04	1.08±0.08	1.03±0.08
24:0	1.70±0.06	1.23±0.04***	1.14±0.03***	1.27±0.11	0.68±0.10 ^a *	1.10±0.13 ^b *
22:6 <i>n</i> -3	12.46±0.25	14.69±0.54 ^a *	11.48±0.64 ^b **	4.66±0.13	6.81±0.37 ^a *	5.03±0.57 ^b *
24:5 <i>n</i> -3	0.53±0.06	0.17±0.01***	0.27±0.01 ^a , ^b *	0.71±0.09	0.30±0.06***	0.49±0.09 ^b *
24:6 <i>n</i> -3	2.32±0.06	2.46±0.11	2.44±0.19	0.72±0.09	0.27±0.04***	0.68±0.10 ^b **

Values are means±SEM from six to 11 different animals and are expressed as mole percent

* $P<0.05$; ** $P<0.01$

^aRepresents significant differences compared to adults

^bRepresents significant differences between old and exceptionally old groups

specific product/substrate ratios. Globally, in brain and spleen, the higher desaturase activities are credited apparently by $\Delta 5$ and $\Delta 6$ desaturases (Table 3). In brain, aging (by comparing adult vs old groups) is associated with a significant increase in $\Delta 9$ and very especially $\Delta 5$ and $\Delta 6$ activities and decrease in $\Delta 8$ activity (Table 3). In spleen, a decreased $\Delta 8$ and $\Delta 9$ activities have been detected in the old group when compared to the adult group, whilst there is a significant increase in $\Delta 5$ desaturase activity (Table 3). These changes induced by aging are not present in exceptionally old animals showing a desaturase activity very similar to the adult group (Table 3). For elongase activities, the old group shows, for brain and spleen, significantly lower ELOVL3, 2/5 and 2 activities and increased ELOVL5 and 2/5($n-3$) activities compared to the adult group (Table 4). No significant differences in elongase activities were detected in exceptionally old animals compared to adult specimens (Table 4). Finally, aging is linked to an increase in peroxisomal β -oxidation in both brain and spleen. By contrast, exceptionally old animals maintain a peroxisomal β -oxidation at levels that showed by the adult group (Table 4).

Because a low rate of generation of endogenous damage is also associated with longevity, the study of the steady-state levels of endogenous protein oxidative damage is pertinent and necessary. The comparison of the brain and the spleen from adult, old and exceptionally old animals revealed that aging and longevity were associated with differences in the

steady-state levels of protein oxidative damage markers. In both organs (brain and spleen), aging is associated with significant increases in (a) the oxidative-derived markers surveyed (in brain: 34% for GSA and 16% for AASA; in spleen: 42% for GSA and 37% for AASA) (Fig. 1) and (b) the lipoxidative damage markers MDAL (65% for brain and 137% for spleen) (Fig. 2) and CML (27% for brain and 55% for spleen) (Fig. 4). The western blot analysis for MDAL (Fig. 3) also shows, in addition to reinforce results obtained by GC/MS, that there is a selective pattern in the main protein targets for lipoxidative damage. In contrast to these changes, (c) the glycooxidative marker CEL is significantly decreased (16% for brain and 21% for spleen) in the old group when compared to the adult group (Fig. 5). Exceptionally old animals show a steady-state levels for all the nonenzymatic protein modification markers no significantly different to the adult group but significantly different with respect to the old group (Figs. 1, 2, 3, 4 and 5).

Discussion

Our main finding is that exceptionally old mice show adult-like steady-state levels of membrane unsaturation and protein oxidative damage in both locations studied, namely brain and spleen, whereas old animals show, in general, increased values when compared to adults and exceptionally old. Of special interest is the

Table 3 Brain and spleen desaturase activities from adult, old and exceptionally old mice

Desaturase activity	Estimated ratio	Brain			Spleen		
		Adult	Old	Exceptionally old	Adult	Old	Exceptionally old
$\Delta 9$ ($n-7$)	C16:1/C16:0	0.03 $\pm$ 0.001	0.03 $\pm$ 0.002	0.03 $\pm$ 0.002	0.07 $\pm$ 0.007	0.06 $\pm$ 0.006	0.06 $\pm$ 0.008
$\Delta 9$ ($n-9$)	C18:1/C18:0	1.04 $\pm$ 0.04	1.28 $\pm$ 0.02**	1.21 $\pm$ 0.05*	1.51 $\pm$ 0.02	0.95 $\pm$ 0.07***	0.90 $\pm$ 0.06***
$\Delta 8$ ($n-6$)	C20:3/C20:2	0.66 $\pm$ 0.02	0.38 $\pm$ 0.03**	0.56 $\pm$ 0.09 ^{b*}	0.70 $\pm$ 0.03	0.51 $\pm$ 0.06**	0.98 $\pm$ 0.10 ^{a*,b**}
$\Delta 5$ ($n-6$)	C20:4/C20:3	12.11 $\pm$ 1.01	28.09 $\pm$ 6.26*	14.20 $\pm$ 0.95 ^{b*}	13.48 $\pm$ 1.72	63.28 $\pm$ 13.79**	16.07 $\pm$ 2.05 ^{b**}
$\Delta 6$ ($n-3$)	C24:6/C24:5	4.56 $\pm$ 0.58	14.63 $\pm$ 1.79***	9.04 $\pm$ 0.89 ^{a*,b**}	1.00 $\pm$ 0.001	1.02 $\pm$ 0.14	1.54 $\pm$ 0.31

Values are means $\pm$ SEM from six to 11 different animals

* $P < 0.05$; ** $P < 0.01$

^a Represents significant differences compared to adults

^b Represents significant differences between old and exceptionally old groups

Table 4 Brain and spleen elongase activities from adult, old and exceptionally old mice

Elongase activity	Estimated ratio	Brain			Spleen		
		Adult	Old	Exceptionally old	Adult	Old	Exceptionally old
ELOVL 3 (<i>n</i> -9)	C20:1/C18:1	0.19±0.01	0.16±0.005 ^{a*}	0.15±0.007 ^{a**}	0.05±0.003	0.04±0.004	0.06±0.008 ^{b*}
ELOVL 6	C18:0/C16:0	1.02±0.03	0.99±0.02	1.02±0.03	0.63±0.01	0.66±0.02	0.73±0.05
ELOVL 1/3/7	C20:0/C18:0	0.04±0.003	0.03±0.001	0.04±0.002	0.06±0.004	0.02±0.006 ^{a**}	0.04±0.004 ^{a*}
ELOVL 1/3/7	C22:0/C20:0	1.10±0.06	1.08±0.04	1.09±0.11	1.52±0.54	1.49±0.21	1.16±0.10
ELOVL 1/3/7	C24:0/C22:0	1.84±0.09	1.64±0.07	1.37±0.09 ^{a**} . ^{b*}	1.29±0.34	1.27±0.11	1.53±0.15
ELOVL 5 (<i>n</i> -6)	C20:2/C18:2	0.74±0.03	1.06±0.10 ^{a*}	0.92±0.09	0.24±0.02	0.15±0.02 ^{a*}	0.18±0.01
ELOVL 2/5 (<i>n</i> -6)	C22:4/C20:4	0.45±0.004	0.41±0.008 ^{a*}	0.39±0.01 ^{a**}	0.20±0.01	0.25±0.01 ^{a*}	0.23±0.007
ELOVL 2/5 (<i>n</i> -3)	C22:5/C20:5	1.20±0.06	1.44±0.14	0.92±0.07 ^{b**}	1.07±0.18	4.44±0.79 ^{a**}	1.79±0.14 ^{b**}
ELOVL 2 (<i>n</i> -3)	C24:5/C22:5	1.00±0.17	0.51±0.06 ^{a**}	1.14±0.09 ^{b**}	0.76±0.12	0.28±0.05 ^{a**}	0.47±0.07 ^{a*,b*}
Peroxisomal beta-oxidation	C22:6/C24:6	5.36±0.23	6.02±0.30	4.80±0.33 ^{b*}	6.69±0.85	29.56±5.83 ^{a**}	7.70±0.66 ^{b**}

Values are means±SEM from six to 11 different animals

* $P<0.05$; ** $P<0.01$

^aRepresents significant differences compared to adults

^bRepresents significant differences between old and exceptionally old groups

fact that exceptionally old mice show decreased peroxidizability index in brain when compared to adult mice, whilst similar values are found on these and old animals. This suggests that changes on the values of markers of lipid susceptibility to oxidation in brain occur at early stages of the aging process, and thus, low values can be regarded as ‘early’ longevity markers. Other remarkable findings include that accumulation of oxidized proteins over time takes place earlier in brain than in spleen, which might show that the age-related nervous-immune impairment could begin in the brain. Moreover, brain has a higher number of postmitotic cells than spleen (De la Fuente and Miquel 2009).

Available evidences support an important role for membrane unsaturation in the aging process (reviewed in Hulbert et al. 2007; Pamplona 2008; Naudi et al. 2011) based on studies showing decreases in membrane fluidity with age and increases in membrane lipid peroxidation during aging in an organ-dependent way mainly due to decreases in mono- and di-unsaturated fatty acids and to increases in highly unsaturated fatty acids like 20:4 and 22:6. In addition to these findings, the animal model senescent accelerated prone mouse (SAM-P) has higher levels of the highly unsaturated arachidonic (20:4) and docosahexaenoic (22:6) acids and lower levels of linoleic acid than SAM-resistant controls (Park et al. 1996;

Matsugo et al. 2000). Furthermore, it has been recently demonstrated that the erythrocyte membranes derived from nonagenarian offspring have a reduced lipid peroxidation and increased membrane integrity to that of the general population (Puca et al. 2008). More important, by perturbing fatty acid composition through diet can be modified animal longevity. Thus, recent studies demonstrate that an increase in membrane unsaturation promotes aging by shortening longevity in worms (Shmookler Reis et al. 2011) and mice (Tsuduki et al. 2011). Additional observations suggesting that membrane unsaturation is related to aging include data showing that strongly unsaturated fatty acids like 20:4 and 22:6 can have detrimental effects in vivo (reviewed in Sanz et al. 2006; Pamplona and Barja 2007). In this line, our results clearly show that brain and spleen are not exceptions, so extending this relationship to two key organs and reinforcing the role of membrane unsaturation in the aging process. Furthermore, our findings add new parameters to be considered, i.e. the increase in peroxidizability index and lipoxidation-derived protein damage during aging, as well as in desaturase activities and peroxisomal β -oxidation.

Several studies using organisms ranging from invertebrates to humans have been performed in order to answer whether levels of oxidatively damaged biomolecules increase with age (Leeuwenburgh et al.

Fig. 1 Protein markers of oxidative damage in both brain (a, upper graphs) and spleen (b, lower graphs) proteins of adult, old and exceptionally old mice. GSA glutamic

semialdehyde, AASA amino adipic semialdehyde. Statistics are indicated inside graphs. Values are mean $\pm$ SEM from six to 11 different animals

1997; Linton et al. 2001; Sohal 2002; Portero-Otin and Pamplona 2006; Gruber et al. 2008; Salmon et al. 2010). Because proteins are major components of biological systems and because proteins play an important role in a variety of cellular functions, an age-related increase in oxidative damage to proteins could be important physiologically to an organism. Levels of modified proteins have been examined in extracellular proteins from lens, skin and articular cartilage and in intracellular proteins from several organs such as brain, heart, liver, skeletal muscle and kidney, among others, of different animal species (specially rodents) and humans ranging in age from infancy to 80 years (reviewed in Portero-Otin and Pamplona 2006). Much of the evidence for accumulation of oxidative damage with age comes from an increase in the protein carbonyl content of

tissues (e.g. brain, kidney and cardiac muscle) (Sohal 2002; Portero-Otin and Pamplona 2006). If only stable oxidation markers are considered, then the evidence for accumulation of oxidative damage with age is contradictory. Therefore, it is evident that more systematic studies with specific markers are needed in order to clarify the observed differences inter-organ and inter-species with age. Our work has been also focused to evaluate possible oxidative changes in proteins during aging and in particular on the data concerning specific protein oxidation products. In this context, our results demonstrate that in the studied organs (brain and spleen), aging is associated with an increase in the steady-state levels of the specific protein oxidation markers GSA and AASA. The differences between organs in the steady-state levels of several markers may

Fig. 2 Protein marker of lipoxidative damage in both brain (**a**, upper graph) and spleen (**b**, lower graph) proteins of adult, old and exceptionally old mice. MDAL malondialdehyde-lysine. Statistics are indicated inside graphs. Values are mean $\pm$ SEM from six to 11 different animals

partly be related to metabolic differences leading to differences in their oxidative states and, therefore, to differences in the rate of free radical production and the antioxidant activities.

In addition to the aging process, available evidences support an important role for membrane unsaturation in the determination of longevity. It is now well-known that the peroxidizability index of membrane fatty acids decreases as longevity increases when different mammalian species are compared (reviewed in Hulbert et al. 2007; Pamplona and Barja 2011) in different tissues including heart, liver and skeletal muscle. A low degree of fatty acid unsaturation also occurs in extraordinarily long-lived animals like birds (Pamplona et al. 1996; 2005a, b; Buttemer et al. 2008),

naked mole-rats (Mitchell et al. 2007), the short-beaker echidna (Hulbert et al. 2008), queen honey bees (Haddad et al. 2007) or *Caenorhabditis elegans* (Shmookler Reis et al. 2011) and correlates with natural variation in longevity even within a species as it occurs in the case of different mice strains (Hulbert et al. 2006). The results obtained in the present study also corroborate that fact in brain and spleen from exceptionally old mice.

Lipid peroxidation is a kind of oxidative damage quantitatively important inside cells. Therefore, a low fatty acid unsaturation degree would be advantageous by strongly decreasing the sensitivity to lipid peroxidation. The low peroxidizability index observed in long-lived species is due to changes in the type of unsaturated fatty acid that participates in the membrane composition. Thus, there is a systematic redistribution between the types of polyunsaturated fatty acids present from the highly unsaturated 22:6 n -3 and 20:4 n -6 in short-lived species to the less unsaturated linoleic acid (18:2 n -6) and, in some cases, linolenic acid (18:3 n -3) in the long-lived ones at mitochondrial and tissue levels. Among these changes, the fatty acid contributing most to lower the peroxidizability index in long-lived animals is 22:6 n -3 (Pamplona and Barja 2011). In order to check if similar changes were occurring in the model studied in the present investigation, we measured the full fatty acid composition. We found a strongly decreased total number of fatty acid double bonds (DBI), as well as PI in brain and spleen from the naturally long-lived mice. The decrease in fatty acid unsaturation in the brain and spleen of the long-lived mice was due to variations in several fatty acids, but the most important changes observed were the decreases in the highly unsaturated 22:6 n -3 and 20:4 n -6. Just like in the case of long-lived animals compared to short-lived ones, the fatty acid most responsible for the decrease in PI was the highly unsaturated 22:6 n -3. The decrease in PI confers the membranes superior resistance to lipid peroxidation and lowers lipoxidation-dependent damage to macromolecules. In agreement with this, previous studies have shown that different tissues of long-lived animals have lower levels of MDAL when compared to short-lived species (Ruiz et al. 2005). Similarly, the decrease in fatty acid unsaturation in the exceptionally old mice of the present study brought about a significant decrease in MDAL in brain and spleen proteins. This finding shows that the relationship between the degree

Fig. 3 Nonenzymatic modification of proteins by malondialdehyde estimated using western blot analyses. Differences in the distribution of protein lipoxidative modifications in whole homogenates from both brain (**a**, upper right graph) and spleen (**b**, lower right graph) samples are shown ($n=4$ for each group).

Left numbers of the blots indicate apparent molecular weight. The left panels show the quantity (in arbitrary units) of these blots by densitometry. Values are expressed as 'relative MDAL levels'. Statistics are indicated inside graphs

of fatty acid unsaturation and lipoxidation-dependent damage to macromolecules like cellular proteins is not restricted to correlations when comparing animals with different longevities.

Concerning the mechanisms responsible for the decrease in peroxidizability index in the case of long-lived animals, the presence of low desaturase activities can be determinant (Pamplona et al. 2002). These enzymes are rate limiting in the multistep pathways of essential fatty acid synthesis from their dietary precursors, 22:6 $n-3$ from 18:3 $n-3$ and 20:4 $n-6$ from 18:2 $n-6$ (Guillou et al. 2010). In the case of our

exceptionally old mice, the highly unsaturated fatty acids 22:6 $n-3$ and 20:4 $n-6$ were also decreased, like in long-lived animals compared to short-lived ones. Accordingly, the estimations of enzymatic activities performed from product/substrate ratios clearly suggest that lower $\Delta 5$ and $\Delta 6$ desaturase activities, as well as peroxisomal β -oxidation, are also a trait of the exceptionally old mice of the present investigation. In accordance with this interpretation, a recent study with a phylogenomic approach to identify the genetic targets of natural selection for elongated longevity in mammals has been published (Jobson

Fig. 4 Protein markers of lipoxidative damage in both brain (a, upper graph) and spleen (b, lower graph) proteins of adult, old and exceptionally old mice. CML carboxymethyl-lysine. Statistics are indicated inside graphs. Values are mean $\pm$ SEM from six to 11 different animals

et al. 2010). In this work, comparing the nonsynonymous and synonymous evolution of 5.7 million codon sites across 25 species shows that genes involved in lipid composition (and particularly desaturation system) have collectively undergone increased selective pressure in long-lived species. So, cellular membrane has apparently been the optimized feature.

When the different markers of protein modification were studied, it was found that exceptionally old mice not only lowered protein lipoxidation (MDAL) but also decreased the two different markers of protein oxidation measured, the specific protein carbonyls GSA and AASA. These two markers are main carbonyl products of protein oxidation (Requena et al. 2001). Concerning those decreases, it must be noted that the

lipid peroxidation products are powerful reactive molecular species. Lipid peroxidation should not be perceived solely in damage to lipids scenario but should also be considered as a significant endogenous source of damage to other cellular macromolecules including proteins. Lipid peroxidation generates products like MDAL or hydroxynonenal, but it also produces secondary free radicals (Spiteller 2010). The decrease observed in fatty acid unsaturation in the exceptionally old group could thus lower the formation of these lipid-derived secondary free radicals and then decrease the levels of oxidated proteins (GSA and AASA). An additional low mitochondrial free radical generation in the exceptionally old mice cannot be discarded, whereas they have been described

Fig. 5 Protein markers of glycoxidative damage in both brain (a, upper graph) and spleen (b, lower graph) proteins of adult, old and exceptionally old mice. CEL carboxyethyl-lysine. Statistics are indicated inside graphs. Values are mean $\pm$ SEM from six to 11 different animals

to preserve better the antioxidant defences in a variety of tissues including those used in the present work (Arranz et al. 2010).

In contrast to the protein oxidation and lipoxidation markers, protein glycooxidation—estimated from the level of CEL—was increased in brain and spleen of the exceptionally old mice. This contrasts with the generalized decrease in oxidation-related parameters brought about by long-lived animals, including the decreases in fatty acid unsaturation, CML, MDAL and protein carbonyls (GSA and AASA). Despite the reason why the glycooxidation-related marker CEL was increased instead of decreased in the present investigation cannot be clarified with our data, this dissociation between marker of glycooxidation and those of lipoxidation or pure oxidation can rely on the chemical origin of CEL. CEL is formed during the reaction of methylglyoxal with lysine residues in proteins (Ahmed et al. 1997). A formation of CEL in reactions of pentoses, ascorbate and other sugars with lysine has been also described (Thornalley 2008). So, CEL provides an index of methylglyoxal concentration in tissues. One of the most important ways of methylglyoxal production occurs from the triose phosphate intermediates in the glycolytic pathway, which include dihydroxyacetone phosphate and glyceraldehyde 3-phosphate by two processes: spontaneous nonenzymatic elimination of the phosphate group, or decomposition of ene-diol triose intermediates that leaks from the active site of triose phosphate isomerase (Thornalley 2008). Thus, an increased or decreased glycolytic flux will be determinant in the intracellular methylglyoxal levels and the subsequent CEL formation. Assuming the key role of glycolytic flux in the cell energy generation, it is plausible to propose that CEL is a biomarker of the cellular bioenergetic status. So, if it would exist an increase in glycolytic intermediates, it could explain why the glycooxidation marker was increased in exceptionally old mice. Consequently, the increased levels of CEL observed in brain and spleen of the exceptionally old animals, like in adult mice, could be considered as a good indicator of a better bioenergetic status of these organs when compared to the old group. Because energy impairments are found in a variety of age-related neurodegenerative processes (Martínez et al. 2010), these findings suggest that old but not long-living subjects would be susceptible to those diseases.

In conclusion, the exceptionally old mice of the present investigation analogously to long-lived animal species (mammals and birds) share a common characteristic: They have tissue macromolecules highly resistant to modification and low rate of generation of endogenous oxidative damage.

Acknowledgements This study was supported in part by I+D grants from the Spanish Ministry of Science and Innovation (BFU2008-04336, BFU2009-11879/BFI, BFU2011-30336), the Spanish Ministry of Health (ISCIII, Red de Envejecimiento y Fragilidad, RD06/0013/0003 and RD06/0013/0012), the Generalitat of Catalunya (2009SGR735) and UCM (ENEROINN-910379) to research groups. Dr. L. Arranz received predoctoral fellowship from the Ministry of Education and Science. The authors are grateful to the anonymous reviewers for criticisms and suggestions, which improved the manuscript.

References

- Ahmed MU, Brinkmann Frye E, Degenhardt TP, Thorpe SR, Baynes JW (1997) N-Epsilon-(carboxyethyl)lysine, a product of the chemical modification of proteins by methylglyoxal, increases with age in human lens proteins. *Biochem J* 324:565–570
- Arranz L, Caamaño JH, Lord JM, De la Fuente M (2010) Preserved immune functions and controlled leukocyte oxidative stress in naturally long-lived mice: possible role of nuclear factor kappa B. *J Gerontol A Biol Sci Med Sci* 65:941–950
- Bielski BHJ, Arudi RI, Sutherland MW (1983) A study of the reactivity of HO₂/O₂ with unsaturated fatty acids. *J Biol Chem* 258:4759–4761
- Buttemer WA, Battam H, Hulbert AJ (2008) Fowl play and the price of petrel: long-living Procellariiformes have peroxidation-resistant membrane composition compared with short-living Galliformes. *Biol Lett* 4:351–354
- Esterbauer H, Schaur RJ, Zollner H (1991) Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* 11:81–128
- Fu MX, Requena JR, Jenkins AJ, Lyons TJ, Baynes JW, Thorpe SR (1996) The advanced glycation end product, Nepsilon-(carboxymethyl)lysine, is a product of both lipid peroxidation and glycooxidation reactions. *J Biol Chem* 271:9982–9986
- Gruber J, Schaffer S, Halliwell B (2008) The mitochondrial free radical theory of ageing—where do we stand? *Front Biosci* 13:6554–6579
- Guillou H, Zdravcov D, Martin PG, Jacobsson A (2010) The key roles of elongases and desaturases in mammalian fatty acid metabolism: insights from transgenic mice. *Prog Lipid Res* 49:186–199
- Haddad LS, Kelbert L, Hulbert AJ (2007) Extended longevity of queen honey bees compared to workers is associated with peroxidation-resistant membranes. *Exp Gerontol* 42:601–609

- Holman RT (1954) Autoxidation of fats and related substances. In: Holman RT, Lundberg WO, Malkin T (eds) Progress in chemistry of fats and other lipids. Pergamon, London, pp 51–98
- Hulbert AJ, Faulks SC, Harper JM, Miller RA, Buffenstein R (2006) Extended longevity of wild-derived mice is associated with peroxidation-resistant membranes. *Mech Ageing Dev* 127:653–657
- Hulbert AJ, Pamplona R, Buffenstein R, Buttemer WA (2007) Life and death: metabolic rate, membrane composition and life span of animals. *Physiol Rev* 87:1175–1213
- Hulbert AJ, Beard LA, Grigg GC (2008) The exceptional longevity of an egg-laying mammal, the short-beaked echidna (*Tachyglossus aculeatus*) is associated with peroxidation-resistant membrane composition. *Exp Gerontol* 43:729–733
- Jobson RW, Nabholz B, Galtier N (2010) An evolutionary genome scan for longevity-related natural selection in mammals. *Mol Biol Evol* 27:840–847
- Knecht KJ, Dunn JA, McFarland KF, McCance DR, Lyons TJ, Thorpe SR, Baynes JW (1991) Effect of diabetes and aging on carboxymethyllysine levels in human urine. *Diabetes* 40:190–196
- la Fuente D, Miquel J (2009) An update of the oxidation-inflammation theory of aging: the involvement of the immune system in oxi-inflamm-aging. *Curr Pharm Des* 15:3003–3026
- Leeuwenburgh C, Wagner P, Holloszy JO, Sohal RS, Heinecke JW (1997) Caloric restriction attenuates dityrosine cross-linking of cardiac and skeletal muscle proteins in aging mice. *Arch Biochem Biophys* 346:74–80
- Linton S, Davies MJ, Dean RT (2001) Protein oxidation and ageing. *Exp Gerontol* 36:1503–1518
- Martínez A, Portero-Otín M, Pamplona R, Ferrer I (2010) Protein targets of oxidative damage in human neurodegenerative diseases with abnormal protein aggregates. *Brain Pathol* 20:281–297
- Matsugo S, Kitagawa T, Minami S, Esashi Y, Oomura Y, Tokumaru S, Kojo S, Matsushima K, Sasaki K (2000) Age-dependent changes in lipid peroxide levels in peripheral organs, but not in brain, in senescence-accelerated mice. *Neurosci Lett* 278:105–108
- Mitchell TW, Buffenstein R, Hulbert AJ (2007) Membrane phospholipid composition may contribute to exceptional longevity of the naked mole-rat (*Heterocephalus glaber*): a comparative study using shotgun lipidomics. *Exp Gerontol* 42:1053–1062
- Naudi A, Jove M, Ayala V, Portero-Otín M, Barja G, Pamplona R (2011) Regulation of membrane unsaturation as antioxidant adaptive mechanism in long-lived animal species. *Free Radic Antiox* 1:3–12
- Pamplona R (2008) Membrane phospholipids, lipoxidative damage and molecular integrity: a causal role in aging and longevity. *Biochim Biophys Acta* 1777:1249–1262
- Pamplona R (2011) Advanced lipoxidation end-products. *Chem Biol Interact* 192:14–20
- Pamplona R, Barja G (2007) Highly resistant macromolecular components and low rate of generation of endogenous damage: two key traits of longevity. *Ageing Res Rev* 6:189–210
- Pamplona R, Barja G (2011) An evolutionary comparative scan for longevity-related oxidative stress resistance mechanisms in homeotherms. *Biogerontology*. doi:10.1007/s10522-011-9348-1
- Pamplona R, Costantini D (2011) Molecular and structural antioxidant defenses against oxidative stress in animals. *Am J Physiol Regul Integr Comp Physiol* 301:R843–R863
- Pamplona R, Prat J, Cadenas S, Rojas C, Perez-Campo R, Lopez-Torres M, Barja G (1996) Low fatty acid unsaturation protects against lipid peroxidation in liver mitochondria from longevous species: the pigeon and human case. *Mech Ageing Dev* 86:53–66
- Pamplona R, Barja G, Portero-Otín M (2002) Membrane fatty acid unsaturation, protection against oxidative stress, and maximum life span: a homeoviscous-longevity adaptation. *Ann N Y Acad Sci* 959:475–490
- Pamplona R, Dalfo E, Ayala V, Bellmunt MJ, Prat J, Ferrer I, Portero-Otín M (2005a) Proteins in human brain cortex are modified by oxidation, glycooxidation, and lipoxidation. Effects of Alzheimer disease and identification of lipoxidation targets. *J Biol Chem* 280:21522–21530
- Pamplona R, Portero-Otín M, Sanz A, Ayala V, Vasileva E, Barja G (2005b) Protein and lipid oxidative damage and complex I content are lower in the brain of parakeets and canaries than in mice. Relation to aging rate. *AGE* 27:267–280
- Park JW, Choi CH, Kim MS, Chung MH (1996) Oxidative status in senescence accelerated mice. *J Gerontol A Biol Sci Med Sci* 51:B337–B345
- Portero-Otín M, Pamplona R (2006) Is endogenous oxidative protein damage involved in the aging process? In: Pietzsch J (ed) Protein oxidation and disease. Research Signpost, Kerala, pp 91–142
- Puca AA, Andrew P, Novelli V, Anselmi CV, Somalvico F, Cirillo NA, Chatgililoglu C, Ferreri C (2008) Fatty acid profile of erythrocyte membranes as possible biomarker of longevity. *Rejuvenation Res* 11:63–72
- Requena JR, Fu MX, Ahmed MU, Jenkins AJ, Lyons TJ, Baynes JW, Thorpe SR (1997) Quantification of malondialdehyde and 4-hydroxynonenal adducts to lysine residues in native and oxidized human low-density lipoprotein. *Biochem J* 322:317–325
- Requena JR, Chao CC, Levine RL, Stadtman ER (2001) Glutamic and amino adipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins. *Proc Natl Acad Sci USA* 98:69–74
- Ruiz MC, Ayala V, Portero-Otín M, Requena JR, Barja G, Pamplona R (2005) Protein methionine content and MDA-lysine adducts are inversely related to maximum life span in the heart of mammals. *Mech Ageing Dev* 126:1106–1114
- Salmon AB, Richardson A, Pérez VI (2010) Update on the oxidative stress theory of aging: does oxidative stress play a role in aging or healthy aging? *Free Radic Biol Med* 48:642–655
- Sanz A, Pamplona R, Barja G (2006) Is the mitochondrial free radical theory of aging intact? *Antioxid Redox Signal* 8(3–4):582–99

- Shmookler Reis RJ, Xu L, Lee H, Chae M, Thaden JJ, Bharill P, Tazearslan C, Siegel E, Alla R, Zimniak P, Ayyadevara S (2011) Modulation of lipid biosynthesis contributes to stress resistance and longevity of *C. elegans* mutants. *Aging* (Albany NY) 3:125–147
- Sohal RS (2002) Role of oxidative stress and protein oxidation in the aging process. *Free Radic Biol Med* 33:37–44
- Spiteller G (2010) Is lipid peroxidation of polyunsaturated acids the only source of free radicals that induce aging and age-related diseases? *Rejuvenation Res* 13:91–103
- Thornalley PJ (2008) Protein and nucleotide damage by glyoxal and methylglyoxal in physiological systems—role in ageing and disease. *Drug Metabol Drug Interact* 23:125–150
- Thorpe SR, Baynes JW (2003) Maillard reaction products in tissue proteins: new products and new perspectives. *Amino Acids* 25:275–281
- Tsuduki T, Honma T, Nakagawa K, Ikeda I, Miyazawa T (2011) Long-term intake of fish oil increases oxidative stress and decreases lifespan in senescence-accelerated mice. *Nutrition* 27:334–337
- Xu L, Davis TA, Porter NA (2009) Rate constants for peroxidation of polyunsaturated fatty acids and sterols in solution and in lysosomes. *J Am Chem Soc* 131:13037–13044