Research article

Protein and lipid oxidative damage and complex I content are lower in the brain of budgerigar and canaries than in mice. Relation to aging rate

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Abstract

What are the mechanisms determining the rate of animal aging? Of the two major classes of endothermic animals, bird species are strikingly long-lived compared to mammals of similar body size and metabolic rate. Thus, they are ideal models to identify longevity-related characteristics not linked to body size or low metabolic rates. Since oxidative stress seems to be related to the basic aging process, we measured specific markers of different kinds of oxidative damage to proteins, like glutamic and amino adipic semialdehydes (GSA and AASA, specific protein carbonyls), N ϵ -(carboxyethyl)lysine (CEL), N ϵ -(carboxymethyl)lysine (CML), and N ϵ -(malondialdehyde)lysine (MDAL), as well as mitochondrial Complex I content and amino acid and membrane fatty acyl composition, in the brain of short-lived mice (maximum life span [MLSP] 3.5 years) compared with those of long-lived budgerigar ‘parakeets’ (MLSP, 21 years) and canaries (MLSP, 24 years). The brains of both bird species had significantly lower levels of compounds formed as a result of oxidative (GSA and AASA), glycoxidative (CEL and CML), and lipoxidative (CML and MDAL) protein modifications, as well as a lower levels of mitochondrial complex I protein. Although it is known that fatty acid unsaturation is lower in many tissues of long-lived compared to short-lived mammals, this is not true in the particular case of brain. In agreement with this, we also found that the brain tissue of budgerigars and canaries contains no fewer double bonds than that of mice. Amino acid composition analyses revealed that bird proteins have a significantly lower content of His, Leu and Phe, as well as, interestingly, of methionine, whereas Asp, Glu, Ala, Val, and Lys contents were higher than in the mammals. These results, together with those previously described in other tissues of pigeons (MLSP, 35 years) compared to rats (MLSP, 4 years), indicate that oxidative damage to proteins, lipids and mitochondrial DNA are lower in birds (very long-lived species) than in short-lived mammals of similar body size. The lower degree of oxidative modification of bird brain proteins was not due to decreases in the target amino acids (lysine for CEL, CML, MDAL, and AASA; and arg and pro for GSA), since these were present in bird brain proteins at higher or similar levels than in those of mice. These results are consistent with the possibility that decreases in oxidative protein modification are caused at least in part by the low rate of mitochondrial oxygen radical generation in these birds, as in all long-lived homeothermic vertebrates investigated so far.

Abbreviations: AASA – aminoadipic semialdehyde; CEL – N ϵ -(carboxyethyl)lysine; CML – N ϵ -(carboxymethyl)lysine; DBI – double bond index; GSA – glutamic semialdehyde; MDAL – N ϵ -(malondialdehyde)lysine; MLSP – maximum life span; PI – peroxidizability index; PUFA – polyunsaturated fatty acids; ROS – reactive oxygen species; SFA – saturated fatty acids; UFA – unsaturated fatty acids

Introduction

What are the mechanisms determining the rate of animal aging? The idea that oxygen radicals (ROS, or reactive oxygen species), especially those of mitochondrial origin are causally related to the basic aging process (Harman 1972), is increasingly receiving support from scientific studies (see Barja 2004a, b for review). This hypothesis is supported by several lines of evidence including comparisons between animal species with different aging rates.

Of the two major classes of endothermic animals, birds are strikingly long-lived compared to their mammalian counterparts; many birds live three to four times longer than mammals of equivalent body mass (Holmes and Austad 1995). In the context of the free radical hypothesis of aging, the slow aging rates typical of the class Aves may seem paradoxical when taking into account their basal metabolic rates (that are at least as high as, and often higher than those of similarly sized mammals), oxygen consumption (birds process more oxygen per g of tissue than do mammals), body temperatures (up to 3 °C higher, depending on the species), and blood glucose levels (two- to four-fold higher) (Holmes and Austad 1995; Holmes et al. 2001). According to the free radical/oxidative stress hypothesis of aging, elevation of these parameters in birds should contribute to accelerated tissue damage due to the presence of higher levels of deleterious by-products of oxidative metabolism (Beckman and Ames 1998), and higher intensities of the Maillard- or carbonyl-amine reactions, which include glycoxidation and lipoxidation reactions (Baynes 2002; Thorpe and Baynes 2003). This suggests that birds may employ special molecular adaptations to prevent or protect against generation of oxidative damage and advanced Maillard end-products or 'AGEs' (Holmes et al. 2001).

In order to examine oxidative stress-related molecular traits associated with the slow aging rate of birds compared to mammals, we used GC/MS methods measuring steady-state levels of markers of various pathways of oxidative protein modification in the brain of mice (MLSP: 3.5 years), budgerigar 'parakeets' and canaries (21 and 24 years, respec-

tively). Our choice of tissue was guided by the fact that brain (despite consisting of heterogeneous tissue) is primarily composed of long-lived, high glucose- and oxygen-consuming postmitotic cells, a feature shared with other critical aging targets such as heart and skeletal muscle, and is of utmost importance for whole-body homeostasis, aging and longevity. It has been proposed that oxidation of proteins is one of the key mechanisms linking oxygen radicals with the basic aging process (Berlett and Stadtman 1997; Stadtman and Berlett 1998). The markers measured were, for protein oxidation: glutamic semialdehyde (GSA) and aminoadipic semialdehyde (AASA), which are specific protein carbonyls; for glycoxidation: N ϵ -(carboxyethyl)lysine (CEL); for mixed glyco- and lipoxidation: N ϵ -(carboxymethyl)lysine (CML); and for lipoxidation: N ϵ -(malondialdehyde)lysine (MDAL). In addition, since complex I is the main mitochondrial site where free-radical production is lowered in animals with extended longevity (as shown in pigeons or calorically restricted rodents; reviewed in Barja 2004a, b), its content was also estimated by immunoblotting. Finally, to assess the role of structural components that can act as substrate targets in determining the degree of protein damage, the amino acid composition and fatty acid profile were also evaluated.

Materials and methods

Animals

Adult mature male mice (*Mus musculus*) of six months of age were obtained from Iffa-Creddo (Lyon, France). Adult mature male budgerigars (*Melopsittacus undulatus*) and canaries (*Serinus canarius*) of two–three years of age were obtained from commercial suppliers in Madrid (Spain). Young adult ages were selected to be within 10–15% of the MLSP of the species. All the animals were acclimated in the laboratory for at least two weeks at 20 $\pm$ 2 °C, under 12:12 L:D illumination conditions and 50–60% relative humidity. Animals had free access to drinking water and food. Mice, budgies and cana-

ries were euthanized by cervical dislocation. Whole brains were washed free of blood, frozen in liquid nitrogen, and stored at -80°C .

Preparation of the brain samples

Brain samples were homogenized in a buffer containing 180 mM KCl, 5 mM 3-[N-morpholino] propanesulfonic acid, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM diethylenetriaminepentaacetic acid and 1 μM butylated hydroxyl toluene, pH 7.3 (Potter-Elvehjem device, at 4°C). After a brief centrifugation (500 g, 5 min) to pellet cellular debris, protein concentrations were measured in the supernatants using the Lowry assay (BioRad Laboratories, München, Germany).

GSA, AASA, CML, CEL and MDAL measurements by gas chromatography/mass spectrometry (GC/MS)

GSA, AASA, CML, CEL and MDAL concentrations in the crude homogenate fraction were measured by GC/MS as previously described (Pamplona et al. 2005). Briefly, samples containing 0.5 mg of protein were delipidated using chloroform:methanol (2:1 v/v) in the presence of 0.01% butylated hydroxytoluene, and proteins were precipitated by adding trichloroacetic acid to 10% (v/v) final concentration, followed by centrifugation. Protein samples were immediately reduced by overnight incubation with 500 mM NaBH_4 in 0.2 M borate buffer at pH 9.2, and containing one drop of hexanol as an anti-foam reagent. Protein was reprecipitated by adding 1 ml of 20% (v/v) trichloroacetic acid, followed by centrifugation. Isotopically labelled internal standards ($[\text{H}_8]$ -lysine (CDN Isotopes); $[\text{H}_4]$ -CML, $[\text{H}_4]$ -CEL, and $[\text{H}_8]$ -MDAL prepared as previously described (Knecht et al. 1991; Ahmed et al. 1997; Requena et al. 1997); and $[\text{H}_5]$ 5-hydroxy-2-aminovaleric acid (for GSA quantification), and $[\text{H}_4]$ 6-hydroxy-2-aminocaproic acid (for AASA quantification), prepared as previously described (Requena et al. 2001), were then added. The samples were hydrolysed at 155°C for 30 min in 1 ml of 6 M HCl, 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 HP-5MS capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm) coupled to a Hewlett-Packard model 5973A mass-selective detector. The injection port was maintained at 275°C ; the temperature program was 5 min at 110°C , then $2^{\circ}\text{C}/\text{min}$ to 150°C , then $5^{\circ}\text{C}/\text{min}$ to 240°C , then $25^{\circ}\text{C}/\text{min}$ 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 (SIM-GC/MS). The ions used were: lysine and $[\text{H}_8]$ -lysine, m/z 180 and 187, respectively; 5-hydroxy-2-aminovaleric acid and $[\text{H}_5]$ 5-hydroxy-2-aminovaleric acid (stable derivatives of GSA), m/z 280 and 285, respectively; 6-hydroxy-2-aminocaproic acid and $[\text{H}_4]$ 6-hydroxy-2-aminocaproic acid (stable derivatives of AASA), m/z 294 and 298, respectively; CML and $[\text{H}_4]$ -CML, m/z 392 and 396, respectively; CEL and $[\text{H}_4]$ -CEL, m/z 379 and 383, respectively; and MDAL and $[\text{H}_8]$ -MDAL, m/z 474 and 482, respectively. The amounts of product were expressed as the μmolar ratio of GSA, AASA, CML, CEL or MDAL/mol lysine.

SDS-PAGE and Immunodetection of CML, CEL, and MDAL

SDS-PAGE was performed according to Laemmli (Laemmli 1970), using a 9% acrylamide as resolving gel. For immunodetection, after SDS-PAGE, proteins were transferred using a Mini Trans-Blot Transfer Cell (Bio-Rad Laboratories, Madrid, Spain) to PVDF membranes (Immobilon-P Millipore, Bedford, MA). Immunodetection was performed using a monoclonal anti-CEL antibody (1:1,000, Transgenic, Inc., Kumamoto, Japan), a monoclonal anti-CML antibody (1:2,000, Transgenic, Inc. 6D12, Kumamoto, Japan), and a polyclonal anti-MDAL antibody (1:2,000, Academy Biomedical Co). Peroxidase-coupled secondary antibodies were used from the Tropix chemiluminescence kit (Bedford, MA). Signal quantification and recording was performed with a CCD camera-based system (Lumi-Imager) from Boehringer Mannheim.

Mitochondrial complex I content

The level of expression of mitochondrial complex I was evaluated using Western blot analysis. Immuno-

detection was performed using a monoclonal antibody specific for the 39,000-Da subunit of complex I (1:2,000, Molecular Probes, Invitrogen Ltd, UK). An antibody to porin (1:1,000, Molecular Probes, Invitrogen Ltd, UK) as a control for total mitochondrial mass was also used in order to determine the proportion of complex I to total mitochondrial mass. Peroxidase-coupled secondary antibodies were used from the Tropix chemiluminescence kit (Bedford, MA). Signal quantification and recording was performed with a CCD camera-based system (Lumi-Imager) from Boehringer Mannheim.

Amino acid analysis

High-purity amino-acid calibration standard for protein hydrolyzates (containing 2.5 μ moles/ml of 0.1 N HCl of 17 protein amino acids, except for L-cysteine, which is supplied at 1.25 μ moles/ml) was obtained from Pierce (Rockford, IL). 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate 10 mM in acetonitrile (AQC, Waters AccQ Fluor reagent) and borate buffer were obtained as a kit from Millipore (Milford, MA). Protein samples of 500 μ g each were precipitated with 10% TCA (final concentration), hydrolyzed in 1 ml 6N HCl for 30 min at 155 °C, and then dried in vacuo. Protein hydrolyzates were resuspended in 100 μ l HCl 4 mM (Pierce, Rockford, IL), and a 10 μ l aliquot of the simple extract or amino acid standard solution (with the internal standard added) was buffered with 70 μ l of borate buffer. The derivatives were formed with 20 μ l of AQC and heated for 10 min at 55 °C. The HPLC system consisted of a Waters model 510 pump, an AccQ-Tag C18 amino acid analysis column (3.9 mm $\times$ 150 mm), a column heater, and a model 470 fluorescence detector set at 250/395 nm as excitation/emission wavelength, respectively (all from Waters). Millennium software (Waters) was used to control system operation and to collect and analyze data.

Eluent A (concentrated sodium acetate buffer) was obtained from Millipore. Mobile phase A was prepared by mixing 100 ml of eluent A (concentrate) with 1,000 ml of water. Mobile phase B was 60% acetonitrile: 40% water. The AccQ-Tag column was thermostated at 37 °C and operated at a flow rate of 1.0 ml/min. The chromatographic system was equilibrated in eluent A and the gradient employed con-

sisted of elution to 93% A in 15 min, then elution to 67% A in 18 min, then washing with 100% B in 2 min for 3 min, and equilibration in 100% A for 27 min. Quantification was performed by using standard curves for each amino acid, and results recalculated to be expressed as mol%. The following indices from amino acid compositional analysis were also calculated: KR (Lys + Arg): positive charge; ED (Glu + Asp): negative charge; KR + ED: total charge; KR-ED: net charge; LVIFM (Leu + Val + Ile + Phe + Met): major hydrophobics; and the groupings AGP (Ala + Gly + Pro) (encoded by CCN, GCN, and GGN codons), and FIKMNY (Phe + Ile + Lys + Met + Asn + Tyr) (encoded by AAN, AUN, UAN, and UUN codons), according to Brendel et al. (Brendel et al. 1992).

Fatty acid analysis

Fatty acyl groups were analyzed as previously described (Pamplona et al. 2005). Brain lipids were extracted from homogenate fractions with chloroform:methanol (2:1, v/v) in the presence of 0.01% (w/v) butylated hydroxytoluene. The chloroform phase was evaporated under nitrogen and the fatty acyl groups were transesterified by incubation in 2.5 ml of 5% (v/v) methanolic HCl for 90 min at 75 °C. The resulting fatty acid methyl esters were extracted by adding 1 ml of saturated NaCl solution and 2.5 ml of *n*-pentane. The *n*-pentane phase was separated and evaporated under nitrogen. The residue was dissolved in 75 μ l of carbon disulphide and 1 μ l was used for GC/MS analysis. Separation was performed in a SP2330 capillary column (30 m $\times$ 0.25 mm $\times$ 0.20 μ m) in a Hewlett Packard 6890 Series II gas chromatograph. A Hewlett Packard 5973A mass spectrometer was used as detector in the electron-impact mode. 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 °C/min to 200 °C, then 5 °C/min to 240 °C, and finally hold at 240 °C for 10 min. Identification of fatty acyl methyl esters was made as previously by comparison with authentic standards and on the basis of mass spectra. Results are expressed as mol%.

The following fatty acyl indices were also calculated: saturated fatty acids (SFA); unsaturated fatty acids (UFA); monounsaturated fatty acids (MUFA);

Figure 1. Brain proteins from birds show significantly lower amounts of GSA and AASA. For details see the Materials and methods section. Values are means $\pm$ SEM from $n = 8$ samples/species. *** $P < 0.001$.

polyunsaturated fatty acids from $n - 3$ and $n - 6$ series (PUFA $n - 3$ and PUFA $n - 6$); average chain length (ACL) = $[(\Sigma\% \text{Total}14 \times 14) + (\Sigma\% \text{Total}16 \times 16) + (\Sigma\% \text{Total}18 \times 18) + (\Sigma\% \text{Total}20 \times 20) + (\Sigma\% \text{Total}22 \times 22)]/100$; double bond index (DBI) = $[(\Sigma\text{mol}\% \text{ monoenoic}) + (2 \times \Sigma\text{mol}\% \text{ dienoic}) + (3 \times \Sigma\text{mol}\% \text{ trienoic}) + (4 \times \Sigma\text{mol}\% \text{ tetraenoic}) + (5 \times \Sigma\text{mol}\% \text{ pentaenoic}) + (6 \times \Sigma\text{mol}\% \text{ hexaenoic})]$, and peroxidizability index (PI) = $[(0.025 \times \Sigma\text{mol}\% \text{ monoenoic}) + (\Sigma\text{mol}\% \text{ dienoic}) + (2 \times \Sigma\text{mol}\% \text{ trienoic})$

$+ (4 \times \Sigma\text{mol}\% \text{ tetraenoic}) + (6 \times \Sigma\text{mol}\% \text{ pentaenoic}) + (8 \times \Sigma\text{mol}\% \text{ hexaenoic})]$.

Statistics

Data were analyzed using a one-way analysis of variance, using the SPSS-PC program (SPSS, Chicago, IL). The minimum level of statistical significance was set at $P < 0.05$ in all the analyses.

Figure 2. Birds show significant lower amounts of glycoxidative (CEL), mixed glyco- and lipoxidative (CML), and lipoxidative (MDAL) stress in brain proteins. For details see the Materials and methods section. Values are means $\pm$ SEM from $n = 8$ samples/species. *** $P < 0.001$.

Figure 3. Distribution of protein oxidative modifications (MDAL, CML, and CEL) in homogenates from brain samples showing a lower immunoreactivity in brain proteins from birds.

Results

Steady-state levels of protein damage

A comparison of brain from long-lived budgerigar 'parakeet' and canary with the short-lived mouse revealed that MLSP was associated with relevant differences in the steady-state levels of protein damage markers. All the five markers surveyed (GSA,

AASA, CML, CEL and MDAL) displayed significantly higher levels in mouse brain proteins than in those of birds, both in parakeets and canaries (Figures 1 and 2). No statistically significant differences were observed between the two bird species (Figures 1 and 2). The Western blot performed to detect CML, CEL and MDAL confirmed the higher protein damage observed in mice than in the birds and exhibited a specific profile of protein non-enzymatic modification (Figure 3).

Figure 4. The level of expression of the ratio of complex I to total mitochondrial mass in brain evaluating the 39,000-Da subunit by Western blot suggested a lower content of complex I in birds compared to mammals. Abbreviations: M, *Mus musculus* (adult male mice); P, *Melopsittacus undulatus* (adult male budgerigar parakeets); C, *Serinus canarius* (adult male canaries).

Mitochondrial complex I content

The level of expression of the brain complex I protein, [calibrated using] a marker of total mitochondrial mass (porin), was performed by evaluating the 39,000-Da complex I subunit using a Western blot. The results also suggested that both birds have a lower content of complex I protein than the mammal (Figure 4).

Amino acid compositional analysis

Amino acid composition of the brain proteins was also compared between mammals and birds (Table 1). The amino acid compositional analysis revealed that the amino acids targets of the protein modifications measured in this study either did not differ significantly among the two bird species and the mouse (as in the case of proline and arginine for the oxidative marker GSA), or were higher in the birds (as in the case of lysine for the glyco- and lipoxidative markers CML, CEL and MDAL, as well

Table 1. Amino acid composition (mol %) in brain proteins from mice, parakeets and canaries.

	Mouse	Parakeet	Canary	P (MvsP)	P (MvsC)	P (PvsC)
Asp	3.28 ± 0.05	3.62 ± 0.03	3.76 ± 0.06	0.000	0.000	0.082
Ser	6.69 ± 0.08	6.59 ± 0.04	6.31 ± 0.06	0.305	0.002	0.012
Glu	4.69 ± 0.12	5.33 ± 0.03	5.68 ± 0.14	0.001	0.000	0.037
Gly	11.35 ± 0.11	11.26 ± 0.09	10.94 ± 0.17	0.623	0.039	0.094
His	4.15 ± 0.08	3.78 ± 0.08	3.50 ± 0.12	0.014	0.000	0.072
Arg + Thr	10.39 ± 0.14	10.73 ± 0.04	10.23 ± 0.12	0.047	0.348	0.009
Ala	6.83 ± 0.10	7.15 ± 0.04	7.61 ± 0.13	0.030	0.000	0.005
Pro	2.79 ± 0.03	2.75 ± 0.04	2.85 ± 0.01	0.451	0.297	0.090
Tyr	5.72 ± 0.10	5.85 ± 0.06	5.54 ± 0.11	0.341	0.223	0.044
Val	7.81 ± 0.04	8.18 ± 0.05	8.21 ± 0.03	0.000	0.000	0.659
Met	1.85 ± 0.01	1.59 ± 0.08	1.71 ± 0.01	0.001	0.053	0.033
Lys	4.77 ± 0.08	5.00 ± 0.07	5.16 ± 0.14	0.135	0.019	0.270
Ile	8.70 ± 0.06	8.67 ± 0.04	8.68 ± 0.05	0.750	0.802	0.957
Leu	10.61 ± 0.11	10.34 ± 0.06	10.33 ± 0.05	0.043	0.042	0.915
Phe	10.30 ± 0.35	9.13 ± 0.09	9.39 ± 0.06	0.002	0.016	0.445
KR	15.17 ± 0.18	15.73 ± 0.05	15.40 ± 0.04	0.004	0.204	0.068
ED	7.98 ± 0.16	8.95 ± 0.07	9.45 ± 0.17	0.000	0.000	0.030
KR+ED	23.15 ± 0.32	24.69 ± 0.09	24.85 ± 0.17	0.000	0.000	0.627
KR-ED	7.18 ± 0.13	6.78 ± 0.08	5.94 ± 0.19	0.054	0.000	0.001
LVIFM	39.29 ± 0.49	37.91 ± 0.14	38.36 ± 0.18	0.009	0.074	0.354
AGP	20.98 ± 0.20	21.16 ± 0.10	21.40 ± 0.12	0.381	0.073	0.299
FIKMNY	34.64 ± 0.43	33.84 ± 0.08	34.29 ± 0.12	0.055	0.392	0.283

Values are mean ± SEM from $n = 8$ specimens each from mice, parakeets and canaries.

M mouse, P parakeet, C canary.

For details see the Materials and Methods section.

as the oxidative marker AASA). The amino acid compositional analysis in birds demonstrated significantly lower content of His, Met, Leu and Phe, whereas Asp, Glu, Ala, and Val were higher in birds than in mammals. Finally, the different parameters calculated using amino acid composition showed a significantly higher negative and total charge in bird brain proteins, together with a lower net charge and lower content of major hydrophobic aminoacids, than in mouse brain proteins. No significant differences in the amino acid groupings FIKMNY and AGP (which are linked to different types of codons), however, were found between the birds and the mouse.

Membrane unsaturation and fatty acid profile

The membrane acyl composition of the mammals and birds studied (Table 2) indicates that their biological membranes maintain a similar fatty acid average chain length (around 18 carbon atoms). Bird brain lipids, however, showed a significantly higher content of saturated fatty acids, and a lower content in unsaturated fatty acid than mammals. Especially relevant was a finding of lower monounsaturated fatty

acid content in both bird species (Figure 5). The slightly higher polyunsaturated fatty acid content detected in birds (PUFA $n - 6$ in parakeets, and PUFA $n - 3$ in canaries) also led to a slightly higher double-bond content (3.4 and 2.2%, respectively) in the birds than in the mouse (Table 2).

Discussion

Birds generally have similar or higher basal metabolic rates, rates of oxygen consumption, body temperatures (although these were only 38.7 °C and 38.4 °C in the birds of this study; Herrero and Barja 1998), and blood glucose levels, on average, than mammals of similar body size. Thus one could in principle expect that, according to older versions of the oxidative stress hypothesis of aging, the higher levels of those parameters in birds should accelerate tissue damage due to generation of deleterious by-products of oxidative reactions. However, birds are very long-lived for their body size relative to mammals. Consequently, the exceptional longevity of

Table 2. Fatty acid composition (mol%) of total lipids in brain from mice, parakeets and canaries.

	Mouse	Parakeet	Canary	P (MvsP)	P (MvsC)	P (PvsC)
14:0	0.29 ± 0.01	0.25 ± 0.008	0.26 ± 0.02	0.084	0.204	0.578
16:0	22.80 ± 0.27	24.98 ± 0.08	25.14 ± 0.22	0.000	0.000	0.605
16:1 n -7	0.62 ± 0.10	0.47 ± 0.03	0.25 ± 0.02	0.106	0.000	0.024
18:0	22.14 ± 0.18	22.25 ± 0.25	23.93 ± 0.15	0.705	0.000	0.000
18:1 n -9	20.72 ± 0.33	14.91 ± 0.20	14.98 ± 0.29	0.000	0.000	0.870
18:2 n -6	0.58 ± 0.01	1.41 ± 0.05	1.06 ± 0.06	0.000	0.000	0.000
20:3 n -6	0.18 ± 0.007	0.23 ± 0.04	0.33 ± 0.03	0.241	0.003	0.050
20:4 n -6	9.42 ± 0.11	10.12 ± 0.15	8.27 ± 0.12	0.002	0.000	0.000
22:4 n -6	2.40 ± 0.03	3.59 ± 0.15	2.22 ± 0.05	0.000	0.170	0.000
22:5 n -6	0.23 ± 0.02	1.25 ± 0.10	0.32 ± 0.01	0.000	0.318	0.000
22:5 n -3	0.19 ± 0.008	0.19 ± 0.008	0.45 ± 0.04	0.999	0.000	0.000
22:6 n -3	19.92 ± 0.11	19.75 ± 0.21	21.98 ± 0.11	0.454	0.000	0.000
24:0	0.29 ± 0.009	0.36 ± 0.01	0.42 ± 0.02	0.041	0.000	0.035
24:1 n -9	0.15 ± 0.005	0.18 ± 0.007	0.32 ± 0.04	0.447	0.001	0.004
ACL	18.64 ± 0.008	18.71 ± 0.003	18.69 ± 0.009	0.000	0.000	0.217
SFA	45.54 ± 0.30	47.85 ± 0.23	49.77 ± 0.32	0.000	0.000	0.000
PUFA	32.95 ± 0.20	36.57 ± 0.17	34.66 ± 0.15	0.000	0.000	0.000
PUFA n -6	12.83 ± 0.16	16.62 ± 0.32	12.22 ± 0.11	0.000	0.052	0.000
PUFA n -3	20.12 ± 0.12	19.95 ± 0.21	22.44 ± 0.11	0.455	0.000	0.000
DBI	192.26 ± 0.81	199.77 ± 0.51	196.53 ± 0.82	0.000	0.001	0.006
PI	210.82 ± 1.12	223.90 ± 0.67	224.72 ± 1.03	0.000	0.000	0.555

Values are mean ± SEM from $n = 8$ specimens of mice, parakeets and canaries. For UFA (unsaturated fatty acids) and MUFA (monounsaturated fatty acids) content, see Figure 5.

M mouse, *P* parakeet, *C* canary, *ACL* average chain length, *SFA* saturated fatty acids, *UFA* unsaturated fatty acids, *PUFA* n -6/ n -3, polyunsaturated fatty acids n -6 or n -3 series, *MUFA* monounsaturated fatty acids; *DBI* double bond index; *PI* peroxidizability index.

For details see the Materials and methods section.

birds as a group suggests that they have evolved molecular adaptations to prevent or protect against oxidative damage and aging.

In principle, oxidative stress could be related to the rate of aging through variations in ROS genera-

tion, ROS elimination, or both. However, various lines of evidence now consistently indicate that levels of antioxidants alone, although perhaps involved in protection against various age-related diseases, do not determine the rate of aging (Barja 2004a, b; Sanz

Figure 5. Unsaturated and monounsaturated fatty acid content in brain lipids from birds and mammals. Values are means ± SEM from $n = 8$ samples/species. *** $P < 0.001$.

et al. 2005a). For this reason, a growing number of studies have now focused on the possible relationship between mitochondrial ROS generation and maximum longevity. All of these investigations have shown that the rate of ROS production by mitochondria isolated from post-mitotic tissues is indeed lower in long-lived than in short-lived species (Barja 2004a). This finding holds for a number of long-lived homeothermic animals, including birds and mammals, independent of their rates of oxygen consumption. This phenomenon can explain why endogenous tissue antioxidants correlate negatively with maximum longevity: long-lived animals have constitutively low levels of antioxidants because they produce ROS at a low rate. The studies in birds are especially illustrative because these animals live much longer than mammals of similar body size and metabolic rate, contradictory to the old "rate-of-living theory of aging" (Pearl 1928). In spite of their high rates of oxygen consumption, birds have low rates of mitochondrial free radical production in brain (Barja et al. 1994; Barja and Herrero 1998) and other major organs. Both small birds and large mammals have low rates of mitochondrial ROS production (Ku and Sohal 1993; Ku et al. 1993; Barja et al. 1994; Herrero and Barja 1997, 1998), in agreement with their slow aging rates, whereas metabolic rate is slow in large mammals but high in small birds. Thus, maximum longevity correlates better with the mitochondrial rate of ROS generation than with overall metabolic rate.

The site in the respiratory chain where ROS production is decreased in long-lived animals has been also studied. Oxygen radical generation by the respiratory chain has been classically attributed to complex III semiquinone (Boveris and Cadenas 2000). However, in agreement with early information obtained using submitochondrial particles (Takeshige and Minakami 1979), complex I also contains an important ROS generator in the form of intact, functional and well-coupled heart and brain mitochondria (Herrero and Barja 1997; Barja and Herrero 1998; Barja 2004a). Furthermore, the respiratory complex responsible for the lower mitochondrial ROS generation by tissues in a long-lived species (the pigeon: MLSP = 35 years) in relation to that of a short-lived one (the rat: MLSP = 4 years) is complex I, not complex III. Most interestingly, this trait appears to be inducible, since caloric restriction (which increases maximum longevity) decreases mitochon-

drial ROS generation in rat liver, heart and brain mitochondria (Gredilla et al. 2001a, b; Sanz et al. 2005a, b) only at the complex I generator, and in as little as seven weeks, in the case of liver (Sanz et al. 2004). In this context, it is especially intriguing that the low complex I content (estimated from the 39 Kd complex I subunit) was observed in this study in which parakeets and canaries were compared to mice, as well as in previously published studies in which pigeons were compared to rats (Saint Pierre et al. 2002). This low complex I content (also confirmed for complex III, data not shown) could be a cause of the low rate of mitochondrial free ROS production of long-lived bird species. Since complex I ROS production is the one shown to be most closely related to aging (Barja 2004a), a decrease in the amount of complex I protein can lead to a decreased rate of ROS generation in slowly aging animals.

ROS can attack many different cellular macromolecules, including proteins, lipids and DNA. Consistent with its localization close to the site of mitochondrial ROS production, brain and heart mtDNA oxidative damage, estimated by levels of 8-hydroxy-2-deoxyguanosine (8-oxodG), correlates negatively with maximum longevity in mammals (Barja and Herrero 2000), as well as when comparing mammals and birds (Herrero and Barja 1999); this correlation is not observed for nuclear DNA. In addition, the levels of 8-oxodG are higher in mtDNA than in nDNA in the brain and heart of all the 11 mammals and bird species we have studied (Barja and Herrero 2000; Herrero and Barja 1999).

The available data concerning protein oxidative damage are not conclusive. Variations in the steady-state levels of oxidatively modified proteins *in vivo* can be due to differences in rates of oxidant generation, antioxidative defenses, protein repair and degradative capacity, or susceptibility of proteins to oxidative modifications, and all these are tissue- or species-dependent. Thus, while in the present study it was clearly observed that brain of bugarigar parakeets and canaries has a lower degree of protein oxidation (specific protein carbonyls GSA and AASA) than that of mice, protein carbonyl contents are similar in pigeon and rat liver mitochondria (Pamplona et al. 1996) and even higher in the skeletal muscle of pigeons compared to rats (Portero-Otin et al. 2004). Further investigation is needed to establish the relationship between protein oxidation and longevity in mammals and birds.

Earlier research on levels of advanced Maillard-derived products (AGEs) in birds have included pentosidine in collagen and glycated hemoglobin (reviewed in Holmes et al. 2001), which do not correspond to intracellular proteins. These studies supported the prediction that birds have a lower steady-state level of these products than mammals. In agreement with these findings, in the present study we found lower steady-state levels of glycoxidation products (CEL and CML) in brain proteins of parakeets and canaries compared to mice. Similar investigations using other organs like skeletal muscle (Portero-Otin et al. 2004) and heart mitochondria (Pamplona et al. 1999) in rats and pigeons, however, disagree with this. Hence the degree of Maillard reaction-derived protein damage also seems to be organ- or species-dependent, like protein oxidative modification.

The MDAL results present a different picture. In this case, lower levels were observed in the brain of both parakeets and canaries compared to mice. Similarly, in every previous study performed to date, MDAL protein levels were always relatively lower in long-lived species, regardless of whether they were birds or mammals (Pamplona et al. 1999, 2000; Portero-Otin et al. 2004; Ruiz et al. 2005). MDAL is formed by covalent attachment of aldehydic functions of malondialdehyde (an important lipid peroxidation product) to lysine protein residues. Thus, the general rule appears to be that the longer the life span of a species, the lower is the level of purely lipoxidation-derived (MDAL) modification of tissue intracellular proteins.

On the other hand, the lower levels of protein oxidative modification of bird brain proteins were not due to decreases in the target amino acids (lys, arg, pro), since these were present in brain proteins at higher or similar levels to those in mice. The results are therefore rather consistent with the possibility that the low levels of protein oxidative modification observed here are due in part to the low rates of mitochondrial ROS generation found in all the long-lived species investigated up to date in homeothermic animals, whether they be birds or mammals (see Barja 2004a for a review).

Although the steady-state levels of the end-products of oxidative attack on proteins seem to increase in postmitotic tissues during aging (Stadtman and Berlett 1998), the oxidative mechanisms that

cause loss of particular cellular/tissue functions have not been clarified. In principle, nonenzymatic protein modifications should consist of spontaneous, random, unprogrammed and uncatalyzed chemical reactions. In addition, as a general rule all proteins can be the target of free radicals and reactive carbonyl species. However, key questions remain as to whether some key cellular or extracellular molecules are preferential targets of these nonenzymatic modifications, and whether the extent of their modification is sufficient to explain impaired cellular and tissue function during aging. In this context, the determination of the steady-state levels for a given marker during the aging process does not allow us to discern if specific proteins are damaged. Other important questions include: Is there a pattern of selectivity in the main protein targets, and is this pattern shared between species? Which proteins, specifically, are damaged, if any? Why are specific protein pools (if any) modified? If there is a mechanism of selective protein damage, what causal role does this play in aging? The Western blot analysis of nonenzymatic protein modification performed in the present study clearly indicates that there is a specific and selective pattern of protein damage.

With respect to the differences between mammals and birds in the amino acid profile: while the possible significance of differences in levels of other amino acids must still be developed on theoretical grounds, the differences in methionine content deserve a special attention. In this study we found that methionine content of brain proteins is lower in the birds (parakeet and canary) than in the mammal (mouse), in agreement with a previous finding of lower methionine protein content in pigeon than in rat skeletal muscle (Portero-Otin et al. 2004), and with our recent finding that the methionine content of heart proteins correlates strongly and negatively with the maximum longevity of different mammalian species (Ruiz et al. 2005). The magnitude of the interspecies differences were smaller in this investigation, however, especially in the case of the canary. This is most interesting, since many recent investigations point to a relationship between methionine and aging (Moskovitz 2001; Ruan et al. 2002; Stadtman et al. 2003, 2005). There are various possible mechanisms by which high methionine content could induce damage. Methionine residues of proteins are the most susceptible to oxidation by ROS (Moskowitz et al.

2001; Slyhenkov et al. 2002), and sensitivity of proteins to oxidative stress increases as a function of the number of methionine residues in the protein (Stadtman et al. 2005). Methionine dietary supplementation also increases iron and lipid peroxidation in rat liver (Mori and Hirayama 2004). Oxidation of methionine residues generates methionine sulfoxide in proteins, which deprives them of their function as methyl donors and may lead to loss of their biological activity (Carp et al. 1982; Ciorba et al. 1997). However, this modification can be repaired by methionine sulfoxide reductase in a thioredoxin-dependent reaction. In this context, it is most interesting that knocking out methionine sulfoxide reductase-A decreases longevity in mice and increases levels of protein carbonyls and sensitivity to hyperoxia in mice (Moskovitz et al. 2001). The opposite manipulation, overexpression of methionine sulfoxide reductase A, increases life span and delays the start of the aging process in *Drosophila* (Ruan et al. 2002). On the other hand, the oxidized form of thioredoxin produced in the reduction of methionine sulfoxide can be converted back to reduced thioredoxin by the enzyme thioredoxin reductase. In agreement with the 'methionine oxidation hypothesis' articulated above, overexpression of thioredoxin reductase seems to increase longevity in mice (Nakamura et al. 2002; Mitsui et al. 2002).

A high methionine content could also be detrimental because it is sequentially converted to metabolites like homocysteine (Mori and Hirayama 2004). Homocysteine levels increase with age in humans and they are a risk factor for aging and many chronic degenerative diseases (Drögue 2001; Durand et al. 2001; Ninomiya et al. 2004; Ferrari 2004). Homocysteine has a free thiol group that can be readily oxidized and generates protein mixed disulfides or thiol bridges between proteins.

Irrespective of the mechanism involved, it is well known that methionine dietary restriction increases maximum longevity in mammals (Richie et al. 1994; Zimmerman et al. 2003), in strong agreement with our finding that long-lived animal species have low levels of methionine in intracellular proteins (Ruiz et al. 2005). One of the most plausible mechanisms by which caloric restriction extends life span is by decreasing mitochondrial ROS production and oxidative damage to mtDNA (Gredilla and Barja 2001a, b; Barja 2004a, b). But it has been recently found that

such decreases also occur after restricting only the intake of protein (Sanz et al. 2004), a manipulation that also increases MLSP, as demonstrated in seven out of eight classic studies (reviewed in Sanz et al. 2005a), with protein restriction perfectly mimicking caloric restriction both qualitatively and quantitatively in this respect. Thus, it is possible that methionine restriction can be responsible for the decrease in ROS production and mtDNA damage observed both in protein and caloric restriction, as well as for the ensuing decrease in aging rate. This possibility warrants further investigation.

In addition to ROS production, another constitutive characteristic of long-lived animals connects aging with oxidative stress: the degree of fatty acid unsaturation of tissue cellular membranes. Unsaturated fatty acids (UFAs) are extremely sensitive to oxidation, their sensitivity to oxidation exponentially increasing as a function of the number of double bonds per fatty acid molecule (Bielski et al. 1983). We have found that the degree of unsaturation of membrane fatty acids is systematically lower in long-lived than in short-lived species, both when comparing mammals (rats or mice) with birds (pigeons, parakeets or canaries) as well as when comparing mammals of different longevity (see Pamplona et al. 2002; Pamplona and Barja 2003; and Barja et al. 2005, for a review). This has held true in a variety of investigations performed on liver mitochondrial phospholipids, total heart, liver and skeletal muscle phospholipids, heart mitochondria, and skeletal muscle. We have coined a term for this phenomenon (which is reminiscent of membrane acclimation to temperature at PUFA level in poikilotherms): we have called it a *homeoviscous longevity adaptation* (Pamplona et al. 2002). Our findings can perhaps explain in part why some avian cells (e.g., primary embryonic fibroblast-like cells) are more resistant to oxidative stress than murine cells (Ogburn et al. 2001).

The finding of negative correlations between the degree of fatty acid unsaturation and maximum longevity applies to most organs including heart, muscle, liver or kidney, but does not seem to apply to mammalian brain. In this particular organ, there is no significant correlation between total double-bond content and maximum mammalian longevity (Couture and Hulbert 1995). For some unknown reason perhaps related to cell excitability (Couture and Hulbert

1995), nervous tissue needs to maintain a certain (relatively high) degree of fatty acid unsaturation in all mammals (both in short-lived and in long-lived ones) (Farkas et al. 2000; Turner et al. 2005). In this investigation we consistently found the same trend in birds. Brain of neither parakeets nor canaries showed a lower double-bond content (DBI) than that of mice. In fact, brain in birds showed marginally higher (2–3%) DBI levels than that of mice, consistent with an expectation of the need for more effective maintenance of a high double-bond content in the brain of long-lived species (similar to that of short-lived mammals), rather than a physiologically meaningful higher level of unsaturation.

In summary, we have found in this investigation that brain of parakeets and canaries has lower levels of specific protein carbonyls (oxidation markers: GSA and AASA), as well as lower glycooxidation (CML and CEL) and lipoxidation (CML and MDAL) protein adducts than brain of mice. This is most relevant when taking into account that the body size is similar in the three model species used, and that the basal rate of oxygen consumption is similar or even higher in the birds. This can be explained at least in part by the well known lower rate of mitochondrial ROS generation of these bird species, as well as pigeons, compared to that of mice or rats. It is also consistent with a lower sensitivity to oxidative damage in birds and mammals and notably, with the lower methionine content of avian vs. mammalian proteins, especially since methionine protein residues are very susceptible to oxidation, and because it is well known that methionine restriction without caloric restriction increases maximum longevity. It is also strongly relevant that the birds seem to have a lower complex I protein content than the mouse, similar to what has been described in previous studies comparing pigeons and rats, since this is the respiratory chain protein complex containing the ROS generator site likely to be most relevant to aging.

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