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Aging Increases $N^{epsilon}$-(Carboxymethyl)lysine and Caloric Restriction Decreases $N^{epsilon}$-(Carboxyethyl)lysine and $N^{epsilon}$-(Malondialdehyde)lysine in Rat Heart Mitochondrial Proteins

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The present investigation studies the effect of aging, short-term and long-term caloric restriction on four different markers of oxidative, glycoxidative or lipoxidative damage to heart mitochondrial proteins: protein carbonyls (measured by ELISA); $N^{epsilon}$-(carboxyethyl)lysine (CEL), $N^{epsilon}$-(carboxymethyl)lysine (CML), and $N^{epsilon}$-(malondialdehyde)lysine (MDA-lys) measured by gas chromatography / mass spectrometry. Aging increased the steady state level of CML in rat heart mitochondria without changing the levels of the other three markers of protein damage. Short-term caloric restriction (six weeks) did not change any of the parameters measured. However, long-term (one year) caloric restriction decreased CEL and MDA-lys in heart mitochondria and did not change protein carbonyls and CML levels. The decrease in MDA-lys was not due to changes in the sensitivity of mitochondrial lipids to peroxidation since the measurements of the fatty acid composition showed that the total number of fatty acid double bonds was not changed by caloric restriction. The decrease in CEL and MDA-lys in caloric restriction agrees with the previously and consistently described finding that caloric restriction agrees with the previously and consistently described finding that caloric restriction lowers the rate of generation of reactive oxygen species (ROS) in rodent heart mitochondria, although in the case of CEL a caloric restriction-induced lowering of glycaemia can also be involved. The CEL and MDA-lys results support the notion that caloric restriction decreases oxidative stress-derived damage to heart mitochondrial proteins.

Keywords: Free radicals; Glycation; Fatty acids; Protein carbonyls; Malondialdehyde; Lipid peroxidation

Abbreviations: ROS, reactive oxygen species; MDA-lys, $N^{epsilon}$-(malondialdehyde)lysine; CEL, $N^{epsilon}$-(carboxyethyl)lysine; CML, $N^{epsilon}$-(carboxymethyl)lysine; AMPs, advanced Maillard products

INTRODUCTION

The production of reactive oxygen species (ROS) by mitochondria is currently considered a major mechanism of aging.[1–3] While a large part of the evidence supporting the mitochondrial free radical theory of aging comes from comparative and experimental studies,[4–6] detailed knowledge of steady-state levels of ROS-induced damage to relevant macromolecular targets at different ages and aging rates is also needed. ROS can damage all kinds of cellular macromolecules. Among them, oxidative damage especially to proteins is important since proteins are essential for the determination and control of tissue structure and function.

Although many reports have previously studied the effect of aging on oxidative damage to tissue proteins, the large majority of those investigations have used only one marker of damage, protein carbonyls. However, the detection of age-related variations can depend on the kind of protein damage marker selected. For instance, 0,0′-dityrosine protein

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crosslinks increase during aging and are lowered by caloric restriction in mouse heart and skeletal muscle but all these changes do not take place when the damage marker selected is o-tyrosine.[7] Furthermore, although various studies have found increases in protein carbonyls with age in tissues,[8–12] other studies could not confirm such findings.[13–15] Considering that protein carbonyls are not a single defined entity, the measurement of different markers using more sensitive and specific techniques is desirable to estimate oxidative protein damage during aging.

During the last decade, the presence of advanced Maillard products (AMPs) like $N^{epsilon}$-(malondialdehyde)lysine (MDA-lys), $N^{epsilon}$-(carboxymethyl)lysine (CML), and $N^{epsilon}$-(carboxyethyl)lysine (CEL) have been characterized in biological proteins.[16–19] All AMPs are products of the chemistry of reactive carbonyl compounds, including enols, enals, and alpha-amino, alpha-hydroxy and alpha-keto aldehydes and ketones.[20,21] Thus, MDA-lys is formed due to attachment of the lipid peroxidation product MDA to lysine residues in proteins,[19] CML can be formed by both lipid peroxidation and glycoxidation,[17] and CEL is the result of glycoxidative damage.[18] A common trait of these three protein adducts is their formation under oxidative conditions[20] and recent information shows that a direct relationship exists between mitochondrial ROS generation and formation of AMPs.[20] Increases in CML and CEL have been observed during aging in extracellular long-lived proteins like cartilage or skin collagen[22–24] or in the also long-lived lens proteins,[18,23] but no information is available for MDA-lys, CML, CEL or any other AMPs during aging or after caloric restriction in the shorter-lived cellular proteins of vital tissues.

In this investigation the effect of aging and caloric restriction (the only manipulation which slows the rate of aging) on the levels of protein carbonyls, MDA-lys, CML and CEL was studied in rat heart mitochondria. The caloric restriction effect was studied both on the short- and the long-term in order to determine the time course of putative caloric restriction-induced modifications in those parameters. Since MDA-lys and CML are under the influence of lipid peroxidation, which increases as a function of the number of the fatty acid double bonds, the complete fatty acid composition was also analyzed. This can discriminate if alterations in the protein damage markers are due or not to modification in the sensitivity to lipid peroxidation secondary to changes in the kinds of fatty acid substrates present. This is the first investigation which studies the effects of aging and caloric restriction on the levels of AMPs in intracellular proteins of a vital post-mitotic tissue and particularly in their mitochondria, the main subcellular site of ROS generation in the heart under normal unstressed conditions.

MATERIALS AND METHODS

Animals and Caloric Restriction Experiments

Wistar rats (males) were caged individually and maintained at 22 ± 2°C, 12:12 L:D cycle, and 55 ± 10% relative humidity. They were fed a standard rodent diet (B & K, Humberside, UK). The rats maintained on caloric restriction had daily access to 60% of the intake of the *ad libitum* fed companion control animals of the same age (40% energy restriction). The short-term dietary restriction experiment was started at 8 weeks of age and continued during 6 weeks until sacrifice for biochemical measurements. At that time two groups of animals of 14 weeks of age were obtained: YL (young controls fed *ad libitum*) and YR (young restricted). The long-term dietary restriction experiment started at 12 months of age and was continued during 1 year until sacrifice. At that time two groups of animals of 24 months of age were obtained: OL (old controls fed *ad libitum*) and OR (old restricted). An additional group of *ad libitum* fed animals, born when OL and OR had 17 months of age, were caged and maintained in parallel and under the same general housing conditions than OL and OR animals during the last seven months of long-term dietary restriction experiment. These animals were 7 months of age at the time of sacrifice, were classified as AL (adult controls fed *ad libitum*) and were used for aging-related comparisons between AL and OL animals. Thus, three groups of animals were available in the long-term experiment: mature adult controls (AL), old controls (OL), and old restricted (OR). All the animals were killed at the laboratory by decapitation.

Isolation of Mitochondria

Just after decapitation, the hearts were processed to obtain functional intact mitochondria using the procedure of Mela and Seitz,[25] specially designed for small amounts of heart muscle, with modifications. This method uses nagarse to liberate mitochondria from tissue myofibrils, eliminates the fibrous structure of the muscle tissue and the contaminating collagen material surrounding the fibers, and takes into account the high Ca^{2+} content and the characteristics of Mg^{2+} binding of the tissue, and allows to obtain mitochondria still functional after several hours. Briefly, dissected ventricles were chopped into small pieces and were homogenized with a loose fitting pestle in 10 ml of isolation buffer (220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) containing 5 mg of nagarse and 25 mg of fatty acid-free albumin. After standing for 1 min, 25 ml of additional isolation buffer containing 25 mg of albumin were added and homogenization was gently performed again with a

tighter fitting pestle. The nuclei and cell debris were removed by centrifugation at 700 g for 10 min. Heart mitochondria were obtained by centrifuging the supernatant twice at 8000 g for 10 min. The mitochondrial yield was (means ± SEM): (A) Short term experiment: 14.20 ± 0.85 mg of mitochondrial protein / mg of initial heart tissue in YL and 14.5 ± 0.76 mg in YR (non significant difference in YL vs. YR, Student's *t* test); (B) Long-term experiment: 11.78 ± 0.67 mg of mitochondrial protein / mg of initial tissue in AL, 10.76 ± 0.32 mg in OL and 10.84 ± 0.46 mg in OR (non-significant difference between the three groups by one-way analysis of variance). The mitochondrial pellets were resuspended in 1 ml of isolation buffer and were frozen at −80°C until analysis of protein carbonyls, protein AMPs and fatty acid composition.

Protein Carbonyl Assay

Protein carbonyl measurements in heart mitochondria were performed by the enzyme-linked immunosorbent assay.[26] Briefly, to 60 μg of mitochondrial proteins (15 μl) 45 μl of 10 mM DNP in 6 M guanidine hydrochloride, 0.5 M potassium phosphate buffer, pH 2.5 were added. After vortex mixing and incubation at room temperature for 45 min, 5 μl of each solution was added to 1 ml PBS pH 7.4. Triplicate 200 μl aliquots (containing 1 μg protein) were added to wells of a Nunc Immuno Plate Maxisorp. Plates were incubated overnight at 4°C, then washed five times with PBS between each of the following steps: incubation with 250 μl / well of PBS containing 0.1% Tween 20 for 1.5 h at room temperature; addition of 200 μl / well of biotinylated anti-DNP antibody (1:1000 dilution in PBS, 0.1% (w / v) Tween 20 solution) for 1 hour at 37°C; addition of 200 μl streptavidin-biotinylated horseradish peroxidase (1:3000 in PBS, 0.1% Tween 20 solution) for one hour at room temperature; and addition of 200 μl of *o*-Phenylenediamine (0.6 mg / ml) / peroxide (30% stock diluted 1:2500) solution in 50 mM Na_2HPO_4 plus 24 mM citric acid. The color was allowed to develop for 25 min before stopping with 100 μl of 2.5 M sulfuric acid and the absorbances were read with a 490 nm filter in a Anthos HTII microplate reader (Izasa, Barcelona, Spain). The absorbances were related to a standard curve ranging from 0 to 2 nmol carbonyl / mg protein. Carbonyl content is determined as nmol carbonyl / mg protein.

CML, CEL and MDA-lys Measurements

CML, CEL and MDA-lys concentrations in mitochondrial proteins were measured by gas chromatography / mass spectrometry. Mitochondrial samples containing 2 mg of protein were delipidated using chloroform:methanol (2:1 v / v), and proteins were precipitated by adding 10% trichloroacetic acid (final concentration) followed by centrifugation. Protein samples were reduced by overnight treatment with 500 mM $NaBH_4$ (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. Isotopically labeled internal standards were then added, the samples were hydrolyzed at 110°C for 24 h in 1 ml of 6N HCl, and then dried *in vacuo*. The *N,O*-trifluoroacetyl methyl ester derivatives of the protein hydrolysate were prepared as previously described.[27] Gas chromatography / mass spectrometry 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 2 min at 150°C, the 5°C / min to 225°C, then 25°C / min to 300°C, and finally held at 300°C for 5 min. Quantification was performed using standard curves constructed from mixtures of deuterated and non-deuterated external standards. The analyses were carried out by selected ion monitoring gas chromatography / mass spectrometry. The ions used were: lysine and [$^2H_8$] lysine, *m/z* 180 and 187, respectively; CML and [$^2H_4$] CML, *m/z* 392 and 396, respectively; CEL and [$^2H_4$] CEL, *m/z* 379 and 383, respectively; and MDA-lys and [$^2H_8$] MDA-lys, *m/z* 474 and 482, respectively. [$^2H_8$] lysine was obtained from CDN Isotopes (CDN Isotopes, Pointe-Claire, Quebec, Canada). CML and [$^2H_4$] CML, CEL and [$^2H_4$] CEL, and MDA-lys and [$^2H_8$] MDA-lys, were prepared as previously described.[18,19,27] The amount of products was expressed as the ratio μmol CML, CEL or MDA-lys / mol lysine.

Fatty Acid Analyses

Mitochondrial lipids were extracted with chloroform: methanol (2:1 v / v) in the presence of 0.01% butylated hydroxytoluene. The chloroform phase was evaporated under N_2, and 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 N_2, redissolved in 75 μl of carbon disulphide and 1 μl was used for gas chromatography / mass spectrometry analysis. Separation was performed in a SP2330 capillary column (30 m × 0.25 mm × 0.20 μm) in a Hewlett–Packard 6890 Series II gas chromatograph (Hewlett–Packard Española, S.A., Barcelona, Spain). A Hewlett–Packard 5973A mass spectrometer was used as detector in

the electron-impact mode. The injection port was maintained at 200°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 held at 240°C for 10 min. Identification of fatty acid methyl esters was made by comparison with authentic standards and on the basis of mass spectra. Results are expressed as mol%. The double bond index (DBI) was calculated as

$$\begin{aligned}\text{DBI} = [&(\sum \text{mol\% Monoenoic} \times 1)\\ &+ (\sum \text{mol\% Dienoic} \times 2)\\ &+ (\sum \text{mol\% Trienoic} \times 3)\\ &+ (\sum \text{mol\% Tetraenoic} \times 4)\\ &+ (\sum \text{mol\% Pentaenoic} \times 5)\\ &+ (\sum \text{mol\% Hexaenoic} \times 6)].\end{aligned}$$

Statistics

Comparisons between ad libitum fed and restricted animals of the same age, as well as between adult and old ad libitum fed animals were statistically analyzed with Student's t-tests. The minimum level of statistical significance was set at $P < 0.05$ in all the analyses.

RESULTS

The effect of short-term (six weeks) caloric restriction on the steady-state concentration of markers of oxidative, glycoxidative, and lipoxidative modification of heart mitochondrial proteins in young immature animals is shown in Table I. Neither protein carbonyls, CML, CEL, or MDA-lys adducts showed statistically significant differences between ad libitum fed and caloric restricted young animals.

TABLE I Markers of oxidative, glycoxidative or lipoxidative stress in rat heart mitochondrial proteins after short-term (6 weeks) caloric restriction. The age of the animals at time of analysis was 14 weeks. Results are means ± SEM from seven different animals. Protein carbonyls are expressed in nm / mg protein; CML, CEL and MDA-lys are expressed in μmol / mol lysine. No significant differences due between ad libitum fed and restricted animals were observed in any protein marker

	Young ad libitum	YR
Protein carbonyls	0.38 ± 0.033	0.30 ± 0.038
CML	1785 ± 210	2347 ± 213
CEL	185 ± 9	181 ± 12
MDA-lys	166 ± 42	169 ± 25

Old control animals did not show significant differences in their levels of protein carbonyls in heart mitochondria when compared with adult controls (Fig. 1). However, CML levels were significantly higher (by 37%) in old than in adult animals fed ad libitum (Fig. 2). The other two markers of protein modification, CEL and MDA-lys, did not show age-related significant variations (Figs. 3 and 4).

The results obtained in the long-term (one year) caloric restriction experiment were different than those found after short-term energy restriction. Similarly to what happened in the short-term experiment, no significant differences in protein carbonyls and CML levels were found between old restricted and old control animals (Figs. 1 and 2). However, long-term caloric restriction significantly decreased the levels of CEL (by 22%) and MDA-lys (by 35%) in old restricted animals when compared to old control fed ad libitum (Figs. 3 and 4).

The fatty acid composition of heart mitochondrial phospholipids is given in Table II. In the short-term experiment, caloric restriction produced small significant changes only in 20:4n-6 and 22:4n-6, and all the other fatty acids showed similar levels between young restricted and young ad libitum fed animals. Furthermore, the small increase in 20:4n-6 in caloric restricted animals was partially compensated by the small decrease in 22:4n-6. The final result was that the total number of double bonds (DBI) was not significantly modified by short-term caloric restriction.

In the long-term experiment the changes observed in fatty acid composition, although somewhat stronger, were of similar nature than in the short-term study (Table II). Thus, old restricted animals showed significant increases in 20:4n-6 in relation to old controls which were partially compensated by significant decreases in 22:6n-3 (the most highly unsaturated fatty acid present), whereas the rest of the fatty acids did not show significant differences. The final result was, as in the short-term experiment, that the DBI was statistically similar in old restricted and old control animals. On the other hand, no differences in any fatty acid or DBI were found when adult and old ad libitum fed animals were compared (Table II).

DISCUSSION

The experiments described in this investigation show that CML levels increase during aging in rat heart mitochondrial proteins. Long-term caloric restriction, which decreases aging rate, lowers the levels of CEL and MDA-lys protein adducts in rat heart mitochondria.

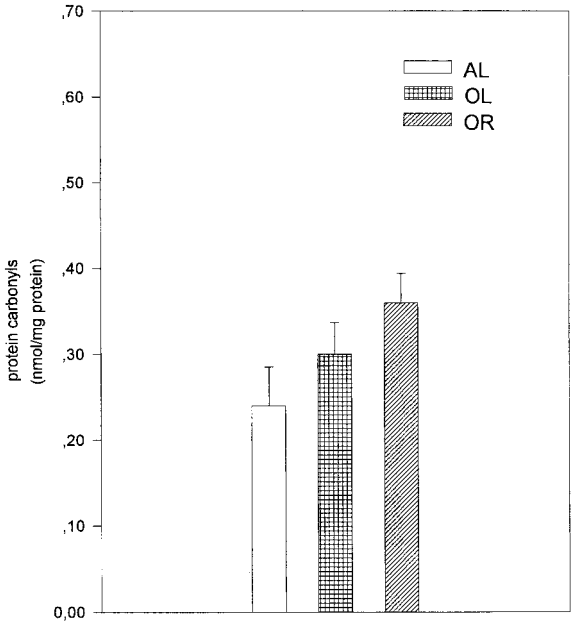

FIGURE 1 Effect of long-term (1 year) caloric restriction and aging on levels of protein carbonyl adducts to rat heart mitochondrial proteins. AL = mature adults ad libitum (7 months of age); OL = old at libitum (24 months of age); OR = old restricted (24 months of age). Values are means ± SEM from seven different animals except for OL (six animals). No significant differences due to age or caloric restriction were found.

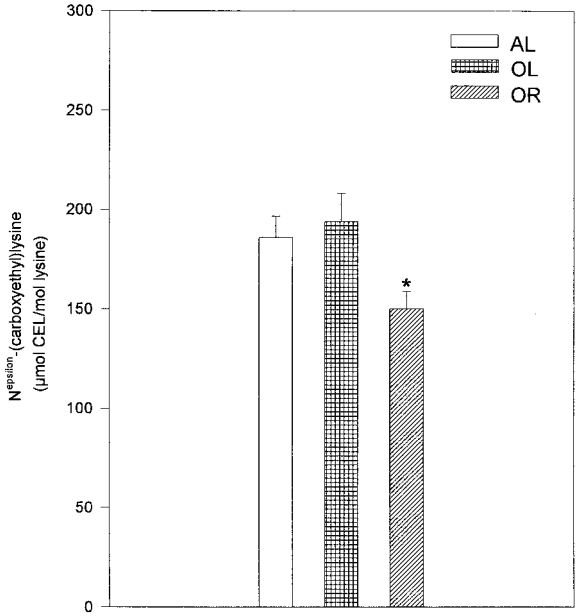

FIGURE 3 Effect of long-term (1 year) caloric restriction and aging on levels of CEL adducts to rat heart mitochondrial proteins. AL = mature adults ad libitum (7 months of age); OL = old at libitum (24 months); OR = old restricted (24 months). Values are means ± SEM from seven different animals except for OL (six animals), *is the significant difference between OR and OL ($P < 0.02$).

Protein oxidative stress has been most commonly estimated in previous studies through the measurement of protein carbonyls. In this investigation we could not detect age related differences in the level of protein carbonyls of rat heart mitochondrial proteins as estimated by an enzyme-linked immunosorbent assay. No previous data are available on age-related variations in protein carbonyls in heart mitochondria. Published studies on age-related variations in tissue protein carbonyls have given contradictory

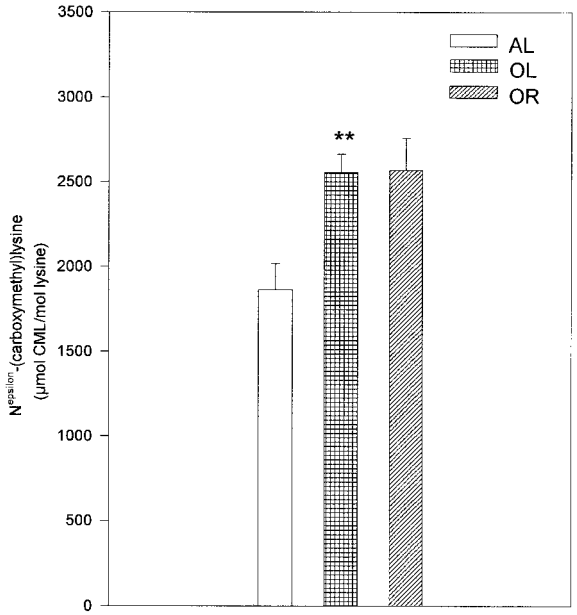

FIGURE 2 Effect of long-term (1 year) caloric restriction and aging on levels of CML adducts to rat heart mitochondrial proteins. AL = mature adults ad libitum (7 months of age); OL = old at libitum (24 months); OR = old restricted (24 months). Values are means ± SEM from seven different animals except for OL (six animals), **is the significant difference between OL and AL ($P < 0.01$).

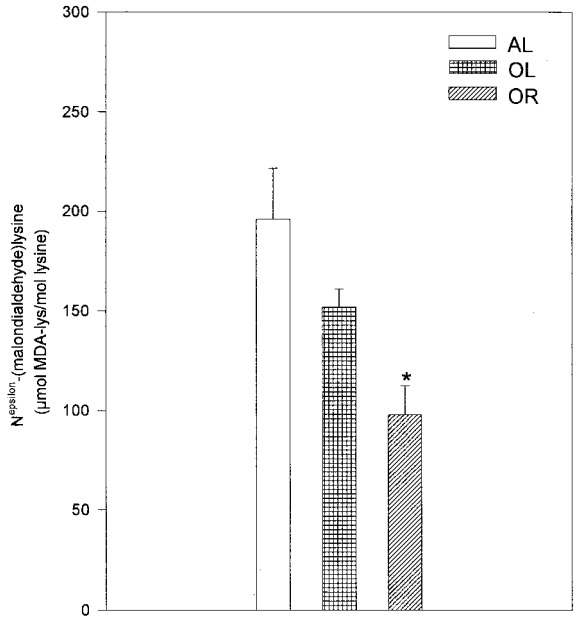

FIGURE 4 Effect of long-term (1 year) caloric restriction and aging on levels of MDA-lys adducts to rat heart mitochondrial proteins. AL = mature adults ad libitum (7 months of age); OL = old at libitum (24 months); OR = old restricted (24 months). Values are means ± SEM from seven different animals except for OL (six animals), *is the significant difference between OR and OL ($P < 0.02$).

TABLE II Fatty acid composition (mol%) of total phospholipids in rat heart mitochondria after short- (6 weeks) and long-term (1 year) caloric restriction. DBI is the ∑% of unsaturated fatty acids × number of double bonds of each unsaturated fatty acid. Values are means ± SEM from seven animals except for OL (six animals). YL are the young ad libitum (14 weeks of age); YR are the young restricted (14 weeks of age); AL are the mature adults ad libitum (7 months of age); OL are the old at libitum (24 months of age); OR are the old restricted (24 months of age). YL and YR correspond to the short-term restriction experiment (6 weeks of CR); AL, OL and OR correspond to the long-term restriction experiment (1 year of CR).

	YL	YR	AL	OL	OR
15:0	1.49 ± 0.08	1.56 ± 0.11	1.58 ± 0.22	1.60 ± 0.14	1.98 ± 0.12
16:0	11.50 ± 0.14	11.68 ± 0.16	10.91 ± 0.22	11.31 ± 0.48	12.24 ± 0.32
18:0	20.64 ± 0.50	21.18 ± 0.42	21.84 ± 0.40	21.96 ± 0.43	20.98 ± 0.37
18:1n-9	8.75 ± 0.78	7.99 ± 0.25	9.33 ± 0.43	9.35 ± 0.21	9.29 ± 0.25
18:2n-6	23.15 ± 1.22	21.25 ± 0.30	15.75 ± 0.90	15.33 ± 0.67	14.67 ± 0.43
20:3n-6	0.48 ± 0.06	0.45 ± 0.02	0.34 ± 0.05	0.42 ± 0.04	0.39 ± 0.02
20:4n-6	19.52 ± 0.63*	21.54 ± 0.21	27.92 ± 0.64	26.94 ± 0.50*	29.23 ± 0.54
22:4n-6	0.48 ± 0.04*	0.34 ± 0.05	1.31 ± 0.13	1.74 ± 0.27	1.31 ± 0.11
22:5n-3	1.25 ± 0.15	1.01 ± 0.07	1.45 ± 0.18	1.57 ± 0.13	1.35 ± 0.10
22:6n-3	12.69 ± 0.84	12.93 ± 0.22	9.52 ± 0.44	9.73 ± 0.14*	8.51 ± 0.36
DBI	219.01 ± 4.28	222.13 ± 0.97	223.26 ± 2.79	222.34 ± 2.24	219.92 ± 2.14

*Significant difference between ad libitum and restricted animals of the same age ($P < 0.05$).

results. Thus, it has been reported that protein carbonyls increase during aging in human fibroblasts,[8] human brain,[9] gerbil brain,[28] mouse brain, heart and kidney,[10,11] mouse muscle mitochondria,[12] and rat brain.[29] Conversely, no age-related changes in protein carbonyls were found in human skeletal muscle,[30] various rat brain areas,[15] mouse heart and lung,[13] and heart, brain, liver and lung.[12] A possible reason for these discrepancies is the tissue studied, since increases during aging are more commonly observed in brain[8–11,28,29] whereas in the case of heart the majority of the studies found,[12,13] like in our case in heart mitochondria, a lack of age-related increases. But differences in the methodologies employed to measure protein carbonyls can be also implicated. A majority of the studies cited above have used the spectrophotometric technique, less sensitive than ELISA, since it is more prone to interference due to variable background absorbance.

A lack of increase in protein carbonyls with age in rat heart mitochondria agrees with the observation that the rate of ROS production of rat heart mitochondria does not increase during aging.[31–33] Heart ROS production is higher in short-lived than in long-lived species,[5] but in each species it is maintained at constant levels throughout aging, continuously attacking with the same intensity mitochondrial macromolecules. This is consistent with its role as a cause of aging, since aging is progressive, thus proceeding at roughly similar rate at all ages. Maintenance of similar rates of formation and elimination of protein carbonyls at young and old age would justify the absence of age-related increases in this parameter. Similar reasons can justify the absence of age-related differences in CEL and MDA-lys found in the present investigation. Previous studies have described increases in CEL and CML during aging in cartilage and skin collagen[22–24] or in the also long-lived lens proteins,[18,23] but these AMPs have never been measured during aging in the shorter-lived intracellular proteins, which are essential for the proper function of the vital tissues. The increase in CML in old animals described in the present investigation constitutes the first description of an age-related accumulation of an AMP product in intracellular tissue proteins. Since mitochondrial ROS production does not increase with age, the rise in the steady-state level of CML in heart mitochondrial proteins observed in this investigation may be due to a decrease in the rate of degradation of carboxymethylated proteins in old animals. The different kinds of age-related changes shown by CML, CEL and MDA-lys in this research can be due to differences in mitochondrial capacities to detoxify the reactive carbonyl intermediates precursors of AMPs. Thus, mitochondrial enzymatic systems which detoxify malondialdehyde,[34] methylglyoxal,[35] or precursors of MDA-lys[20] have been described. In contrast, in the case of glyoxal, the primary precursor of CML formation,[20] no specific detoxification system has been found. Thus, the combined effect oxidative state and detoxification activities (if present) may be involved in the increase in CML during aging observed in this work, and in the absence of age-related differences in MDA-lys and CEL. These last results do not eliminate the possibility that increases in MDA-lys and CEL can still occur at more advanced ages.

Dietary restriction constitutes the only experimental manipulation which increases maximum longevity, slows down the rate of aging, and delays the onset of most age-related diseases. While the mechanism of action of caloric restriction on the rate of aging is not known, there is increasing evidence that it consists, at least in part, a decrease in oxidative stress.[2–4] Previous studies

have described that caloric restriction decreases the age-related accumulation of chemically unidentified AMPs[36–38] or CML[39,40] in collagen (an extracellular protein) during aging in rodents. In this investigation we show for the first time that caloric restriction decreases two chemically defined AMPs, CEL and MDA-lys, in rat heart mitochondrial proteins. We also show that the decrease in MDA-lys is not due to changes in lipid susceptibility to peroxidative damage, since the total number of double bonds was not affected by caloric restriction. Previous work has shown that caloric restriction decreases the rate of ROS generation of mouse[10] and rat[33] heart mitochondria. Taking into account that free radicals are unstable molecules which tend to react near their sites of generation it is logical to think that the decreases in CEL and MDA-lys in caloric restriction are due, at least in part, to the decreased rate of mitochondrial ROS generation of caloric restricted animals. In the case of CEL, a glycoxidation product, the well known decrease in lifetime glycemia induced by caloric restriction[41] can be also causally involved. A further observation supporting a causal connection between the changes in ROS production and in CEL and MDA-lys is the observation that the caloric restriction-induced decrease in these AMPs occurred after long-term but not after short-term caloric restriction. In agreement with that causal connection, we have recently found that caloric restriction also decreases the rate of ROS generation of rat heart mitochondria after 1 year but not after 6 weeks of caloric restriction.[33] A direct relationship between free radical production and CEL formation has also been recently described.[20]

Concerning protein carbonyls, previous studies in other organs have shown caloric restriction-induced decreases.[10–12,28,42,43] Similar to the case of the age-related variations in protein carbonyls described above, tissue or methodological differences can also be responsible for the different result obtained here. In any case, since caloric restriction consistently decreases the rate of ROS production of rat heart mitochondria, the absence of caloric restriction-induced decrements in protein carbonyls and CML found in the present investigation suggests that the rate of degradation of proteins containing those two markers of damage is also down regulated in the mitochondria of caloric restricted animals.

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