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Dietary Protein Restriction Decreases Oxidative Protein Damage, Peroxidizability Index, and Mitochondrial Complex I Content in Rat Liver

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Caloric restriction (CR) decreases oxidative damage, which contributes to the slowing of aging rate. It is not known if such decreases are due to calories themselves or specific dietary components. In this work, the ingestion of proteins of Wistar rats was decreased by 40% below that of controls. After 7 weeks, the liver of the protein-restricted (PR) animals showed decreases in oxidative protein damage, degree of membrane unsaturation, and mitochondrial complex I content. The results and previous information suggest that the decrease in the rate of aging induced by PR can be due in part to decreases in mitochondrial reactive oxygen species production and DNA and protein oxidative modification, increases in fatty acid components more resistant to oxidative damage, and decreased expression of complex I, analogously to what occurs during CR. Recent studies suggest that those benefits of PR could be caused, in turn, by the lowered methionine intake of that dietary manipulation.

THE oxidative stress/free radical theory of aging is currently receiving considerable support from comparative and experimental studies (1–3). It is well known that caloric restriction (CR) slows down the rate of aging of phylogenetically distant species (ranging from invertebrates to primates), increases both their mean and maximum life spans, and delays the occurrence of many degenerative diseases (3–7). However, the basic mechanisms underlying the effects of CR on aging and maximum life span are still uncertain.

Many studies have previously shown that CR decreases mitochondrial reactive oxygen species (ROS) generation and oxidative damage to DNA, protein, and lipids (8–14). Low levels of these two features are also constitutively exhibited by long-lived species when compared to short-lived ones (3,15). These findings associate CR with the oxidative stress/free radical theory of aging, and offer a plausible mechanism by which CR could slow down the rate of aging.

However, it has never been reported whether CR-induced decreases in protein oxidative damage and protective changes in fatty acid unsaturation are due to the reduction in calories themselves or to diminished intake of specific dietary components. In the present work we test the possibility that protein restriction (PR) is responsible, at least in part, for the main effects on molecular oxidative damage of CR. Although a general consensus was reached in the last decade that the life extension effect of CR is related to the reduction in calories themselves, variations in the proportions of the main dietary components could also modulate life span (16,17). The large majority of the available experiments (16 of 18) described significant increases in mean and maximum life span of rats after restricting only the dietary intake of proteins (18–20). The mean increase in maximum longevity in these studies could explain 30%–50% of the increase in longevity elicited by CR. We have used a dietary protocol in which protein ingestion was reduced while the intake and quality of carbohydrates and fat was maintained as in control animals. This protocol avoids confusing the effects of PR with those of increasing the percentage of other dietary components, mainly carbohydrates (21,22). CR-induced decreases in mitochondrial ROS production and oxidative damage have been previously demonstrated by us both in liver (8,12,14) and heart (13,23) of Wistar rats. However, whereas heart usually needs long-term restriction to exhibit these changes (23), the liver shows quicker effects and can be detected already after only 7 weeks of treatment (8,14). Therefore, we have selected this organ for the present study of PR because it could allow the demonstration of changes in a short time. The markers measured were, for protein oxidation: glutamic semialdehyde (GSA) and aminoadipic semialdehyde (AASA), which are specific protein carbonyls generated in vitro by metal-catalyzed oxidation; for glycoxidation: Nε-(carboxyethyl)lysine (CEL); for mixed glyco- and lipoxidation: Nε-(carboxymethyl)lysine (CML); and for lipoxidation: Nε-(malondialdehyde)lysine (MDAL). In addition, because complex I is the main mitochondrial site where free-radical production is lowered in long-lived species and calorically restricted animals (3), its concentration was also inferred by immunoblotting. Finally, to assess the role of structural components that can act as substrate targets in

determining the degree of nonenzymatic protein modification, the amino acid composition and fatty acid profile were also evaluated.

Material and Methods

Animals and Diets

Male Wistar rats (250 g body weight) were obtained from Iffa-Creddo (Lyon, France). The animals were caged individually and maintained in a 12-hour light/dark cycle at 22 ± 2°C and 50 ± 10% relative humidity. Control animals were fed ad libitum the semipurified American Institute of Nutrition diet AIN-93G: 39.7486% cornstarch, dextrinized cornstarch 13.20%, sucrose 10.00%, casein 20.00%, soybean oil 7.00%, Alpha-Cel (nonnutritive bulk) 5% (International Fiber Corporation, North Tonawanda, NY), mineral mix 3.5%, vitamin mix 1.0%, L-cystine 0.3%, choline bitartrate 0.25%, and tert-butylhydroquinone 0.0014%. The diet given to the PR animals was a modified AIN-93G ''PR'' diet. Its protein content was reduced whereas its content in sucrose, soybean oil, and all the rest of its components was appropriately increased. Its composition was: cornstarch 39.7486%, dextrinized cornstarch 13.20%, sucrose 15.4719%, casein 13.04%, soybean oil 7.61%, Alpha-Cel 5.44%, mineral mix 3.80%, vitamin mix 1.09%, L-cystine 0.326%, choline bitartrate 0.272%, and tert-butylhydroquinone 0.0015%. This diet was given each day to the PR animals in an amount equal to 92% of the amount of food eaten by the controls (see Table 1). The final result is that PR animals ingested daily 40% less protein than did the controls, whereas the total amount of carbohydrates, fat, and the rest of dietary components eaten was the same in controls and PR animals. With this procedure, PR animals ingested 8.5% fewer calories than the controls did. The daily amount of protein casein eaten by the PR animals was substantially higher than the minimum daily requirement (24). The mean body weight at the end of the dietary experiment was 354 g in controls and 342 g in PR animals. After 7 weeks of dietary treatment, the animals were decapitated. The liver was immediately processed to isolate mitochondria by differential centrifugation, whereas whole liver samples were stored at −80°C.

Table 1. Composition of Diets Ingested by Experimental Groups

Component	Amounts Ingested in the Control Group (g/100 g)	Amounts Ingested in the PR Group (g/92 g of PR diet)*
Cornstarch	39.7486	36.5687
Casein	20.00	12.00
Dextrinized corn starch	13.20	12.144
Sucrose	10.00	14.234
Soybean oil	7.00	7.00
Alphacel, nonnutritive bulk	5.00	5.00
Mineral mix	3.50	3.50
Vitamin mix	1.0	1.00
L-Cystine	0.30	0.30
Choline bitartrate	0.25	0.25
Tert-butylhydroquinone	0.0014	0.0014
Total	100 g	92 g

Note: *Protein-restricted (PR) animals received each day a modified AIN93G ''PR'' diet (see Materials and Methods for its detailed composition). The amount ingested of that diet per animal per day was equal to 92% of the mean amount of food ingested per day by the control animals. The final result was that PR animals ingested daily 40% less protein than the controls did, whereas the total amount of carbohydrates, fat, and the rest of dietary components eaten was the same in control and PR animals. Thus, the 12 g of casein ingested by the PR group represents 40% protein restriction compared with the control group (20 g of casein); in the case of the total carbohydrates ingested (the sum of cornstarch, dextrinized cornstarch, and sucrose: 62.94 g in both cases), it was the same in both the control and the PR groups, as for the rest of dietary components except for casein. See Materials and Methods section for more details.

Amino Acid Analysis by High-Performance Liquid Chromatography

Amino acid analyses were measured by high-performance liquid chromatography (HPLC) as previously described (25). Briefly, high-purity amino-acid calibration standard for protein hydrolyzates (containing 2.5 μmol/mL of 0.1 N HCl of 17 protein amino acids, except for L-cysteine, which is supplied at 1.25 μmol/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 μg each were precipitated with 10% trichloroacetic acid (TCA) (final concentration), hydrolyzed in 1 mL of 6N HCl for 30 minutes at 155°C, and then dried in vacuo. Protein hydrolyzates were resuspended in 100 μL of HCl 4 mM (Pierce), and a 10-μL aliquot of the simple extract or amino acid standard solution (with the internal standard added) was buffered with 70 μL of borate buffer. The derivatives were formed with 20 μL of AQC and heated for 10 minutes at 55°C. The HPLC system consisted of a Waters model 510 pump, an AccQ-Tag C18 amino acid analysis column (3.9 mm × 150 mm), a column heater, and a model 470 fluorescence detector set at 250/395 nm as excitation/emission wavelength, respectively (all obtained 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 1000 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 used consisted of elution to 93% A in 15 minutes, then elution to 67% A in 18 minutes, then washing with 100% B in 2 minutes for 3 minutes, and equilibration in 100% A for 27 minutes. Quantification was performed by using standard curves for each amino acid, and results were 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 and colleagues (26).

Fatty Acid Analysis by Gas Chromatography/Mass Spectrometry

Fatty acyl groups were analyzed as previously described (27). Both whole and mitochondrial lipids were extracted into 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 minutes 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 μL of hexane, and 1 μL was used for gas chromatography/mass spectrometry (GC/MS) 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. 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 minutes 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 minutes. Identification of fatty acyl methyl esters was made 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); polyunsaturated fatty acids from n-3 and n-6 series (PUFAn-3 and PUFAn-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) = [(1 × Σmol% monoenoic) + (2 × Σmol% dienoic) + (3 × Σmol% trienoic) + (4 × Σmol% tetraenoic) + (5 × Σmol% pentaenoic) + (6 × Σmol% hexaenoic)], and peroxidizability index (PI) = [(0.025 × Σmol% monoenoic) + (1 × Σmol% dienoic) + (2 × Σmol% trienoic) + (4 × Σmol% tetraenoic) + (6 × Σmol% pentaenoic) + (8 × Σmol% hexaenoic)].

Desaturase activities were estimated from specific product/substrate ratios: For Δ9 desaturase activity, 18:1n-9/18:0 ratio; for Δ5 desaturase activity, 20:4n-6/20:3n-6 ratio; and for Δ6 desaturase activity. 22:6n-3/18:3n-3 ratio.

Mitochondrial Complex I and IV

The concentration of mitochondrial complexes I (the main mitochondrial free radical source) and IV (a key component of the respiratory chain, but not free radical generator) was estimated using western blot analysis of representative subunits of these complexes. Immunodetection was performed using a monoclonal antibody specific for the NDUFA9 subunit of complex I, and subunit I of complex IV (1:500/1:2000; Molecular Probes, Invitrogen Ltd, U.K.). An antibody to porin (1:1000; Molecular Probes) as a control for total mitochondrial mass was also used to determine the proportion of complex I and IV referred 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.

GSA, AASA, CML, CEL, and MDAL Measurements by GC/MS

GSA, AASA, CML, CEL, and MDAL concentrations were measured by GC/MS as previously described (28). Briefly, samples containing 500 μg of either whole or mitochondrial 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 1 drop of hexanol as an antifoam reagent. Protein was reprecipitated by adding 1 mL of 20% (v/v) trichloroacetic acid, followed by centrifugation. Isotopically labeled internal standards ($[^2H_8]$lysine, $[^2H_4]$CML, $[^2H_4]$CEL, $[^2H_8]$MDAL, $[^2H_5]$5-hydroxy-2-aminovaleric acid [for GSA quantification] and $[^2H_4]$6-hydroxy-2-aminocaproic acid [for AASA quantification]) were then added. The samples were hydrolyzed at 155°C for 30 minutes in 1 mL of 6 M HCl, and were dried in vacuo. The N,O-trifluoroacetyl methyl ester derivatives of the protein hydrolysates were prepared as described (28). GC/MS analyses were carried out on a Hewlett-Packard model 6890 gas chromatograph equipped with an HP-5MS capillary column (30 m × 0.25 mm × 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 minutes 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 held at 300°C for 5 minutes. Quantification was performed by external standardization using standard curves constructed from mixtures of deuterated and nondeuterated standards. Analyses were carried out by selected ion-monitoring GC/MS (SIM-GC/MS). The ions used were: lysine and $[^2H_8]$lysine, *m/z* 180 and 187, respectively; 5-hydroxy-2-aminovaleric acid and $[^2H_5]$5-hydroxy-2-aminovaleric acid (stable derivatives of GSA), *m/z* 280 and 285, respectively; 6-hydroxy-2-aminocaproic acid and $[^2H_4]$6-hydroxy-2-aminocaproic acid (stable derivatives of AASA), *m/z* 294 and 298, respectively; CML and $[^2H_4]$CML, *m/z* 392 and 396, respectively; CEL and $[^2H_4]$CEL, *m/z* 379 and 383, respectively; and MDAL and $[^2H_8]$MDAL, *m/z* 474 and 482, respectively. The amounts of product were expressed as the micromolar ratio of GSA, AASA, CML, CEL, or MDAL to lysine.

Statistics

Comparisons between ad libitum–fed and PR animals were statistically analyzed with Student's *t* tests. The minimum level of statistical significance was set at $p < .05$ in all the analyses.

Results

Amino Acid Compositional Analysis

Amino acid composition of total and mitochondrial proteins was compared between control and PR groups (Tables 2 and 3). Supporting a profound effect in liver nitrogen metabolism, the amino acid compositional analysis

Table 2. Amino Acid Compositional Analysis of Whole Liver Proteins in Ad Libitum-Fed and Protein-Restricted Rats

	Whole Liver		
Amino Acid	Control (mol%)	Protein Restricted (mol%)	*p*
Asp	2.99 ± 0.20	2.71 ± 0.04	.210
Ser	5.63 ± 0.09	5.64 ± 0.07	.992
Glu	3.85 ± 0.23	3.62 ± 0.05	.375
Gly	9.50 ± 0.39	10.10 ± 0.10	.178
His	3.44 ± 0.11	3.94 ± 0.06	.005
Arg + Thr	10.92 ± 0.29	11.71 ± 0.24	.072
Ala	6.01 ± 0.19	4.93 ± 0.10	.001
Pro	2.27 ± 0.02	1.77 ± 0.03	.001
Tyr	4.75 ± 0.33	5.76 ± 0.31	.062
Val	8.59 ± 0.10	8.59 ± 0.11	.990
Met	1.41 ± 0.09	1.13 ± 0.03	.024
Lys	5.75 ± 0.31	4.90 ± 0.07	.030
Ile	8.94 ± 0.06	8.54 ± 0.06	.003
Leu	13.89 ± 0.12	13.82 ± 0.14	.731
Phe	11.99 ± 0.16	12.75 ± 0.11	.006
KR	16.67 ± 0.38	16.61 ± 0.26	.892
ED	6.84 ± 0.43	6.34 ± 0.09	.287
KR + ED	23.52 ± 0.67	22.95 ± 0.28	.459
KR − ED	9.83 ± 0.46	10.27 ± 0.28	.445
LVIFM	44.82 ± 0.20	44.87 ± 0.12	.859
AGP	17.80 ± 0.22	16.81 ± 0.19	.011
FIKMNY	35.84 ± 0.27	35.83 ± 0.27	.967

Note: Values are means ± standard error of the mean from $n = 6$ samples. 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; AGP (Ala + Gly + Pro) = encoded by CCN, GCN, and GGN codons; FIKMNY (Phe + Ile + Lys + Met + Asn + Tyr) = encoded by AAN, AUN, UAN, and UUN codons.

Table 3. Amino Acid Compositional Analysis of Liver Mitochondrial Proteins in Ad Libitum-Fed and Protein-Restricted Rats

	Mitochondria Liver		
Amino Acid	Control (mol%)	Protein Restricted (mol%)	*p*
Asp	2.41 ± 0.12	2.58 ± 0.09	.298
Ser	5.70 ± 0.04	5.91 ± 0.04	.010
Glu	3.07 ± 0.14	3.32 ± 0.10	.186
Gly	10.28 ± 0.13	10.48 ± 0.13	.326
His	3.14 ± 0.11	3.21 ± 0.09	.667
Arg + Thr	12.01 ± 0.19	11.59 ± 0.29	.256
Ala	5.18 ± 0.19	5.53 ± 0.22	.270
Pro	2.08 ± 0.04	2.03 ± 0.07	.609
Tyr	5.92 ± 0.48	5.40 ± 0.39	.430
Val	8.98 ± 0.13	8.72 ± 0.13	.212
Met	1.34 ± 0.06	1.28 ± 0.05	.523
Lys	4.45 ± 0.24	4.78 ± 0.19	.331
Ile	9.45 ± 0.04	9.28 ± 0.05	.056
Leu	13.52 ± 0.08	13.37 ± 0.11	.349
Phe	12.39 ± 0.19	12.43 ± 0.26	.897
KR	16.47 ± 0.06	16.37 ± 0.19	.643
ED	5.48 ± 0.26	5.90 ± 0.20	.233
KR + ED	21.95 ± 0.32	22.28 ± 0.27	.458
KR − ED	10.99 ± 0.20	10.46 ± 0.28	.174
LVIFM	45.70 ± 0.22	45.11 ± 0.32	.175
AGP	17.55 ± 0.11	18.05 ± 0.23	.092
FIKMNY	35.98 ± 0.21	35.79 ± 0.15	.483

Note: Values are means ± standard error of the mean from $n = 6$ samples. 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 hidrophobics; AGP (Ala + Gly + Pro) = encoded by CCN, GCN, and GGN codons; FIKMNY (Phe + Ile + Lys + Met + Asn + Tyr) = encoded by AAN, AUN, UAN, and UUN codons.

revealed in whole liver a significant lower content of Ala, Pro, Met, Lys, and Ile, and a higher content for His and Phe in PR animals compared to control, yet no changes were detected for global parameters such as charge, hydrophobicity, and grouping. In contrast, liver mitochondria were very resistant to changes induced by diet, as no differences in either amino acid composition or indices were detectable between groups.

Membrane Unsaturation and Fatty Acid Profile

PR altered the fatty acid composition of total liver and liver mitochondria (Tables 4 and 5), so that the total number of double bonds (DBI) and PI was significantly decreased in both cases (Figure 1). The fatty acids mainly responsible for the decrease in DBI and PI were basically the same. PR significantly increased fatty acids with a lower content of double bonds (one or two) such as the monounsaturated fatty acid (18.1) and linoleic acid (18:2) and decreased the highly unsaturated arachidonic (20:4) and docosahexaenoic acids (22:6). The membrane acyl composition (Tables 4 and 5) indicated that the membranes maintain a similar fatty acid average chain length (around 18 carbon atoms) in the PR and control groups.

These changes may be explained by differences in the activity of desaturase enzymes. These enzymatic activities may be estimated from specific product/substrate ratios. Accordingly, PR led to higher delta-9 desaturase activity, and lower delta-5 and delta-6 activities (Table 6).

Mitochondrial Complex I and IV Content

The concentration of the peptides representative of mitochondrial respiratory complexes I and IV, adjusted by the porin content, was significantly decreased by PR to 30% of that of controls (Figure 2).

Steady-State Levels of Protein Oxidative Damage

A comparison of liver from control versus treated rats revealed that PR was associated with relevant differences in the steady-state levels of protein oxidative damage markers. All the five markers surveyed (GSA, AASA, CML, CEL, and MDAL) displayed significantly lower levels in liver proteins from PR animals than in those of control rats, in both whole liver and in the mitochondrial fraction (Figures 1, 3, and 4). The magnitude of change for whole liver ranged from 10% for CML to 20% for CEL, around 14% for GSA, 16% for AASA, and 18% for MDAL, suggesting an overall lowered status of protein oxidative damage induced by PR. For liver mitochondrial proteins, the magnitude of detected changes was slightly higher than that for whole liver. Thus, PR decreased the steady-state levels of the different markers as follow: 20% for GSA, 24% for AASA, 20% for CEL, 7% for CML, and 25% for MDAL.

Discussion

In this work it is shown that restricting the dietary intake of protein in mature animals without strongly decreasing the

Table 4. Fatty Acid Composition and Derived Indices in Liver Samples From Ad Libitum-Fed and Protein-Restricted Rats

Fatty Acid	Control (mol%)	Protein Restricted (mol%)	*p*
14:0	0.19 ± 0.01	0.17 ± 0.01	.176
16:0	20.11 ± 0.33	20.40 ± 0.42	.598
16:1n-7	1.06 ± 0.12	1.57 ± 0.15	.037
18:0	16.75 ± 0.45	14.01 ± 0.47	.003
18:1n-9	12.45 ± 0.75	16.37 ± 0.57	.003
18:2n-6	17.25 ± 1.07	22.53 ± 0.73	.004
18:3n-3	0.20 ± 0.01	0.21 ± 0.02	.817
20:2n-6	0.30 ± 0.01	0.42 ± 0.19	.562
20:3n-6	0.22 ± 0.02	0.60 ± 0.10	.010
20:4n-6	23.78 ± 1.06	18.05 ± 0.59	.002
20:5n-3	0.46 ± 0.07	0.18 ± 0.02	.009
22:4n-6	0.35 ± 0.01	0.25 ± 0.02	.013
22:5n-6	0.31 ± 0.04	0.51 ± 0.12	.182
22:5n-3	0.72 ± 0.04	0.48 ± 0.06	.017
22:6n-3	5.78 ± 0.30	4.19 ± 0.23	.003
ACL	18.35 ± 0.02	18.15 ± 0.02	.001
SFA	37.05 ± 0.69	34.59 ± 0.36	.014
UFA	62.94 ± 0.69	65.40 ± 0.34	.014
MUFA	13.52 ± 0.85	17.95 ± 0.68	.004
PUFA	49.41 ± 0.62	47.46 ± 0.54	.047
PUFAn-3	7.17 ± 0.23	5.07 ± 0.29	.001
PUFAn-6	42.23 ± 0.66	42.38 ± 0.53	.869

Notes: Values shown are means ± standard error of the mean from $n = 6$ samples.

ACL = average chain length; SFA = saturated fatty acid; UFA = unsaturated fatty acid; MUFA = monounsaturated fatty acid; PUFA n-3/n-6 = polyunsaturated fatty acid n-6 or n-3 series.

Table 5. Fatty Acid Composition and Derived Indices in Liver Mitochondria From Ad Libitum-Fed and Protein-Restricted Rats

Fatty Acid	Control (mol%)	Protein Restricted (mol%)	*p*
14:0	0.18 ± 0.01	0.15 ± 0.01	.194
16:0	17.31 ± 0.44	18.20 ± 0.42	.177
16:1n-7	1.11 ± 0.07	1.70 ± 0.28	.069
18:0	17.19 ± 0.24	17.21 ± 0.36	.959
18:1n-9	9.09 ± 0.17	11.81 ± 0.21	.001
18:2n-6	19.27 ± 0.66	20.22 ± 0.45	.266
18:3n-3	0.18 ± 0.04	0.29 ± 0.02	.055
20:2n-6	0.35 ± 0.01	0.72 ± 0.09	.003
20:3n-6	0.27 ± 0.02	0.72 ± 0.08	.001
20:4n-6	26.61 ± 0.44	22.12 ± 0.28	.001
20:5n-3	0.35 ± 0.02	0.21 ± 0.02	.004
22:4n-6	0.16 ± 0.008	0.13 ± 0.007	.026
22:5n-6	0.28 ± 0.04	0.28 ± 0.02	.959
22:5n-3	0.79 ± 0.04	0.67 ± 0.03	.070
22:6n-3	6.79 ± 0.44	5.50 ± 0.22	.028
ACL	18.49 ± 0.01	18.33 ± 0.01	.001
SFA	34.69 ± 0.50	35.57 ± 0.53	.262
UFA	65.30 ± 0.50	64.42 ± 0.53	.262
MUFA	10.20 ± 0.18	13.51 ± 0.46	.001
PUFA	55.09 ± 0.56	50.90 ± 0.65	.001
PUFAn-3	8.12 ± 0.41	6.67 ± 0.25	.014
PUFAn-6	46.97 ± 0.95	44.23 ± 0.57	.033

Notes: Values shown are means ± standard error of the mean from $n = 6$ samples.

ACL = average chain length; SFA = saturated fatty acid; UFA = unsaturated fatty acid; MUFA = monounsaturated fatty acid; PUFA n-3/n-6 = polyunsaturated fatty acid n-6 or n-3 series.

caloric intake lowers both total and mitochondrial protein oxidative damage, membrane unsaturation by the modulation of desaturase enzymatic activities, and mitochondrial respiratory complex I content in the liver of a mammal. These results contribute to clarify how CR decreases tissue oxidative stress and slows down aging. They can be also illustrative for future prospects to postpone aging without the unpleasant and stressful side effects of strong decreases in the dietary intake of energy (29).

It is generally considered that the anti-aging effect of CR is due to the decreased intake of calories rather than to specific dietary components, although variations in the proportions of the main dietary constituents seem also to affect life span (16,17). Concerning the effects of PR on aging, consideration of published studies performed in mammals shows that increases in longevity are more common than any other effect. Thus, some authors found that decreasing the casein content of the diet from 42% to 18% decreased the survival of male rats (30). However, many other investigators found earlier that low protein diets increase the life span of rats (20), increase the mean and maximum life span of Fisher 344 rats (21,31), increase the life span of C57BL/6J and hybrid F1 mice (32,33), prolong life expectancy in BALB/c mice (34), and prolong the life of DBA/2f mice (35). In some of these studies the diet was changed only with respect to protein (35) (as in the present experiments), whereas in other cases the decrease in protein was balanced by an increase in dietary carbohydrate (21). In some cases it was observed that the life-span extension effect (15%) of the low protein diets was smaller than that of CR diets, and it was concluded that the retardation of aging was due to restriction of energy intake rather than to a specific nutrient (36). Other authors have found that PR can increase the life span of Wistar rats by as much as 25% (20).

The dietary protein component is increased in some CR protocols, to insure that both CR and control animals consume equal amounts of protein (37). Life span is also increased in CR rodents consuming these diets, despite the fact that the total protein intake is the same in these CR animals as in their controls. In principle, these results might indicate that a reduction in dietary protein is not required for life-span extension with CR. However, many classic studies have investigated whether PR can also modify longevity in rodents (20,21,31–35). A recent review of these PR studies (38) shows that in 16 of the 18 life-long aging experiments available in mice and rats, PR increased maximum longevity. The 16 positive experiments found a mean increase in maximum longevity of 19.2% for a mean degree of PR of 68%. If the effect of PR on longevity is, as in the case of CR, proportional to the intensity of PR, this effect would translate into a mean increase in maximum longevity of 11.5% at 40% PR, whereas the increase in maximum longevity induced by CR is usually higher (around 40% increase). This result suggests that CR increases maximum longevity through: i) the decrease in protein intake (which can explain 30%–50% of the increase in longevity); or ii) through other mechanisms, e.g., the lowered intake of the calories themselves, which would explain the rest of that increase (available studies on lipid and carbohydrate restriction, although very scarce, do not support a role for

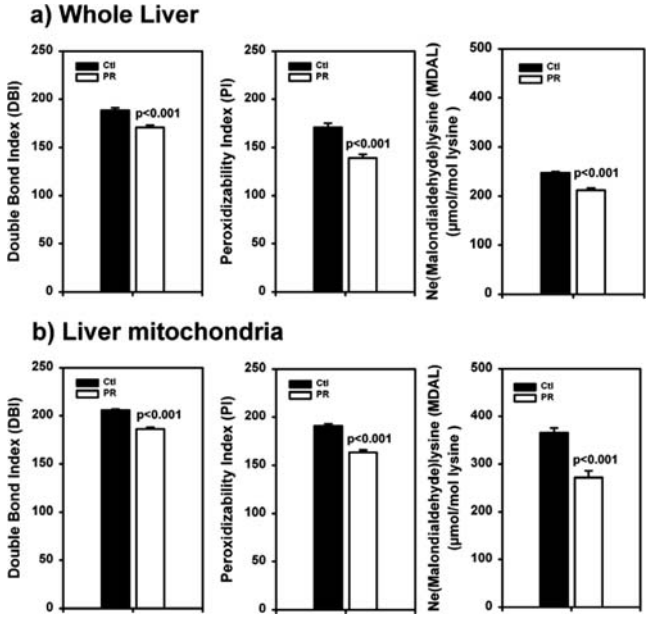

Figure 1. Proteins from protein-restricted animals show significant decreases in lipid peroxidation–derived protein damage, as shown by measurements of Nε-(malondialdehyde)lysine (MDAL). This is associated with a decrease in double bond index (DBI) and peroxidizability index (PI). Data from whole liver (*top*), and liver mitochondria (*bottom*). Values shown are means ± standard error of the mean from $n = 5$–7 samples. Units: µmol/mol lysine. Significant differences are indicated.

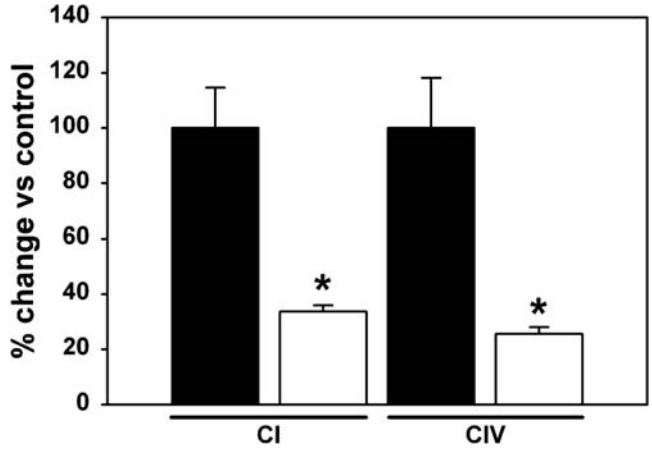

Figure 2. Concentration of protein complexes I and IV in liver mitochondria from ad libitum–fed and protein-restricted rats. Values are means ± standard error of the mean from $n = 5$–7 samples. Significant differences are indicated. *Filled bars* = controls; *open bars* = protein restricted. CI = Mitochondrial Complex I; CIV = Mitochondrial Complex IV.

these components in life extension). The second mechanism likely explains why CR experiments in which protein intake is not modified can also increase rodent longevity. The variability in the degree of life extension observed in different 40% CR experiments can be so great as the increase in longevity due to PR alone (around 12%). That variability could explain, for instance, that a particular experiment of 40% CR without PR can lead to a similar increase in longevity than could another particular typical experiment of 40% total CR (without modifying dietary components), and this variability would still be compatible with the existence of a real effect of PR on longevity.

Taken together, the available data (20,21,31–35,38) suggest that PR can be responsible for part of the life span prolonging effects of CR. This would be also consistent with the observation that methionine or tryptophan restriction increases the maximum life span of rats independently of energy restriction (39,40). The remaining effects of CR on the rate of aging could be related to decreases in other dietary components or in the calories themselves through different additional mechanisms. CR and PR share many common effects in addition to life prolongation, including delays in puberty, decreases in growth rate, changes in metabolic rate, boosting of cell-mediated immunity, lowering of cholesterol, or decreases in preneoplastic lesions and tumors (22,41). Low-protein diets also decrease insulin-like growth factor-1 levels and decelerate glomerulosclerosis in mice (42), delay the occurrence of chronic nephropathy and cardiomyopathy in rats (43), and protect rat liver against exposure to toxic chemicals (44).

Table 6. Hepatic Desaturase Activities From Rats Fed on Control and Protein-Restricted Diet

Desaturase Enzymes	Product/Substrate Ratio	Calculated From	Control	Protein Restricted	$p <$
Δ9	18:1n-9/18:0	Liver	0.74 ± 0.06	1.17 ± 0.07	.002
		mtLiver	0.52 ± 0.01	0.68 ± 0.02	.001
Δ5	20:4n-6/20:3n-6	Liver	109.98 ± 9.25	36.83 ± 10.04	.001
		mtLiver	103.65 ± 12.1	32.69 ± 3.94	.001
Δ6	22:6n-3/18:3n-3	Liver	28.80 ± 3.02	20.15 ± 1.52	.034
		mtLiver	47.85 ± 9.93	20.05 ± 2.27	.021

Note: Values shown are means ± standard error of the mean from $n = 6$ samples. mtLiver = mitochondrial liver.

Although the large majority of the studies involving CR use 40% restriction in energy intake, some investigations have shown that 25%–35% reduction in energy is still able to increase the survival of rats (45,46). In contrast, graded dose studies indicate that at least 20% or higher energy restriction is necessary to decrease oxidative DNA damage (47). These minimum degrees of energy restriction are much higher than the one present in the PR rats in our investigation (8.5%). In contrast, we have recently found that 8.5% CR does not change mitochondrial ROS production (Pamplona and Barja, 2006, unpublished results), whereas it is known that 40% CR decreases it (8). Thus, the results obtained in the present investigation are related to the restriction in proteins themselves and not to the marginal decrease in energy intake. Restriction of protein intake can be responsible for part of the aging-delaying effect of CR through the decreases in complex I content, protein oxidative damage, and membrane unsaturation that we have observed in the present study, along with recent findings demonstrating that PR also decreases the mitochondrial ROS production and oxidative DNA damage (48). In addition, previous studies have also shown that PR decreases protein carbonyls in rat liver and lowers lipid peroxidation in rat kidney (22). The changes in liver and

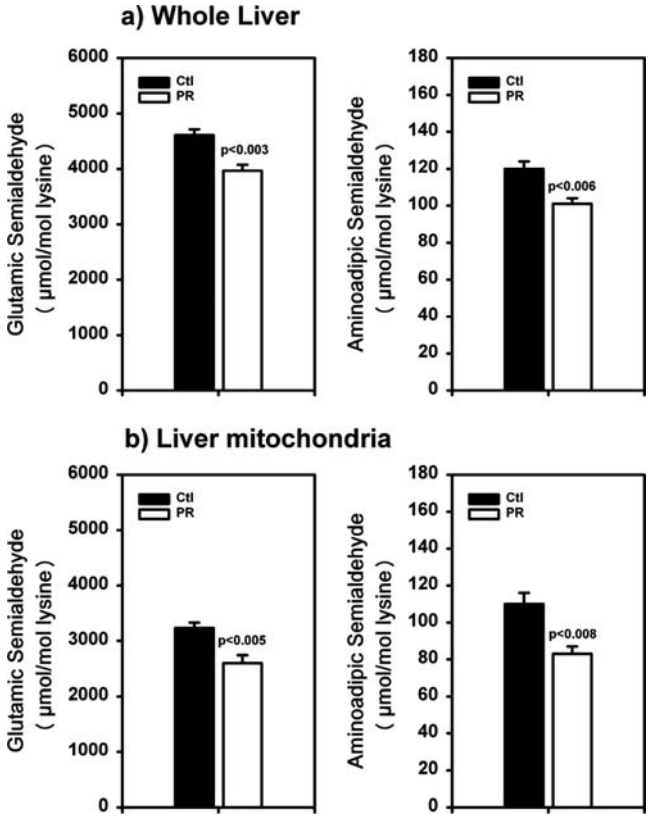

Figure 3. Proteins from protein-restricted (PR) animals show significant decreases in the amounts of glutamic semialdehyde and aminoadipic semialdehyde, specific protein carbonyl markers, compared to the ad libitum–fed control (Ctl) group in both whole liver (*top*) and liver mitochondria (*bottom*). Values shown are means ± standard error of the mean from $n = 5$–7 samples. Units: µmol/mol lysine. Significant differences are indicated.

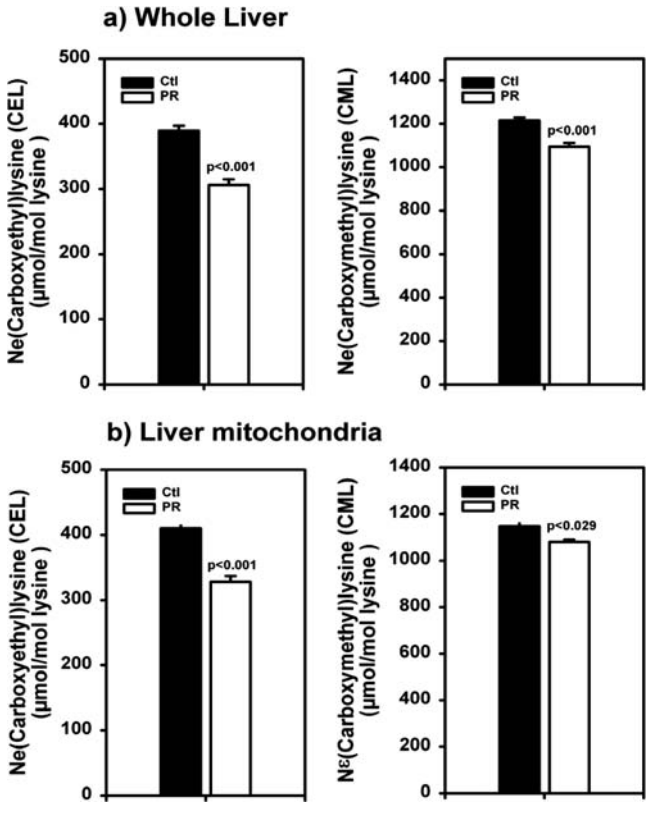

Figure 4. Proteins from protein-restricted (PR) animals show significant decreases in the amounts of Nε-(carboxyethyl)lysine (CEL) and Nε-carboxymethyl(lysine) (CML) arising from mixed glyco- and lipoxidation reactions, compared to the ad libitum–fed control (Ctl) group in both whole liver (*top*) and liver mitochondria (*bottom*). Values shown are means ± standard error of the mean from $n = 5$–7 samples. Units: µmol/mol lysine. Significant differences are indicated.

mitochondria fatty acid compositions with dietary PR deserve special mention. In typical 40% CR experiments, the total amount of food ingested is limited by 40%, and thus ingestion of proteins is also decreased by 40%. Accordingly, if PR changes fatty acid unsaturation, as evidenced by present results, in principle similar changes in fatty acid composition should occur in such kinds of CR experiments. In agreement with this, previous studies from some of us reported qualitatively similar—although quantitatively less intense—changes in hepatic mitochondrial fatty acid composition in rats calorie restricted for 28 months (11), although not after 18 or 6 months (11), than in the present PR experiment. Because in the present study PR was applied for 7 weeks, it is unlikely that the time of restriction could be responsible for differences between that CR experiment and the present PR experiment. Differences in factors such as rat strain, dietary protein source, time treatment, and tissue-organ examined likely modulate the intensity of the changes in fatty acid composition during dietary restriction experiments in general. Comparing the previous CR study (11) with the present investigation some of these differences are apparent. In the CR study (11) Brown-Norway rats were used instead of Wistar (this investigation), in the CR experiment the procedure of restriction was ''to limit food intake such that food body weight was maintained at 50%–55% of ad libitum'' (11) instead of 40% PR (this study), and access to food of ad libitum–fed rats was limited to between 10 AM and 3 PM to synchronize the control rats to the same feed/fast cycle as the CR rats (11) instead of free access to food of ad libitum–fed rats during the 24 hours (present investigation). In any case it is important to note that in other CR studies (49–52), decreases in fatty acid unsaturation of similar duration and magnitude than those found here for PR have been obtained, and these changes were also mainly due, as in our case, to decreases in the highly unsaturated (and thus easily oxidizable) 22:6n-3 and 20:4n-6 fatty acids and to increases in the less unsaturated (and thus much more oxidation resistant) 18:1n-9 and 18:2n-6 fatty acids. Interestingly, these are also the main fatty acids varying in the same sense when comparing short-lived with long-lived mammals (51,53).

Some clues about the mechanisms by which PR decreases oxidative stress are already available. So, it is known that 7 weeks of PR decreases the rate of ROS generation of liver mitochondria independently of CR (48), and that neither lipid restriction (54) nor carbohydrate restriction (55) decrease ROS production or oxidative DNA damage.

Furthermore, very recent studies from our laboratory indicate that the same occurs after methionine restriction (a manipulation that also increases maximum longevity) without changing other dietary components (56). Thus, it is possible that the decrease in mitochondrial ROS production (and thus in oxidative damage) induced by PR is due to the lowered methionine intake of PR (and CR) animals. It can be speculated that methionine restriction can induce these changes through a decreased thiolization of complex I (the ROS generator involved in the decrease in ROS production during CR) due, for instance, to a decrease in homocysteine levels (various studies have shown that thiolization of complex I increases the rate of ROS production) because homocysteine is a metabolite derived from methionine. Alternatively, restriction of methionine or of other essential amino acids can be limiting for the synthesis of some particular protein involved in the modulation of complex I ROS production (38).

All these works suggest that the decrease in protein intake causes the changes detected during CR, and that mitochondrial ROS generation and endogenous oxidative damage to macromolecules are key determinants in the slowing of aging rate in both restriction paradigms. Noteworthy, PR led to changes in amino acid composition in whole liver, but not in mitochondria, suggesting the existence of mitochondria-specific homeostatic mechanisms for maintaining amino acid composition, despite dietary stress.

The decrease in complex I content can be particularly relevant. Previous experiments concerning localization of the site where PR lowers ROS production demonstrated that this decrease occurs, similarly to CR (8,9), at complex I. Concerning the mechanisms involved in the decrease in ROS generation, it was not due to a simple decrease in oxygen consumption, but a decrease in the free radical leak—the percentage of total electron flow directed to ROS generation—related to the degree of electronic reduction of the complex I ROS generator (48). This mechanism agrees with previous studies in the liver of PR (57) and CR (9) rats. Thus, the mitochondria of PR animals have a respiratory chain more efficient in avoiding ROS generation because they produce a smaller amount of ROS per unit of electron flow. The decrease in complex I content, suggested by our results, in the PR animals can represent an additional adaptation to decrease free radical production and the derived molecular damage.

Summary

CR and PR decrease mitochondrial ROS production, membrane unsaturation, and endogenous DNA, protein, and lipid oxidative damage. The decreases in ROS generation that occur at complex I are related to an increase in the efficiency of the respiratory chain in avoiding free radical production in both models, as well as to a decrease in complex I content or a different stoichiometry of its subunits. All these results suggests that part of the decrease in aging rate induced by CR can be due to the decreased intake of proteins acting through the modulation of oxidative conditions.

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