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Effect of the degree of fatty acid unsaturation of rat heart mitochondria on their rates of H_2O_2 production and lipid and protein oxidative damage

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Abstract

Previous comparative studies have shown that long-lived animals have lower fatty acid double bond content in their mitochondrial membranes than short-lived ones. In order to ascertain whether this trait protects mitochondria by decreasing lipid and protein oxidation and oxygen radical generation, the double bond content of rat heart mitochondrial membranes was manipulated by chronic feeding with semi-purified AIN-93G diets rich in highly unsaturated (UNSAT) or saturated (SAT) oils. UNSAT rat heart mitochondria had significantly higher double bond content and lipid peroxidation than SAT mitochondria. They also showed increased levels of the markers of protein oxidative damage malondialdehyde-lysine, protein carbonyls, and N^e-(carboxymethyl)lysine adducts. Basal rates of mitochondrial oxygen radical generation were not modified by the degree of fatty acid unsaturation, but the rates of H_2O_2 generation stimulated by antimycin A were higher in UNSAT than in SAT mitochondria. These results demonstrate that increasing the degree of fatty acid unsaturation of heart mitochondria increases oxidative damage to their lipids and proteins, and can also

Abbreviations: SAT – group fed saturated diet; UNSAT – group fed unsaturated diet; MDA – malondialdehyde; MDA-Lys – MDA-lysine; CML – N^e-(carboxymethyl)lysine.

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increase their rates of mitochondrial oxygen radical generation in situations in which the degree of reduction of Complex III is higher than normal. These observations strengthen the notion that the relatively low double bond content of the membranes of long-lived animals could have evolved to protect them from oxidative damage.

Keywords: Polyunsaturated fatty acids; Free radical generation; Protein carbonyls; Ageing; Malondialdehyde; Lipid peroxidation; Hydrogen peroxide; Carboxymethyllysine

1. Introduction

There is increasing evidence that oxidative stress, specially that coming from mitochondria, is implicated in the ageing process (Sohal and Weindruch, 1996; Harman, 1998; Barja, 1999a; Barja and Herrero, 2000). An important part of this evidence comes from comparative animal studies. Among oxidative stress-related parameters, which can putatively cause ageing, two correlate with the maximum longevity of animal species in the appropriate (inverse) sense: the rate of mitochondrial free radical generation and the degree of fatty acid unsaturation. Thus, long-lived animals, either mammals or birds, consistently have low levels of mitochondrial H_2O_2 production (Ku et al., 1993; Barja, 1999a) and low degrees of fatty acid unsaturation (Pamplona et al., 1996, 1998, 1999a,b,c, 2000), both in tissues and in mitochondria, when compared to short-lived ones.

Those inverse correlations are specially strong when studied in post-mitotic vital tissues like heart. However, since correlation does not implicate causality, experimental studies are needed to demonstrate it. Furthermore, while these two traits can collaborate independently to decrease mitochondrial oxidative damage in long-lived animals, they can also be mechanistically interconnected. Mitochondrial free radical production occurs at the inner mitochondrial membrane, which is composed of proteins and lipids. Thus, the low rate of free radical generation of the mitochondria of long-lived animals can be due to the possession of different polypeptides in the respiratory chain in relation to those of short-lived animals, but it can also be due to differences in membrane lipid composition. Fatty acids are major components of membranes and specific interactions between particular fatty acids and membrane proteins affecting their functions have been described (Brenner, 1984; Spector and Yorek, 1985; Lee, 1991, 1995), the functionality of the proteins being dependent on the lipidic medium (e.g. fatty acid unsaturation) in which they are embedded. Thus, the low rate of mitochondrial free radical generation of long-lived animals could also be due in part to the low double bond content of their mitochondria.

In order to clarify those questions, the degree of fatty acid unsaturation of heart mitochondria was experimentally manipulated by chronically feeding rats with semi-purified diets rich in saturated or unsaturated lipids. In these lipid-modified mitochondria, respiratory activities, basal and stimulated mitochondrial H_2O_2 generation in States 4 and 3 and at different respiratory Complexes, lipid peroxidation, protein carbonyls, and the tissue markers of lipoxidation-derived protein

damage malondialdehyde (MDA)-lysine and N^{e}-(carboxymethyl)lysine (CML) (Reddy et al., 1995; Requena et al., 1997) were measured. The possible effect of in vivo modification of the double bond content of the mitochondrial membranes on mitochondrial H_2O_2 generation and mitochondrial levels of protein carbonyls, MDA-lysine and CML have not been previously studied.

2. Material and methods

2.1. Materials

Biotin-conjugated rabbit IgG polyclonal antibody raised against a 2,4-dinitrophenylhydrazine (DNP) conjugate of Keyhole limpet haemocyanin (anti-DNP) was obtained from Molecular Probes (Eugene, OR); rabbit anti-DNP antiserum was from Dako (V401, Dako, Carpenteria, CA); streptavidin-biotinylated horseradish peroxidase was from Amersham International (Buckinghamshire, UK); and [$^2H_8$]lysine from MSD Isotopes (Rahway, NJ). Unless otherwise stated, other biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). CML, [$^2H_4$]CML, MDA-lys and [$^2H_8$]MDA-lys were prepared as previously described (Knecht et al., 1991; Requena et al., 1997). The rabbit antiserum used for CML immunoblotting was obtained as described by Reddy et al. (1995).

2.2. Animals and diets

Two hundred and fifty gram male Wistar rats were obtained from Iffa-Creddo (Lyon, France) and were divided in two groups which were fed ad libitum during 7 weeks under 12:12 L:D illumination conditions at thermoneutrality with semi-purified diets (American Institute of Nutrition AIN-93G diet; ICN, Costa Mesa, USA) rich in unsaturated (UNSAT group) or saturated (SAT group) oils. The composition of the UNSAT diet was cornstarch 36.75%, purified casein high nitrogen 20%, dextrinized corn starch 13.2%, sucrose 10%, menhaden oil 10%, alphacel non-nutritive bulk 5%, AIN-93G mineral mix 3.5%, AIN-93 vitamin mix 1%, L-cystine 0.3%, choline bitartrate 0.25% and T-butyl hydroquinone 0.0014%. The composition of the SAT diet was identical to that of the UNSAT diet except that 9.5% hydrogenated coconut oil plus 0.5% corn oil were present as fat source instead of 10% menhaden oil. A small amount (0.5%) of corn oil was included in the SAT diet in order to avoid long-term unsaturated fatty acid deficiency leading to homeostatic compensatory increases in mead acid (20:3n-9) which, reaching as high as 15% of tissue fatty acids, could lead to a lack of final differences between the two dietary groups in the total number of double bonds (DBI) of heart mitochondria. The diets were maintained frozen at -75°C through the 7 weeks of experimentation and small aliquots were unfrozen each day to feed the animals. Food intake and body weight were statistically similar in the SAT and UNSAT groups throughout the experiment. A complete fatty acid analysis of the lipids of the SAT and UNSAT diets was performed after the end of the dietary experiments

and is given in Table 1. The DBI of these diets, 11.46 in SAT and 214.51 in UNSAT, was respectively lower and higher than that obtained previously analysing a standard rat diet (DBI = 112; Pamplona et al., 1999c).

2.3. Mitochondrial respiration and H_2O_2 production

Animals were sacrificed by decapitation and the hearts were processed directly to obtain and assay mitochondria from fresh tissues by the procedure of Mela and Seitz (1979) with modifications. Hearts were quickly chilled, ventricles were separated from remaining vessels and rings of fat, chopped into small pieces, rinsed several times and homogenized with a loose fitting pestle in 10 ml of isolation buffer (220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Tris–ClH, pH 7.4)

Table 1
Fatty acid composition (mol%) and general fatty acid indexes of the dietary fats[a]

	Saturated diet	Unsaturated diet	*P*
8:0	0.54 ± 0.07	0.00 ± 0.00	<0.0001
10:0	4.15 ± 0.30	0.00 ± 0.00	<0.0001
11:0	5.34 ± 0.16	0.00 ± 0.00	<0.0001
12:0	39.18 ± 0.75	0.00 ± 0.00	<0.0001
13:0	0.46 ± 0.00	0.00 ± 0.00	<0.0001
14:0	21.07 ± 0.09	9.14 ± 0.15	<0.0001
15:0	0.01 ± 0.00	0.46 ± 0.00	<0.0001
16:0	10.88 ± 0.03	19.94 ± 0.08	<0.0001
16:1n-7	0.00 ± 0.00	11.90 ± 0.08	<0.0001
17:0	0.07 ± 0.00	2.16 ± 0.00	<0.0001
18:0	11.14 ± 0.07	4.27 ± 0.01	<0.0001
18:1n-9	2.60 ± 0.00	12.81 ± 0.17	<0.0001
18:2n-6	4.30 ± 0.15	1.55 ± 0.01	<0.0001
18:3n-3	0.07 ± 0.00	1.95 ± 0.01	<0.0001
18:4	0.00 ± 0.00	3.98 ± 0.05	<0.0001
20:0	0.12 ± 0.00	0.00 ± 0.00	<0.0001
20:1	0.00 ± 0.00	1.51 ± 0.01	<0.0001
20:2n-6	0.00 ± 0.00	0.08 ± 0.00	<0.0001
20:3n-6	0.00 ± 0.00	0.40 ± 0.02	<0.0001
20:4n-6	0.00 ± 0.00	0.91 ± 0.00	<0.0001
20:5n-3	0.00 ± 0.00	12.55 ± 0.05	<0.0001
22:5n-3	0.00 ± 0.00	2.34 ± 0.01	<0.0001
22:6n-3	0.00 ± 0.00	13.97 ± 0.06	<0.0001
SFA	93.00 ± 0.15	35.99 ± 0.04	<0.0001
UFA	6.99 ± 0.15	64.00 ± 0.04	<0.0001
PUFAn-3	0.07 ± 0.00	30.82 ± 0.01	<0.0001
PUFAn-6	4.30 ± 0.15	2.87 ± 0.01	<0.0001
DBI	11.46 ± 0.30	214.51 ± 0.02	<0.0001

[a] Values are means ± SEM from four independent measurements in each diet. SFA – saturated fatty acids; UFA – unsaturated fatty acids; PUFA – polyunsaturated fatty acids; DBI – double bond index. For calculations of these general indexes of fatty acid composition, see Section 2. *P* – significant difference of fatty acid parameter between both diets.

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. After homogenization, the pH was checked and readjusted to 7.4 if needed. The nuclei and cell debris were removed by centrifugation at 700*g* during 10 min in a RC5C Sorvall centrifuge using a SS-34 rotor. Heart mitochondria were obtained after centrifugation of the supernatant at 8000*g* during 10 min. The mitochondrial pellets were resuspended in 1 ml of isolation buffer. All the above procedures were performed at 5°C. Mitochondrial protein was measured by the Biuret method. The final mitochondrial suspensions were maintained over ice and were immediately used for the oxygen consumption and H_2O_2 production measurements, which were completed in less than 2 h. The rest of each suspension was frozen at −75°C to be used later for analysis of fatty acids and lipid and protein oxidative damage.

The oxygen consumption of heart mitochondria was measured at 37°C in a water-thermostatized incubation chamber with a Clark-type O_2 electrode and O_2 control box (CB1-D Hansatech, Norfolk, UK) in incubation buffer (145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.1% albumin, pH 7.4) with 0.25 mg of mitochondrial protein per ml and 2.5 mM pyruvate/2.5 mM malate as substrates, in the absence (State 4) and in the presence (State 3) of 500 μM ADP. The mitochondrial respiratory control ratio did not statistically differ between the two experimental groups (5.6 ± 0.33 in SAT and 5.5 ± 0.37 in UNSAT groups).

Mitochondrial H_2O_2 production was measured kinetically following the linear increase in fluorescence (excitation at 312 nm, emission at 420 nm) due to oxidation of homovanillic acid by H_2O_2 in the presence of horseradish peroxidase (Barja, 1999b) in a LS50B computer-controlled Perkin–Elmer fluorometer (Beaconsfield, UK). Reaction conditions were 0.25 mg of mitochondrial protein per ml, 6U/ml of horseradish peroxidase, 0.1 mM homovanillic acid, 50 U/ml of superoxide dismutase, and 2.5 mM pyruvate/2.5 mM malate in incubation buffer (145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.1% albumin, pH 7.4) at 37°C. Conversion of fluorescence units to nanomoles of H_2O_2 was performed with a standard curve using glucose plus glucose oxidase as H_2O_2 generator. When included in the assay, specific respiratory chain inhibitors or ADP were present at the following concentrations: 2 μM rotenone, 2 μM antimycin A, 10 μM myxothiazol and 500 μM ADP.

2.4. Fatty acid analyses

Lipids from diet samples and heart mitochondrial fractions 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 organic phase was separated, evaporated under N_2, redissolved in 75 μl of carbon disulphide and 1 μl was submitted to gas chromatog-

raphy/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 (Hewlett-Packard SA, Barcelona, Spain). 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 acid methyl esters was made by comparison with authentic standards and on the basis of mass spectra. Results are expressed as mol%.

2.5. Protein carbonyls

Mitochondrial protein carbonyls were measured by enzyme-linked immunosorbent assay (ELISA), following the method previously described by Winterbourn and Buss (1999). 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 were added to 1 ml PBS pH 7.4. From this, 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% Tween 20 solution) for 1 h at 37°C; addition of 200 μl streptavidin-biotinylated horseradish peroxidase (1:3000 in PBS, 0.1% Tween 20 solution) for 1 h at room temperature. Finally, 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 were then added and the colour was allowed to develop for 25 min before stopping with 100 μl of 2.5 M sulphuric acid. Absorbances at 490 nm were recorded 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 prepared by mixing varying proportions of ascorbic acid/ferrous ammonium sulphate-oxidized bovine serum albumin (BSA) and $NaBH_4$-reduced BSA. The carbonyl content of oxidized BSA was calibrated colorimetrically. The blank consisted of DNP reagent added to PBS without protein. The background absorbance for DNP reagent was subtracted from all other absorbances. Carbonyl content was expressed as nmol carbonyl/mg protein.

2.6. N^e-(carboxymethyl)lysine and malondialdehyde-lysine measurement by gas chromatography/mass spectrometry

CML and MDA-lys concentrations in mitochondrial proteins were measured by GC/MS as previously described (Pamplona et al., 1999d). Mitochondrial samples containing 2 mg of proteins were delipidated using chloroform:methanol (2:1 v/v) in the presence of 0.01% butylhydroxytoluene, and proteins from the methanolic phase precipitated by adding 10% trichloroacetic acid (final concentration) and

subsequent centrifugation. Protein samples were reduced overnight by incubation with 500 mM $NaBH_4$ in 0.2 M borate buffer, pH 9.2, containing 1 drop of hexanol as an anti-foam reagent. Proteins were then reprecipitated by adding 1 ml of 20% trichloroacetic acid and subsequent centrifugation. Isotopically labelled internal standards were then added, and the samples were hydrolysed at 110°C for 24 h in 1 ml of 6 N 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 × 0.25 mm × 0.25 μm) coupled to a Hewlett-Packard model 5973A mass selective detector (Hewlett-Packard SA, Barcelona, Spain). The injection port was maintained at 275°C; the temperature program was 2 min at 150°C, then 5°C/min to 225°C, then 25°C/min to 300°C, and finally hold at 300°C for 5 min. Quantification was performed by external standardization using standard curves constructed from mixtures of deuterated and non-deuterated standards. Analyses were carried out by selected ion monitoring GC/MS. 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; and MDA-lysine and [$^2H_8$]MDA-lys, m/z 474 and 482, respectively. The amount of products is expressed as the ratio μmol CML or MDA-lys per mol lysine.

2.7. Immunoblotting of protein-bound 2,4-dinitrophenylhydrazones and N^e-carboxymethylated proteins

Immunoblotting of protein-bound 2,4-dinitrophenylhydrazones and N^e-carboxymethylated heart mitochondrial proteins were performed as previously described (Portero-Otín et al., 1999; Pamplona et al. 1999d). SDS-PAGE was performed according to Laemmli (1970), using 5% and 9% acrylamide concentrations as stacking and resolving gels, respectively, followed by Coomassie Blue staining. For protein carbonyl immunodetection, samples were derivatized with DNP as previously described (Portero-Otín et al., 1999) prior to electrophoresis. Briefly, to 15 μl mitochondrial samples adjusted to 4 mg of protein/ml, SDS was added to a final concentration of 6%, and, after heating at 95°C for 3 min, 20 μl of 10 mM DNP in 10% trifluoroacetic acid were added. After 15 min at room temperature, 20 μl of a solution containing 2 M Tris base, 30% glycerol and 15% β-mercaptoethanol were added for neutralization and sample preparation prior to loading onto SDS-gels. After SDS-PAGE, proteins were transferred to PVDF membranes (Immobilon-P Millipore, Bedford, MA) using a Semi-dry system (Semi-Phor, Hoefer Scientific Instruments, San Francisco, CA). Membranes were then blocked by overnight incubation at 4°C with 2% casein in PBS pH 7.35 containing 0.1% Tween 20. Subsequently, membranes were incubated for 1 h at room temperature with anti-DNP (1:5000 in the above buffer), or with anti-CML (1:1000 in PBS pH 7.35 containing 0.1% Tween 20 and 0.25% skim milk). Immunodetection, in both cases, was performed using the Tropix chemiluminescence kit (Bedford, MA). Luminescence was detected with a Lumi-Imager equipment (Boehringer, Mannheim, Germany).

2.8. Lipid peroxidation

Lipid peroxidation levels of heart mitochondria were measured by a thiobarbituric acid test specially adapted to tissue samples (Uchiyama and Mihara, 1978) in the presence of 0.07 mM butylhydroxytoluene, added as an antioxidant to avoid artifactual lipid peroxidation during the assay. Malondialdehyde-bis(dimethylacetal) (Merck, Germany) was used as standard and the results were expressed as nanomoles of MDA/mg of mitochondrial protein.

2.9. Calculations and statistical methods

Saturated fatty acids (SFA) = Σmol% (8:0 + ··· + 20:0); unsaturated fatty acids (UFA) = Σmol% (16:1 + 18:1 + 18:2 + 18:3 + 18:4 + 20:1 + 20:2 + 20:3 + 20:4 + 20:5 + 22:5 + 22:6); polyunsaturated fatty acid n-3 (PUFAn-3) = Σmol% (18:3 + 18:4 + 20:5 + 22:5 + 22.6); polyunsaturated fatty acid n-6 (PUFAn-6) = Σmol% (18:2 + 20:2 + 20:3 + 20:4); double bond index (DBI) = [(Σmol% Monoenoic × 1) + (Σmol% Dienoic × 2) + (Σmol% Trienoic × 3) + (Σmol% Tetraenoic × 4) + (Σmol% Pentaenoic × 5) + (Σmol% Hexaenoic × 6)]. All statistics were obtained using the SPSS software (SPSS, Chicago). Normality of variables was assessed using the Kolmogorov–Smirnoff test. Differences between study groups were analysed by the Student *t* test. The 0.05 level was selected as the point of minimal statistical significance in every comparison.

3. Results

The fatty acid composition of heart mitochondria obtained from SAT and UNSAT animals is given in Table 2. Many fatty acids showed significant differences between the two groups. The DBI indicates the total number of double bonds of all the fatty acids taken together. Although the difference in DBI was greater between the two different diets (Table 1) than between the SAT and UNSAT mitochondria (Table 2), the DBI was significantly higher in UNSAT than in SAT heart mitochondria. This was due in part to the lack of reactive homeostatic increases in mead acid (20:3n-9) which was not detected in the mitochondrial analyses. The higher DBI of the UNSAT mitochondria was mainly due to their higher content in highly unsaturated fatty acids of the n-3 series like 22:6, 22:5 and 20:5, whereas in SAT mitochondria fatty acids with a lower number of double bonds (20:4n-6, 18:2n-6 and 18:1n-9) were more abundant.

No significant differences in State 4 and State 3 oxygen consumption of heart mitochondria were observed between SAT and UNSAT groups (Table 3). The basal rates of mitochondrial H_2O_2 production were also not affected by the degree of fatty acid unsaturation of heart mitochondria either in State 4 or State 3 (Table 4). However, pyruvate/malate-supported H_2O_2 production stimulated by antimycin A (which comes from Complexes I and III; see Section 4), was significantly higher in UNSAT than in SAT mitochondria (Fig. 1). Pyruvate/malate-supported H_2O_2

Table 2
Fatty acid composition (mol%) of total phospholipids in heart mitochondria of rats fed saturated or unsaturated diets[a]

	SAT	UNSAT	*P*
12:0	0.24 ± 0.04	0.00 ± 0.00	<0.0001
14:0	11.30 ± 0.71	12.59 ± 0.50	
15:0	1.64 ± 0.08	2.15 ± 0.03	<0.0001
16:0	10.44 ± 0.16	12.33 ± 0.45	<0.002
16:1n-7	1.16 ± 0.04	1.16 ± 0.17	
17:0	0.85 ± 0.03	0.85 ± 0.04	
18:0	22.99 ± 0.23	21.24 ± 0.28	<0.0001
18:1n-9	10.24 ± 0.35	7.09 ± 0.41	<0.0001
18:2n-6	9.48 ± 0.61	4.86 ± 0.30	<0.0001
18:3n-3	0.11 ± 0.01	0.19 ± 0.08	
20:3n-6	0.89 ± 0.08	0.73 ± 0.03	
20:4n-6	20.79 ± 0.67	10.77 ± 0.46	<0.0001
20:5n-3	0.09 ± 0.00	4.01 ± 0.19	<0.0001
22:5n-3	0.48 ± 0.03	1.85 ± 0.06	<0.0001
22:6n-3	9.24 ± 0.32	20.10 ± 0.65	<0.0001
SFA	47.49 ± 0.50	49.19 ± 0.49	<0.04
UFA	52.50 ± 0.50	50.80 ± 0.49	<0.04
PUFAn-3	9.93 ± 0.33	26.16 ± 0.80	<0.0001
PUFAn-6	31.17 ± 0.20	16.38 ± 0.28	<0.0001
DBI	174.91 ± 2.57	213.87 ± 4.98	<0.0001

[a] Values are means ± SEM from seven animals. SAT – group of animals fed with the saturated diet; UNSAT – group of animals fed with the unsaturated diet. For other abbreviations see Table 1. *P* – significant difference of fatty acid parameter between SAT and UNSAT groups.

production stimulated by rotenone (which comes only from Complex I), did not show significant differences between the two groups (Fig. 1). After double inhibition with antimycin A plus myxothiazol a lack of difference in H_2O_2 production (also coming exclusively from Complex I in this experiment) was observed again between the two groups (Fig. 1).

Oxidized and carboxymethylated proteins of heart mitochondrial fractions were visualized by immunoblotting analyses after SDS-PAGE (Fig. 2). The anti-DNPH signal was less marked in the SAT than in the UNSAT group, suggesting that the

Table 3
Oxygen consumption of heart mitochondria from rats fed saturated or unsaturated diets[a]

	SAT	UNSAT
State 4 (pyr/mal)	21.7 ± 1.9 (7)	20.5 ± 2.3 (7)
State 3 (pyr/mal + ADP)	81.0 ± 6.2 (7)	80.8 ± 7.1 (7)

[a] Values are means ± SEM from the number of animals indicated in parenthesis. Values are nanomoles of O_2/min/mg of mitochondrial protein. No significant differences were found between the SAT and UNSAT dietary groups.

Table 4
H_2O_2 production of heart mitochondria from rats fed saturated or unsaturated diets[a]

	SAT	UNSAT
State 4 (pyr/mal)	0.78 ± 0.09 (7)	0.82 ± 0.08 (7)
State 3 (pyr/mal + ADP)	0.71 ± 0.06 (7)	0.84 ± 0.11 (7)

[a] Values are means ± SEM from the number of animals indicated in parenthesis. Values are nanomoles of H_2O_2/min/mg of mitochondrial protein. No significant differences were found between the SAT and UNSAT dietary groups.

steady-state level of protein carbonyls is directly related to the degree of unsaturation of the mitochondrial membrane. This effect of the mitochondrial fatty acid double bond content on the immunoblotting pattern was quantitatively confirmed by the ELISA analysis described below (Fig. 3).

Lipid peroxidation of heart mitochondria was significantly different between the two groups, the values in the UNSAT group reaching 255% of those in the SAT group (Fig. 3). Three different markers of protein oxidative damage also showed significant differences as a function of the degree of unsaturation of mitochondrial fatty acids. MDA-lys was the protein marker more profoundly affected since its levels in the UNSAT group were 186% of those present in the SAT group (Fig. 3). Protein carbonyls were also stimulated by fatty acid unsaturation, UNSAT levels reaching 153% of those present in the SAT group (Fig. 3). CML was the protein modification marker less profoundly affected. Even so, CML values were again significantly higher in UNSAT than in SAT mitochondria (Fig. 3).

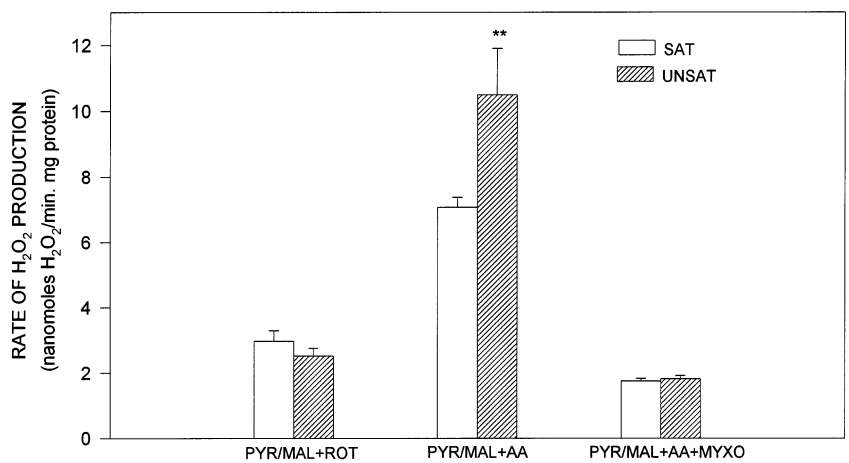

Fig. 1. Stimulated rates of H_2O_2 production in heart mitochondria of rats fed saturated (SAT) or unsaturated (UNSAT) diets. Pyruvate/malate (PYR/MAL) was used as substrate in the presence of rotenone (ROT), antimycin A (AA), or AA + myxothiazol (AA + MYXO). In the presence of ROT or AA + MYXO the H_2O_2 comes from Complex I, whereas in the presence of AA it comes from Complex I and Complex III. Values are means ± SEM. (**) Significantly different ($P < 0.01$) from SAT group.

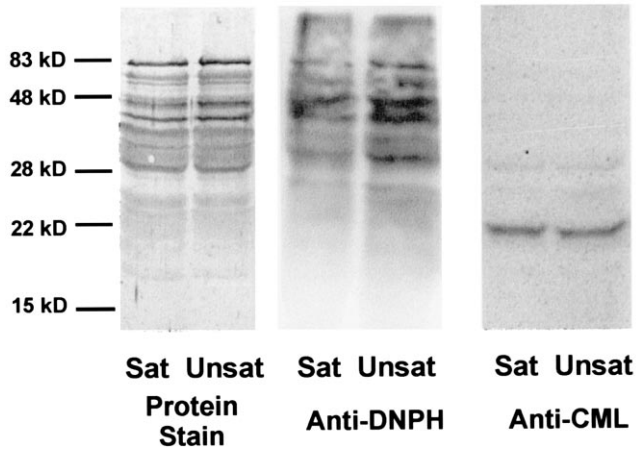

Fig. 2. Detection of oxidized and carboxymethylated proteins in heart mitochondria of rats fed saturated (SAT) or unsaturated (UNSAT) diets by SDS-PAGE. Left – Coomassie Blue protein stain; middle – anti-DNPH immunostaining; right – anti-CML immunostaining. Molecular weight markers are shown on the left of protein stain panel. The blots shown are representative of three independent analyses in each group.

4. Discussion

In this investigation the double bond content of heart mitochondria was successfully manipulated in vivo by long-term maintenance of the animals with diets strongly differing in fatty acid unsaturation. Previous studies have shown that long-lived animals have lower levels of fatty acid unsaturation in heart tissue and heart mitochondria in relation to short-lived ones (Pamplona et al., 1999a,b,c). According to the equation describing the quantitative relationship between heart double bond content and mammalian longevity (Pamplona et al., 1999c), the magnitude of the decrease in fatty acid unsaturation obtained here from the UNSAT to the SAT group is equivalent to the decrease in DBI observed from the rat (longevity = 4 yr) to a mammal showing a longevity more than three-fold higher (12.2 yr). Besides, our previous comparative studies (Pamplona et al., 1999a,b,c) showed that the lower double bond content of the heart of long-lived animals in relation to that of short-lived ones was not due to differences in total saturated or unsaturated fatty acids, but to a lower degree of unsaturation (mainly due to substitution of PUFAn-3 by PUFA n-6). This is similar to what was observed here when comparing the SAT and UNSAT groups. Thus, the experimental manipulation developed in this investigation successfully mimics quantitatively and qualitatively the differences in fatty acid unsaturation observed between mammals of different longevities.

It is known that the degree of in vitro lipid peroxidation increases as a function of the degree of unsaturation of the fatty acid substrates present (North et al., 1994; Bondy and Marwah, 1995). It is then logical to expect that the relatively low fatty

acid double bond content of the mitochondria of long-lived animals will afford them protection against lipid peroxidation. This is supported by the experiments described in this report. While increases in lipid peroxidation in rats fed diets rich in PUFAn-3 had already been described in total heart tissue (Javouhey-Donzel et al., 1993), it is shown here that the increased double bond content of UNSAT rat heart mitochondria led to a strong increase in their levels of lipid peroxidation.

Whereas the low fatty acid unsaturation of long-lived animals protects their mitochondria against lipid peroxidation, it was interesting to investigate if it could also be responsible in part for their lower rates of oxygen radical production (Ku et al., 1993; Barja, 1999a) due to lipid–protein interactions occurring at the inner mitochondrial membrane, the place where reactive oxygen species are generated. Previous studies adding fatty acids to the external medium of isolated mitochondria have described increases (Turrens et al., 1991; Bondy and Marwah, 1995; Cocco et al., 1999) or decreases (Korshunov et al., 1998) in mitochondrial $O_2^{\cdot -}$ or H_2O_2 generation by different types of fatty acids. At variance with those reports, in our case it is the fatty acid composition of the mitochondrial membrane itself what was modified in vivo. Under basal conditions, the rates of pyruvate/malate H_2O_2 production of heart mitochondria were not modified by changing the double bond contact of the mitochondrial membrane, and the same was true for the mitochondrial State 4 and 3 oxygen consumption and the respiratory control ratios. The effects of dietary fatty acids have been mainly studied in liver mitochondria, and there is considerable controversy as to whether they actually alter mitochondrial

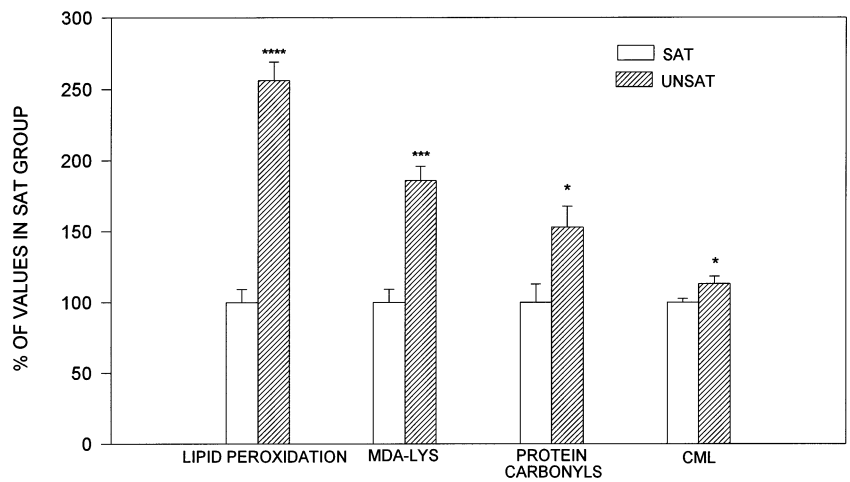

Fig. 3. Lipid and protein peroxidation markers in heart mitochondria of rats fed saturated (SAT) or unsaturated (UNSAT) diets. Values (means ± SEM) are shown as percentage of the mean of the SAT group. Absolute values: Lipid peroxidation (1.62 ± 0.15 in SAT and 4.13 ± 0.21 in UNSAT; nanomoles of MDA/mg protein); MDA-LYS (250 ± 23 in SAT and 465 ± 25 in UNSAT; μmole/mol lys); Protein carbonyls (0.38 ± 0.05 in SAT and 0.58 ± 0.06 in UNSAT; nanomole/mg protein); CML (1979 ± 50 in SAT and 2234 ± 104 in UNSAT; μmol/mol lys). Asterisks represent significant differences between the SAT groups: (*) $P < 0.05$; (***) $P < 0.0001$; (****) $P < 0.000001$.

bioenergetics, reports demonstrating fatty acid-induced decreases in State 4 or State 3 respiration and respiratory control (Divakaran and Venkataram, 1977; Stillwell et al., 1997; Pepe et al., 1999) being offset by reports demonstrating no or contradictory effects on the same parameters under similar conditions (Stancliff et al., 1969; Rafael et al., 1984; Toyomizu et al., 1992).

The situation observed for the stimulated rates of H_2O_2 generation was different in some cases to that found for basal rates. Previous studies have shown that heart mitochondria produce oxygen radicals only at Complexes I and III of the respiratory chain (Herrero and Barja, 1997a,b; Barja, 1999a), their capacity increasing as a function of the degree of reduction of the free radical generator sites. We have thus studied H_2O_2 production in the presence of respiratory inhibitors, which increase the degree of reduction of the respiratory chain on the substrate side of their sites of action, in order to ascertain if those two respiratory Complexes change their capacity of free radical generation after modifying the degree of fatty acid unsaturation of the mitochondria. In the presence of antimycin A, pyruvate/malate H_2O_2 production was significantly higher in the UNSAT than in the SAT mitochondria. Since antimycin A inhibits electron flow at Complex III, under these conditions the oxygen radicals can come from any of the two respiratory complexes situated on the substrate side of the inhibitor, i.e., Complexes I or III. However, pyruvate/malate-supported H_2O_2 generation stimulated by rotenone, which blocks electron flow from Complex I to the ubiquinone pool, was similar in SAT and UNSAT mitochondria. This indicates that Complex I do not change its capacity for oxygen radical generation as a function of the double bond content of the mitochondrial membrane. This was also corroborated by the lack of differences in pyruvate/malate-supported H_2O_2 production between the SAT and the UNSAT groups in the presence of antimycin A plus myxothiazol. Addition of myxothiazol to antimycin A-treated mitochondria eliminates any contribution of Complex III to free radical generation. Thus, the lack of differences between the two groups in the rate of H_2O_2 production with antimycin A plus myxothiazol (which can come only from Complex I) indicates again that Complex I does not change its rate of free radical generation in response to fatty acid unsaturation. All these results, taken together, indicate that, although the basal rate of free radical generation is not modified, the capacity for H_2O_2 production is higher in UNSAT mitochondria, and that this difference occurs at the level of the Complex III generator. This could be relevant throughout ageing during episodes of increased H_2O_2 production due to conditions which increase the degree of reduction of Complex III. On the other hand, the lack of modulation of the basal rates of mitochondrial oxygen radical generation by an exogenous parameter (the dietary fatty acid unsaturation) is theoretically what would be expected if the basal rate of free radical generation contributes to determine the ageing rate of each animal species (an endogenous process).

While the low double bond content of SAT mitochondria primarily protects them from lipid peroxidation, it can have also an indirect impact mainly on nearby macromolecules since some free radicals, like hydroxyl radical, tend to react strongly near their sites of generation. Thus, inner membrane proteins would be

primary targets. Mitochondrial protein modification was studied using three different markers of protein oxidative damage, MDA-lysine, protein carbonyls and CML. MDA is a lipid peroxidation product which is able to bind covalently to lysine protein residues generating MDA-lysine adducts (Requena et al., 1997). In agreement with a prominent role of lipoxidation-derived protein modification, MDA-lysine was the protein marker showing the stronger increase in the UNSAT when compared to the SAT group. This shows that increasing the double bond content of heart mitochondrial membranes increases oxidative damage of mitochondrial proteins most probably as a consequence of in vivo increases in mitochondrial lipid peroxidation. An increase in oxidative damage of UNSAT mitochondrial proteins was also suggested by the results of the anti-DNP and anti-CML based immunoblotting analysis. Strikingly, CML-immunostaining was restricted almost completely to one band, at approximately 22 kDa, suggesting a high specificity of CML modification in heart mitochondria. Current works are in progress to elucidate the identity of this band. In contrast, a more general immunostaining was observed when anti-DNP was used. The presence of higher protein carbonyls and CML levels in the UNSAT than in the SAT group was confirmed by quantifying their levels by ELISA and GC/MS assays respectively. Previous studies have shown that in vitro incubation of beef heart submitochondrial particles or rat liver mitochondria with free radical generating systems gives rise to protein carbonyl formation in parallel with lipid peroxidation (Forsmark-Andrée et al., 1995; Reinheckel et al., 1998). While part of these carbonyls were products of direct protein oxidation, part of their generation was also mediated by lipid-derived peroxyl ($LOO^{\bullet}$) and alkoxyl ($LO^{\bullet}$) radicals (Forsmark-Andrée et al., 1995), which is possible taking into account the close proximity between the lipids and the proteins in the membrane. Our results demonstrate that this process occurs in heart mitochondria in vivo, since mitochondrial protein carbonyls were elevated as a consequence of increasing the double bond content of the mitochondrial membranes. A recent study has also found that polyunsaturated fatty acids increase protein carbonyls in vitro in a way dependent on the degree of unsaturation of the fatty acid used (Refsgaard et al., 2000). In agreement with the increase in MDA-lysine and protein carbonyls, the CML levels of the mitochondrial membranes were also higher in the UNSAT than in the SAT group. It is known that CML can originate either through lipid peroxidation or glycoxidation processes (Knecht, 1991; Reddy et al., 1995). This origin from carbohydrate in addition to lipid oxidative damage can be responsible for the observation that CML was the protein marker showing the smaller quantitative increases in the UNSAT in relation to the SAT mitochondria. Analogously, differences in liver CML between animal species with different longevities and degrees of fatty acid unsaturation were also much smaller than their differences in liver MDA-lysine (Pamplona et al., 2000). Other factors such as the turnover rates of the modified proteins (Pamplona et al., 1999d) can be also implicated in the magnitude of the response of the different markers of protein oxidative damage to the modification of fatty acid unsaturation.

In summary, the results of this investigation demonstrate that experimentally increasing the fatty acid double bond content of the mitochondrial membranes

increases their levels of lipid and protein oxidative damage. This further strengthens the notion that the low double bond content of the mitochondria from long-lived animals in relation to that of short-lived ones protects them from lipid and lipoxidation-derived protein oxidative damage. However, the possibility cannot be discarded that other putative changes induced by the dietary treatment, different from their impact on lipid composition, might be also responsible for the variations observed in lipid and protein oxidative damage. Our results also suggest that, whereas the rate of mitochondrial oxygen radical generation mainly depends on the protein rather than on the lipid components of the inner mitochondrial membrane, the degree of fatty acid unsaturation can also have some influence. Thus, the relatively lower double bond content of long-lived animals can also help to limit their rates of mitochondrial oxygen radical generation in situations in which the degree of reduction of Complex III is higher than normal.

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