# Methionine restriction decreases mitochondrial oxygen radical generation and leak as well as oxidative damage to mitochondrial DNA and proteins

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ABSTRACT **Previous studies have consistently shown that caloric restriction (CR) decreases mitochondrial reactive oxygen species (ROS) (mitROS) generation and oxidative damage to mtDNA and mitochondrial proteins, and increases maximum longevity, although the mechanisms responsible for this are unknown. We recently found that protein restriction (PR) also produces these changes independent of energy restriction. Various facts link methionine to aging, and methionine restriction (MetR) without energy restriction increases, like CR, maximum longevity. We have thus hypothesized that MetR is responsible for the decrease in mitROS generation and oxidative stress in PR and CR. In this investigation we subjected male rats to exactly the same dietary protocol of MetR that is known to increase their longevity. We have found, for the first time, that MetR profoundly decreases mitROS production, decreases oxidative damage to mtDNA, lowers membrane unsaturation, and decreases all five markers of protein oxidation measured in rat heart and liver mitochondria. The concentration of complexes I and IV also decreases in MetR. The decrease in mitROS generation occurs in complexes I and III in liver and in complex I in heart mitochondria, and is due to an increase in efficiency of the respiratory chain in avoiding electron leak to oxygen. These changes are strikingly similar to those observed in CR and PR, suggesting that the decrease in methionine ingestion is responsible for the decrease in mitochondrial ROS production and oxidative stress, and possibly part of the decrease in aging rate, occurring during caloric restriction.—Sanz, A., Caro, P., Ayala, V., Portero-Otin, M., Pamplona, R., Barja, G. Methionine restriction decreases mitochondrial oxygen radical generation and leak as well as oxidative damage to mitochondrial DNA and proteins. *FASEB J.* 20, 1064–1073 (2006)**

*Key Words: mitochondria · methionine restriction · caloric restriction · free radicals · aging · DNA damage · oxidative damage*

The results of many experimental and comparative studies are consistent with the validity of the mitochondrial oxygen radical theory of aging (1,2). These studies suggest that the mitochondrial rate of generation of ROS (mitROS generation) plays a main role. Many investigations have shown that mitROS generation is lower in long-lived than in short-lived animal species and that caloric restriction (CR) consistently decreases the rate of mitROS production (reviewed in ref 3). In these two models, low rates of mitROS generation are accompanied by lower levels of oxidative damage to mitochondrial DNA (mtDNA) and proteins (2,4–6).

Although numerous studies have documented the decrease in MitROS production and oxidative damage to mitochondrial macromolecules in CR, the dietary factor that causes these beneficial changes is unknown. Recent studies are beginning to clarify this subject. Thus, while lipid restriction does not change mitROS production (7), we recently found that protein restriction decreases mitROS production in rat liver (8) independent of energy intake, although the protein component responsible for this is not known. This decrease occurs specifically at complex I, lowers the percent free radical leak (FRL) in the respiratory chain, and decreases oxidative damage to mtDNA in rat liver (8). All these changes also occur in CR with a similar magnitude, suggesting that restriction of protein intake can be responsible for the well-known decreases in mitROS production and oxidative stress that take place in CR. On the other hand, although it has been believed that the antiaging effect of CR is due to the decreased intake of calories themselves rather than to decreases in specific dietary components, recent findings question this consensus (9). Moreover, classic studies have repeatedly found that protein restriction increases maximum longevity in rats and mice (10–13), although the magnitude of this increases are around half that of CR. In addition, it has been observed that methionine restriction (MetR) without energy restriction increases maximum longevity in rats and mice

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doi: 10.1096/fj.05–5568com



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(14,15). Various other kinds of recent investigations also point to a relationship between methionine and longevity (16–19).

Taking into account all these findings, we hypothesized that the restriction in methionine intake can be the cause of the decrease in mitROS generation and oxidative stress observed in CR and in protein restriction in rodents, and so can be responsible for around half of the increase in maximum longevity observed in caloric restriction. To test that hypothesis, in the present investigation we subjected male rats to exactly the same dietary protocol of MetR that is known to increase rat maximum longevity (14). The time of restriction used (6–7 wk) was the same that induces decreases in mitROS generation in the liver of rats subjected to both caloric restriction (20) and protein restriction (8). In liver and heart mitochondria of these methionine-restricted rats, we measured the rates of mitROS generation, mitochondrial oxygen consumption, FRL, the concentration of complexes I and IV, five oxidative damage markers of protein oxidation (the specific protein carbonyls glutamic and aminoadipic semialdehydes, GSA and AASA), glycoxidation (carboxyethyl-lysine CEL and carboxymethyl-lysine CML) and lipoxidation (malondialdehydelysine MDAL, and CML), as well as oxidative damage to mitochondrial DNA. Since it is known that the degree of unsaturation of phospholipids can affect markers of protein lipoxidation, the full fatty acid composition was measured in liver and heart mitochondria of methionine-restricted and control animals.

## MATERIALS AND METHODS

### Animals and diets

Male Wistar rats of 250 g of body wt were obtained from Iffa-Creddo (Lyon, France). The animals were caged individually and maintained in a 12:12 (light:dark) cycle at 22 ± 2°C and 50 ± 10% relative humidity. The semipurified diets used in this investigation were prepared by MP Biochemicals (formerly ICN), Irvine, CA, USA. Control animals were fed *ad libitum* a semipurified diet based on the American Institute of Nutrition AIN-93G diet: 31.80% cornstarch, 31.80% sucrose, 5.00% dextrin, 8.00% corn oil, 5.00% alphacel non-nutritive bulk, 1.12% L-arginine, 1.44% L-lysine, 0.33% L-hystidine, 1.11% L-leucine, 0.82% L-isoleucine, 0.82% L-valine, 0.82% L-threonine, 0.18% L-tryptophan, 0.86% L-methionine, 2.70% L-glutamic acid, 1.16% L-phenylalanine, 2.33% glycine, 0.20% choline bitartrate, 1.00% AIN vitamin mix, and 3.50% AIN mineral mix. The methionine-restricted rats received the same diet except that L-methionine was 0.17% and L-glutamic acid was 3.39%. Control animals received daily the same amount of food that the methionine-restricted animals had eaten as a mean during the previous day (pair feeding). Using this diet and protocol, it has been demonstrated that methionine restriction increases maximum longevity in rats (14). After 6–7 wk of dietary treatment the animals were sacrificed by decapitation. The liver and heart were immediately processed to isolate mitochondria, and liver and heart samples were stored at −80°C for the assays of oxidative damage to mtDNA.

### Mitochondria isolation

Rat liver and heart mitochondria were obtained from fresh tissue. The liver was rinsed and homogenized in 60 ml of isolation buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EDTA, pH 7.35). The nuclei and cell debris were removed by centrifugation at 1000 *g* for 10 min. Supernatants were centrifuged at 10,000 *g* for 10 min and the resulting supernatants were eliminated. The pellets were resuspended in 40 ml of isolation buffer without EDTA and centrifuged at 1000 *g* for 5 min. Mitochondria were obtained after centrifugation of the supernatants at 10,000 *g* for 10 min. After each centrifugation step, any overlaying layer of fat was eliminated. Heart mitochondria were isolated by differential centrifugation as described previously (4) except that the centrifugations were performed at 1000 *g* for 10 min to eliminate nuclei and cellular debris and at 10,000 *g* to pellet mitochondria. The mitochondrial pellets were resuspended in 1 ml of isolation buffer without EDTA. 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 used immediately for measurements of oxygen consumption and $H_2O_2$ production.

### Mitochondrial $H_2O_2$ production

The rate of mitochondrial $H_2O_2$ production was assayed by measuring the 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 (21). 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 (SOD), and 2.5 mM pyruvate/2.5 mM malate, 2.5 mM glutamate/2.5 mM malate, or 5 mM succinate + 2 μM rotenone as substrates, added at the end to start the reaction to the 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, in a total vol of 1.5 ml. Unless otherwise stated, the assays with succinate as substrate were performed in the presence of rotenone in order to avoid the backward flow of electrons to Complex I. In some experiments rotenone (2 μM) or antimycin A (2 μM) were also included in the reaction mixture to assay maximum rates of Complex I or Complex III $H_2O_2$ generation. Duplicated samples were incubated for 15 min at 37°C; the reaction was stopped by transferring the samples to a cold bath and adding 0.5 ml of stop solution (2.0 M glycine, 2.2M NaOH, 50 mM EDTA), and the fluorescence was read in a LS50B Perkin-Elmer fluorometer. Known amounts of $H_2O_2$ generated in parallel by glucose (Glc) oxidase with Glc as substrate were used as standards. Since the SOD added in excess converts all $O_2^{\cdot-}$ excreted by mitochondria (if any) to $H_2O_2$, the measurements represent the total ($O_2^{\cdot-}$ plus $H_2O_2$) rate of mitochondrial ROS production.

### Mitochondrial oxygen consumption

The oxygen consumption of liver and heart mitochondria was measured at 37°C in a water-thermostatized incubation chamber with a computer-controlled Clark-type $O_2$ electrode (Oxygraph, Hansatech, UK) in 0.5 ml of the same incubation buffer used for $H_2O_2$ measurements. The substrates used were complex I- (2.5 mM pyruvate/2.5 mM malate or 2.5 mM glutamate/2.5 mM malate) or complex II-linked (5 mM succinate+2 μM rotenone). The assays were performed in the absence (State 4, resting) and in the presence (State 3, phosphorylating) of 500 μM ADP.

### Mitochondrial free radical leak

The $H_2O_2$ production and $O_2$ consumption of liver and heart mitochondria were measured in parallel in the same samples under similar experimental conditions. This allowed the calculation of the fraction of electrons out of sequence, which reduce $O_2$ to ROS at the respiratory chain (the percent free radical leak, FRL) instead of reaching cytochrome oxidase to reduce $O_2$ to water. Since two electrons are needed to reduce 1 mol of $O_2$ to $H_2O_2$ whereas four electrons are transferred in the reduction of 1 mol of $O_2$ to water, the percent free radical leak was calculated as the rate of $H_2O_2$ production divided by twice the rate of $O_2$ consumption, and the result was multiplied by 100.

### Measurement of 8-oxodG in mtDNA

Mitochondrial DNA (mtDNA) was isolated by the method of Latorre et al. (22) adapted to mammals (23). The isolated nuclear and mitochondrial DNAs were digested to deoxynucleoside level by incubation at 37°C with 5 U of nuclease P1 (in 20 μl of 20 mM sodium acetate, 10 mM $ZnCl_2$, 15% glycerol, pH 4.8) for 30 min and 1 U of alkaline phosphatase (in 20 μl of 1 M Tris-HCl, pH 8.0) for 1 h (24). All aqueous solutions used for mtDNA isolation, digestion, and chromatographic separation were prepared in HPLC-grade water. Steady-state oxidative damage to mtDNA was estimated by measuring the concentration of 8-oxodG, referred to that of the nonoxidized base (deoxyguanosine, dG). 8-oxodG and dG were analyzed by HPLC with on-line electrochemical and UV detection, respectively. The nucleoside mixture was injected into a reverse-phase Beckman Ultrasphere ODS column (5 μm, 4.6 mM×25 cm) and was eluted with a mobile phase containing 2.5% acetonitrile and 50 mM phosphate buffer pH 5.0. A Waters 510 pump at 1 μl/min was used. 8-oxodG was detected with an ESA Coulochem II electrochemical coulometric detector (ESA, Inc. Bedford, MA, USA) with a 5011 analytical cell run in the oxidative mode (225 mV/20 nA), and dG was detected with a Bio-Rad model 1806 UV detector at 254 nM. For quantification, peak areas of dG standards and of three concentration calibration pure 8-oxodG standards (Sigma) were analyzed during each HPLC run. A comparison of areas of 8-oxodG standards injected with and without simultaneous injection of dG standards ensured that no oxidation of dG occurred during the HPLC run.

### GSA, AASA, CML, CEL, and MDAL measurements in mitochondrial proteins

GSA, AASA, CML, CEL, and MDAL concentrations were measured by GC/MS as described previously (25). Briefly, samples containing 500 μg of 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 (CDN Isotopes, Pointe-Claire, Quebec, Canada) were then added; $[^2H_4]$-CML, $[^2H_4]$-CEL, and $[^2H_8]$-MDAL were prepared as described previously (26–28); $[^2H_5]$5-hydroxy-2-aminovaleric acid (for GSA quantification) and $[^2H_4]$6-hydroxy-2-aminocaproic acid (for AASA quantification) were prepared as described previously (29). The samples were hydrolyzed at 155°C for 30 min in 1 ml of 6 M HCl and dried *in vacuo*. The *N,O*-trifluoroacetyl methyl ester derivatives of the protein hydrolysates were prepared as described previously (26). 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. The injection port was maintained at 275°C; the temperature program was 5 min at 110°C, then 2°C/min to 150°C, then 5°C/min to 240°C, then 25°C/min to 300°C, and finally held at 300°C for 5 min. 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 μmolar ratio of GSA, AASA, CML, CEL, or MDAL/mol lysine.

### Mitochondrial complex I and IV

The concentration of mitochondrial complexes I and IV was estimated using Western blot analysis. Immunodetection was performed using a monoclonal antibody specific for the NDUFA9 subunit of complex I and subunit I of complex IV (1:500 /1:2,000, Molecular Probes, Invitrogen Ltd., Abingdon, Oxon, UK). An antibody to porin (1:1,000, Molecular Probes, Invitrogen Ltd.) 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, USA). Signal quantification and recording was performed with a CCD camera-based system (Lumi-Imager) from Boehringer Mannheim (Mannheim, Germany).

### Fatty acid analysis

Fatty acyl groups were analyzed as described previously (25). Tissue lipids were extracted from homogenate fractions with chloroform:methanol (2:1, v/v) in the presence of 0.01% (w/v) butylated hydroxytoluene. The chloroform phase was evaporated under nitrogen and the fatty acyl groups were transesterified by incubation in 2.5 ml of 5% (v/v) methanolic HCl for 90 min at 75°C. The resulting fatty acid methyl esters were extracted by adding 1 ml of saturated NaCl solution and 2.5 ml of n-pentane. The n-pentane phase was separated and evaporated under nitrogen. The residue was dissolved in 75 μl of carbon disulfide and 1 μl was used for 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 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 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) = [(Σ%Total14×14) + (Σ%Total16×16) + (Σ%Total18×18) + (Σ%Total20×20) + (Σ%Total22×22)]/100; double bond index (DBI) = [(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) + (Σmol% dienoic) + (2×Σmol% trienoic) + (4×Σmol% tetraenoic) + (6×Σmol% pentaenoic) + (8×Σmol% hexaenoic)].

### Statistics

Data were analyzed by Student's *t* tests. The minimum concentration of statistical significance was set at $P < 0.05$ in all the analyses.

## RESULTS

Methionine restriction significantly decreased the rate of $H_2O_2$ production of heart mitochondria with pyruvate/malate as substrates to values 64% those of control animals (**Fig. 1**, upper panel). With succinate as substrate no significant differences between control and MetR heart mitochondria were observed (Fig. 1, upper panel). However, when the experiments with succinate as substrate were repeated in the absence of rotenone, the rate of $H_2O_2$ generation of the MetR group was significantly lower than that of controls.

In liver mitochondria, methionine restriction also significantly decreased the rate of $H_2O_2$ generation to 46% and 43% of control values with pyruvate/malate and with glutamate/malate as substrates, respectively (Fig. 1, lower panel). In the presence of succinate, hepatic mitochondrial $H_2O_2$ production also was significantly lower in MetR than in control mitochondria.

Maximum rates of $H_2O_2$ generation were assayed using appropriate combinations of substrates and inhibitors of the respiratory chain (**Table 1**). The rate of $H_2O_2$ production of heart mitochondria was significantly decreased by methionine restriction in the presence of pyruvate/malate+rotenone (full reduction of complex I) but not in the presence of succinate+antimycin A (full reduction of complex III). In the case of liver mitochondria, no significant differences in $H_2O_2$ generation between control and MetR groups were observed with pyruvate/malate+rotenone, glutamate/malate+rotenone or succinate+antimycin A (Table 1).

The rate of $O_2$ consumption of heart mitochondria was significantly increased by MetR with pyruvate/malate in state 3 but not in state 4 (**Table 2**). In the presence of succinate, nonsignificant trends to increase heart mitochondrial $O_2$ consumption were found that showed marginal significance ($P < 0.07$ in state 4 and $P < 0.08$ in state 3).

In liver mitochondria, the rate of $O_2$ consumption was significantly increased by MetR with glutamate/malate and with succinate in both state 4 and state 3, as well as with pyruvate/malate in state 3 (Table 2). Only with pyruvate/malate in state 4 did the increase in $O_2$ consumption fail to reach statistical significance.

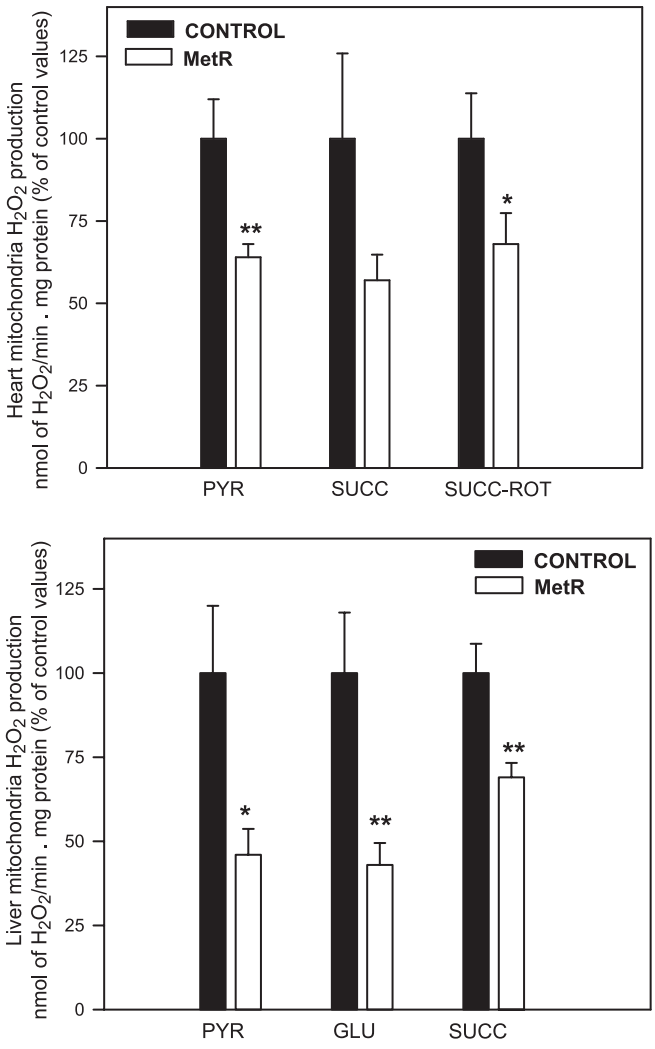


**Figure 1.** Rates of $H_2O_2$ production of heart and liver mitochondria from control and methionine-restricted rats. PYR = pyruvate/malate; SUCC = succinate; SUCC-ROT = succinate without rotenone; GLU = glutamate/malate; MetR = methionine-restricted. Control values in heart mitochondria: 0.25 ± 0.03 (PYR); 0.77 ± 0.20 (SUCC); 7.24 ± 1.00 (SUCC-ROT). Control values in liver mitochondria: 0.13 ± 0.03 (PYR); 0.93 ± 0.17 (GLU); 0.460 ± 0.04 (SUCC). Values are means ± SE from 6–8 different animals. Asterisks represent statistically significant differences between control and methionine-restricted animals (*$P<0.05$; **$P<0.01$).

The percentage of electrons in the respiratory chain directed to ROS generation (the free radical leak, FRL) in heart mitochondria was significantly decreased by MetR with pyruvate/malate (to 43% of controls; **Fig. 2**) but not with succinate (results not shown). In liver mitochondria, MetR significantly decreased the FRL both with the complex I (pyruvate/malate and glutamate/malate) and the complex II (succinate) linked substrates used to values 28–39% those of controls (Fig. 2).

Methionine restriction significantly decreased the concentration of complex I and complex IV in both heart and liver mitochondria (**Fig. 3**).

TABLE 1. *Maximum $H_2O_2$ production in the presence of respiratory chain inhibitors in heart and liver mitochondria from control and methionine-restricted rats*[a]

| | Control | Methionine restricted |
|---|---|---|
| *Heart:* | | |
| Pyr+Rot | 3.53 ± 0.32 | 2.73 ± 0.17* |
| Succ+AA | 23.17 ± 3.40 | 19.54 ± 2.59 |
| *Liver:* | | |
| Pyr+Rot | 0.56 ± 0.07 | 0.69 ± 0.06 |
| Glu+Rot | 0.82 ± 0.09 | 0.74 ± 0.08 |
| Succ+AA | 3.21 ± 0.33 | 3.39 ± 0.34 |

[a]Pyr, pyruvate/malate; Succ, succinate; Rot, rotenone; AA, antimycin A. Values are means ± SE from 7–8 different animals and are expressed in μmol of $H_2O_2$/min. mg mitochondrial protein. *($P<0.05$ between control and methionine-restricted groups).

The levels of the oxidative damage marker 8-oxodG in mtDNA were significantly decreased by MetR to 48% of controls in liver and to 65% of controls in heart (**Fig. 4**).

All the protein markers of oxidative, glycoxidative, and lipoxidative damage were significantly decreased by MetR in both liver and heart mitochondria (**Table 3**). In heart mitochondrial proteins GSA, AASA, CEL, and CML were decreased to 67–82% of control values, whereas in the case of MDAL the values found in MetR were 50% those of controls (Table 3). In liver mitochondria, GSA, CEL, and CML were decreased by MetR to 70–85% of controls whereas AASA and MDAL were lowered to 68% and 63% of controls, respectively.

Methionine restriction altered the fatty acid composition of liver (**Table 4**) and heart (**Table 5**) mitochondria, so that the total number of double bonds (DBI) was significantly decreased in both cases. In both kinds of mitochondria, the fatty acids mainly responsible for the decrease in DBI were the same. Thus, methionine restriction significantly increased the fatty acids with no or a small number of double bonds 18:0, 18:1n-9, and 18:2n-6 whereas it significantly decreased the main highly unsaturated ones 20:4n-6 and 22:6n-3 (Tables 4 and 5).

TABLE 2. *Oxygen consumption of heart and liver mitochondria from control and methionine-restricted rats*[a]

| | Control | Methionine restricted |
|---|---|---|
| *Heart mitochondria:* | | |
| Pyr (E4) | 26.2 ± 4.8 | 33.7±5.0 |
| Pyr (E3) | 123 ± 16 | 185 ± 3* |
| Succ (E4) | 86 ± 18 | 125 ± 15[a] |
| Succ (E3) | 137 ± 30 | 218 ± 48[b] |
| *Liver mitochondria:* | | |
| Pyr (E4) | 5 ± 1.2 | 6 ± 1.0 |
| Pyr (E3) | 15 ± 1.5 | 20 ± 2.3* |
| Glu (E4) | 7 ± 1.2 | 10 ± 1.2* |
| Glu (E3) | 59 ± 7.4 | 93 ± 6.8** |
| Succ (E4) | 17 ± 2.0 | 30 ± 2.9** |
| Succ (E3) | 88 ± 10.2 | 129 ± 9.4** |

[a]Pyr, pyruvate/malate; Glu, Glutamate/malate; Succ, succinate; E4, state 4 (substrate alone); E3, state 3 (substrate+ADP). Values are means ± SE from 7–8 different animals and are expressed in nanomoles of $O_2$/min. mg mitochondrial protein. Asterisks represent statistically significant differences between control and methionine-restricted groups (*$P<0.05$; **$P<0.01$); [a] ($P<0.07$); [b] ($P<0.08$).

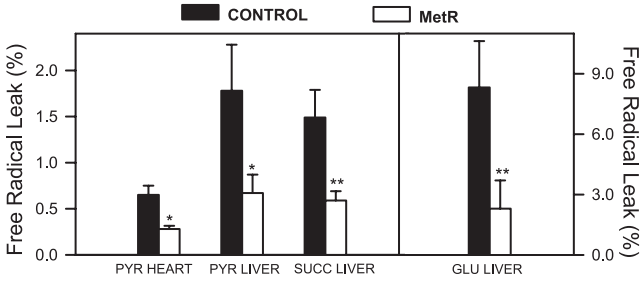


**Figure 2.** Free radical leak (%) of heart and liver mitochondria from control and methionine-restricted rats. The FRL is the percentage of the total electron electron flow in the respiratory chain directed to oxygen radical generation (see Materials and Methods). PYR, pyruvate/malate; SUCC, succinate; GLU, glutamate/malate; MetR, methionine-restricted. Values are means ± SE from 7–8 different animals. Asterisks represent statistically significant differences between control and methionine-restricted animals (*$P<0.05$; **$P<0.01$).

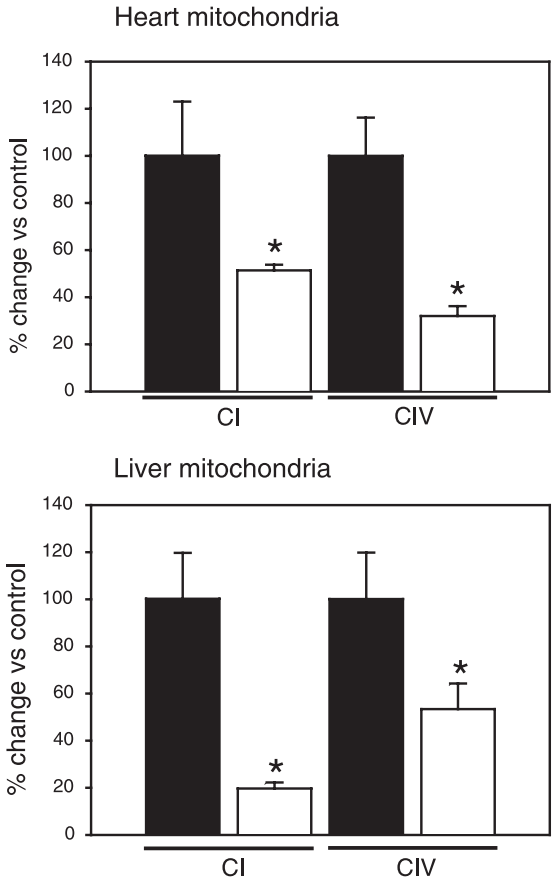


**Figure 3.** Concentration of protein complexes I and IV in heart and liver mitochondria from control and methionine-restricted rats. Values are means ± SE from 4–5 different animals. Asterisks represent statistically significant differences between control and methionine-restricted animals ($P<0.05$).

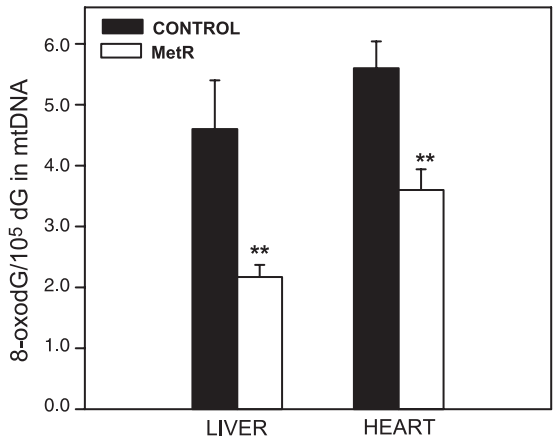


**Figure 4.** Oxidative damage to mitochondrial DNA (8-oxodG) in heart and liver from control and methionine-restricted rats. MetR, methionine-restricted. Values are means ± SE from 6–7 different animals. Asterisks represent statistically significant differences between control and methionine-restricted animals (**$P<0.01$).

## DISCUSSION

In this work it is shown for the first time that methionine restriction decreases mitochondrial ROS generation and oxidative damage to mitochondrial DNA and proteins. This strongly suggests that the decrease in methionine intake is the cause of these effects consistently found in many investigations on caloric restriction in rodents.

Caloric restriction is the better known experimental manipulation that decreases aging rate, but its mechanisms of action are unknown. Many studies have consistently shown that CR decreases mitROS generation (3), suggesting this can be one of the main mechanisms. But the dietary component responsible for this change was unknown. A recent study from our laboratory found that protein restriction (PR) without strong caloric restriction also decreases mitROS generation at complex I, lowers FRL, and decreases 8-oxodG in mtDNA (8) in rat liver mitochondria in exactly the same quantitative and qualitative way as CR, whereas neither lipid restriction (7) nor carbohydrate restriction (unpublished results) modify the rate of mitROS production. Looking for the protein component responsible for these effects, we focused on methionine in the present investigation since various sources of data currently relate methionine to aging. First, it is known that MetR without energy restriction increases maximum life span in rats and mice (14,15), similar to what was found in PR (10–13) although the PR effect on life span is smaller than that of CR. Recent investigations suggest that the increase in maximum longevity induced by MetR always occurs irrespective of the particular rat strain selected (30). Second, it has been found that the protein methionine content is inversely

TABLE 4. *Fatty acid analysis of liver mitochondria from control and methionine-restricted rats*[a]

| | Liver mitochondria | | |
|---|---|---|---|
| | Control | Methionine restriction | *P* |
| 14:0 | 0.23 ± 0.003 | 0.21 ± 0.01 | 0.224 |
| 16:0 | 16.11 ± 0.31 | 15.04 ± 0.24 | 0.022 |
| 16:1n-7 | 0.75 ± 0.09 | 0.48 ± 0.04 | 0.022 |
| 18:0 | 18.14 ± 0.22 | 20.48 ± 0.31 | 0.000 |
| 18:1n-9 | 7.13 ± 0.40 | 8.73 ± 0.57 | 0.064 |
| 18:2n-6 | 20.20 ± 0.49 | 21.93 ± 0.46 | 0.031 |
| 18:3n-3 | 0.28 ± 0.01 | 0.26 ± 0.01 | 0.368 |
| 20:3n-6 | 0.42 ± 0.07 | 0.30 ± 0.03 | 0.139 |
| 20:4n-6 | 28.05 ± 0.44 | 25.23 ± 0.29 | 0.000 |
| 22:4n-6 | 1.08 ± 0.12 | 1.01 ± 0.07 | 0.623 |
| 22:5n-6 | 0.55 ± 0.04 | 0.46 ± 0.03 | 0.120 |
| 22:5n-3 | 0.64 ± 0.04 | 0.56 ± 0.02 | 0.124 |
| 22:6n-3 | 6.36 ± 0.16 | 5.25 ± 0.11 | 0.000 |
| ACL | 18.56 ± 0.01 | 18.48 ± 0.01 | 0.000 |
| SFA | 34.48 ± 0.13 | 35.73 ± 0.28 | 0.006 |
| UFA | 65.51 ± 0.13 | 64.26 ± 0.28 | 0.006 |
| MUFA | 7.88 ± 0.37 | 9.22 ± 0.57 | 0.109 |
| PUFA | 57.62 ± 0.39 | 55.03 ± 0.57 | 0.007 |
| PUFAn-3 | 7.29 ± 0.17 | 6.08 ± 0.11 | 0.000 |
| PUFAn-6 | 50.32 ± 0.29 | 48.94 ± 0.61 | 0.107 |
| DBI | 211.17 ± 1.37 | 196.48 ± 1.14 | 0.000 |
| PI | 196.48 ± 1.94 | 176.54 ± 1.64 | 0.000 |

[a]ACL, acyl chain length; SFA, saturated fatty acids; UFA, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DBI, double bond index; PI, poliunsaturation index. Values are means ± SE from 5–7 different animals and are expressed as mol%.

TABLE 3. *Protein markers of oxidative, glycoxidative, and lipoxidative damage in liver and heart mitochondria from control and methionine-restricted rats*[a]

| | Liver mitochondria | | Heart mitochondria | |
|---|---|---|---|---|
| | Control | MetR | Control | MetR |
| GSA | 4193 ± 96 | 3168 ± 137*** | 5004 ± 121 | 3950 ± 131*** |
| AASA | 137 ± 7 | 93 ± 2*** | 212 ± 13 | 143 ± 23* |
| CEL | 369 ± 8 | 258 ± 10*** | 572 ± 22 | 409 ± 25** |
| CML | 1201 ± 17 | 1021 ± 30*** | 1768 ± 35 | 1441 ± 43*** |
| MDAL | 387 ± 14 | 245 ± 8*** | 452 ± 17 | 228 ± 14*** |

[a]GSA, glutamic semialdehyde; AASA, aminoadipic semialdehyde; CEL, carboxyethyl-lysine; CML, carboxymethyl-lysine; MDAL, malondialdehyde-lysine. MetR, methionine-restricted. Values are means ± SE from 5–7 different animals and are expressed as μmol/mol lysine. Asterisks represent statistically significant differences between control and methionine-restricted groups (*$P<0.05$; **$P< 0.01$; ***$P<0.001$).

TABLE 5. *Fatty acid analysis of heart mitochondria from control and methionine-restricted rats*[a]

| | Heart mitochondria | | |
|---|---|---|---|
| | Control | Methionine restriction | *P* |
| 14:0 | 0.21 ± 0.01 | 0.18 ± 0.002 | 0.037 |
| 16:0 | 11.77 ± 0.06 | 11.05 ± 0.11 | 0.001 |
| 16:1n-7 | 0.29 ± 0.03 | 0.20 ± 0.02 | 0.063 |
| 18:0 | 23.30 ± 0.30 | 24.59 ± 0.36 | 0.029 |
| 18:1n-9 | 6.10 ± 0.03 | 7.24 ± 0.22 | 0.001 |
| 18:2n-6 | 16.69 ± 0.42 | 18.37 ± 0.19 | 0.014 |
| 20:4n-6 | 27.88 ± 0.14 | 26.18 ± 0.40 | 0.003 |
| 22:4n-6 | 2.85 ± 0.07 | 2.15 ± 0.15 | 0.003 |
| 22:5n-6 | 1.31 ± 0.08 | 1.29 ± 0.04 | 0.890 |
| 22:5n-3 | 1.43 ± 0.04 | 1.32 ± 0.02 | 0.064 |
| 22:6n-3 | 8.11 ± 0.05 | 7.39 ± 0.22 | 0.009 |
| ACL | 18.85 ± 0.007 | 18.77 ± 0.006 | 0.000 |
| SFA | 35.29 ± 0.30 | 35.83 ± 0.35 | 0.283 |
| UFA | 64.70 ± 0.30 | 64.16 ± 0.35 | 0.283 |
| MUFA | 6.39 ± 0.06 | 7.44 ± 0.24 | 0.002 |
| PUFA | 58.30 ± 0.33 | 56.71 ± 0.14 | 0.005 |
| PUFAn-3 | 9.55 ± 0.07 | 8.71 ± 0.24 | 0.009 |
| PUFAn-6 | 48.75 ± 0.38 | 48.00 ± 0.12 | 0.140 |
| DBI | 225.21 ± 0.74 | 214.97 ± 1.08 | 0.000 |
| PI | 221.26 ± 0.93 | 206.74 ± 1.24 | 0.000 |

[a]ACL, acyl chain length; SFA, saturated fatty acids; UFA, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DBI, double bond index; PI, poliunsaturation index. Values are means ± SE from 5–7 different animals and are expressed as mol%.

related to maximum life span in mammals (16), which makes sense since methionine is among the protein amino acids most susceptible to oxidation by ROS (17). Third, methionine dietary supplementation damages many vital organs (like cardiovascular system and liver) and increases oxidative stress (31,32), and similar negative effects have been found in rats fed high protein diets (31,33). Fourth, knocking out methionine sulfoxide reductase-A (an enzyme that reduces methionine sulfoxide back to methionine through a thioredoxin-dependent reaction) lowers maximum longevity and increases protein carbonyls in mice (17), whereas overexpression of this enzyme in *Drosophila* increases its life span and delays aging (18). Fifth, overexpression of thioredoxin increases longevity in mice (19). Sixth, long-lived mutant Ames dwarf mice seem to have altered methionine metabolism (34).

In agreement with our hypothesis, we found in this investigation that MetR, using the same dietary protocol that increases maximun longevity in rats (14), also decreases mitROS production, FRL, and oxidative damage to mtDNA, similar to what has been observed in PR and CR (3,4,20). The decrease in mitROS production occurred in both liver and heart mitochondria and was quantitatively strong. In the case of heart mitochondria, the decrease in ROS generation occurred only at complex I since it occurred with pyruvate/malate but not with succinate (+rotenone) as substrate, and it also took place when the assay with succinate was performed in the absence of rotenone (which allows backward flow of electrons from succinate to the complex I ROS generator). In heart mitochondria it has been found that the decrease in ROS generation in CR also occurs exclusively at complex I (4). In the case of liver mitochondria, MetR decreased ROS generation both at complex I and complex III, since the decreases occurred with the complex II-linked substrate (succinate) as well as with the complex I-linked ones (pyruvate/malate and glutamate/malate). In 40% CR, decreases in ROS generation have been detected only at complex I in rat liver mitochondria (20).

Concerning the mechanism responsible for the decrease in mitROS production during MetR, there are various possibilities. A simple mechanism can be a decrease in the concentration of the respiratory complex/es responsible for ROS generation. This has been described in relation to aging in comparisons between rats and pigeons, in which the lower rate of mitROS production of the long-lived species (the pigeon) has been attributed to its lower complex I content (35). This could also be a mechanism during food restriction, since variations in the levels of mitochondrial protein complexes have been described as a result of CR (36). To test this, we measured the concentration of complex I and IV in control and MetR animals. Complex I contains the ROS generator site mainly related to aging, whereas complex IV is known not to produce ROS and was also measured as a control. Similar results were found in both heart and liver mitochondria. MetR decreased the concentration of complexes I and IV. This strong decrease in complex I content in MetR, as in the pigeon vs. rat comparison, can contribute to the decrease observed in mitROS production.

Even if the decrease in complex I helps to decrease ROS generation, it cannot be the only explanation of this change for various reasons. First, while ROS production with substrate alone decreases in MetR, when maximum rates of ROS production were assayed with appropriate combinations of substrate plus inhibitor (pyruvate/malate plus rotenone and succinate plus antimycin A), in almost every case the difference in ROS generation between control and MetR mitochondria disappeared. With substrate alone the respiratory chain is only partially reduced, so that ROS production depends both on the amount of ROS generators present as well as on the degree of electronic reduction of these generators (the higher their degree of reduction, the higher will be their rate of ROS production). But in the presence of the inhibitors, the ROS generators are fully reduced, so that the degree of reduction of the generators is no longer a variable. If the decrease in ROS production observed in MetR were only due to decreases in the concentration of ROS generators, a lower ROS production would be observed in the presence of those substrate+inhibitor combinations in MetR than in the controls. Since this was not the case, it seems that the degree of reduction of the ROS generators is involved in the decreases in ROS production observed in MetR with substrate alone.

That intrinsic changes at the concentration of the ROS generators occur in MetR is also indicated by the

strong decrease in percent free radical leak (FRL) observed in MetR. MetR mitochondria not only produce less ROS per unit time, but also produce less ROS per unit elecron flow in the respiratory chain, similar to what has been observed in CR (4,20) and PR (8) rats as well as in long-lived vs. short-lived animal species (36). In all these models of slow aging, the mitochondria are more efficient since they have a lower rate of ROS generation without the need to decrease oxygen consumption (and thus energy production), an interesting capability. In the case of MetR, not only was the consumption of oxygen not decreased, but it was even increased, providing more clues concerning the mechanisms of decrease in ROS generation. Contrary to intuitive thinking, when in the same individual the rate of mitochondrial $O_2$ consumption increases it tends to decrease (instead of increase) ROS generation due to two different effects. On the one hand, the degree of reduction of the respiratory chain decreases, which tends to decrease ROS generation. On the other hand, the increased oxygen consumption locally lowers the $pO_2$, and this lowers ROS production since the $K_M$ of the ROS generators (which have low affinity for $O_2$) falls within the range of physiological tissue $pO_2$, meaning that ROS production is $pO_2$ dependent at physiological tissue oxygen partial pressures. For these two reasons, the increase in oxygen consumption of the MetR mitochondria helps lower their rate of ROS production while decreasing their FRL (increase in efficiency in avoiding ROS generation).

Another possibility is that the decrease in ROS production is related to a mild uncoupling induced by the MetR protocol. This is suggested by the general trend to increased oxygen consumption of the MetR mitochondria, which suggests that the proton gradient was lowered by MetR. This would be relevant since it is known that high proton gradients raise ROS production at least at complex III. In any case, such changes in oxygen consumption do not occur in 40% CR and 40% PR (4,8,20). The reason for this difference could be the particular MetR protocol used in our investigation: methionine was restricted by 80% instead of 40% (as in the previous CR and PR studies), and it was substituted for glutamate in the diet. More studies are needed to ascertain whether the decrease in ROS production still occurs and the increases in oxygen consumption disappear when MetR is performed at 40% and without substituting it for dietary glutamate.

Another mechanism can also be involved in the decrease in mitROS production in MetR. It has been reported that addition of oxidized glutathione to mitochondrial complex I increases its rate of superoxide radical generation (37), and the same seems to occur after addition of homocysteine to rat heart mitochondria (38), suggesting that MitROS production can be regulated by thiol agents. Toxic effects of dietary methionine have been related to its conversion to and increase in homocysteine levels (31,39). Homocysteine has a free thiol group that can react with protein thiols, leading to protein mixed disulfides. Thus, MetR could decrease ROS generation through decreases in homocysteine, which would decrease thyolization of complex I. Homocysteine levels also increase with age in humans and represent a risk factor for aging and free radical-associated degenerative diseases (40).

Concerning oxidative damage, in this investigation it was found that MetR decreases 8-oxodG levels in mtDNA in both liver and heart. This also occurs in the same rat organs after both CR (4,20) and PR (8). This further supports the idea that the decrease in methionine ingestion is responsible for the decrease in mitochondrial oxidative stress observed in CR and PR. The decrease in 8-oxodG in MetR also agrees with the lower mitROS generation observed in this dietary manipulation, similar to what has been found in CR (4,20) and PR (8). Similar reasoning can be applied to the strong decreases in all of the five different markers of oxidative damage to mitochondrial proteins measured in this study. Decreases in GSA, MDAL, CML, and CEL have been observed in heart mitochondrial proteins of CR rats (5,6). Of these protein markers, some of them (CML and MDAL) are dependent on lipid peroxidation. Since lipid peroxidation increases strongly as a function of the number of double bonds per fatty acid, we measured the full fatty acid composition of heart and liver mitochondria. We found that in both tissues MetR significantly decreases the total number of double bonds. Thus, part of the decrease in CML and MDAL can be secondary to this decrease in the number of fatty acid double bonds. But this cannot be the full explanation because other protein markers, not dependent on lipid peroxidation (GSA, AASA and CEL), were also decreased by MetR, and because the quantitative decrease in double bonds was relatively small compared to that in MDAL and CML. Thus, it seems that the decrease in mitROS generation induced by MetR leads to a generalized decrease in oxidation, glycoxidation, and lipoxidation of mitochondrial proteins.

Concerning the particular fatty acid modified by MetR, the most important changes observed (quantitatively) were the same in both tissues. MetR decreased the highly unsaturated fatty acids 20:4n-6 and 22:6n-3, and substituted them for the much less unsaturated 18:2n-6, 18:1n-9, and 18:0. It is striking that this is essentially the kind of difference that has been observed when comparing animals with different longevities. Long-lived animals have less unsaturated membranes in tissues and mitochondria than short-lived ones, mainly due to a decrease in 22:6n-3 and 20:4n-6 and to an increase in 18:2n-6, leading to a lower lipid peroxidation and lipoxidation-dependent damage to macromolecules (41).

In summary, it is shown here for the first time that methionine restriction, similar to both caloric and protein restriction, decreases mitochondrial ROS generation, increases the efficiency of the respiratory chain in avoiding electron leak to oxygen, and lowers steady-state oxidative damage to mitochondrial DNA and proteins. This suggests that the decrease in methionine

ingestion can be the single molecular component responsible for the decrease in mitochondrial ROS generation and oxidative stress that occurs during caloric restriction, and thus for part of the decrease in aging rate elicited by this dietary manipulation, although a role for other dietary amino acids cannot be discarded without further investigation. FJ

This study was supported in part by I+D grants from the Spanish Ministry of Science and Technology (BFI2003–01287), the Generalitat of Catalunya (Departament d'Universitats, Recerca, i Societat de la Informació, DURSI, ref. 2005SGR00101), and the Spanish Ministry of Health (FIS 02–0891, 04–0355, and 05–2241) to R.P., from Fundacio la Caixa to R.P. and M.P.O, and from the Spanish Ministry of Science and Education (BFU2005–02584) to G.B. We thank David Argiles for excellent technical assistance. P. Caro and A. Sanz received a predoctoral fellowship from the Ministry of Science and Technology and from the Complutense University, respectively.

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*Received for publication December 12, 2005.*
*Accepted for publication January 24, 2006.*