

# Forty Percent Methionine Restriction Decreases Mitochondrial Oxygen Radical Production and Leak at Complex I During Forward Electron Flow and Lowers Oxidative Damage to Proteins and Mitochondrial DNA in Rat Kidney and Brain Mitochondria

Pilar Caro,<sup>1</sup> Jose Gomez,<sup>1</sup> Ines Sanchez,<sup>1</sup> Alba Naudi,<sup>2</sup> Victoria Ayala,<sup>2</sup> Monica Lopez-Torres,<sup>1</sup> Reinald Pamplona,<sup>2</sup> and Gustavo Barja<sup>1</sup>

## Abstract

Eighty percent dietary methionine restriction (MetR) in rodents (without calorie restriction), like dietary restriction (DR), increases maximum longevity and strongly decreases mitochondrial reactive oxygen species (ROS) production and oxidative stress. Eighty percent MetR also lowers the degree of membrane fatty acid unsaturation in rat liver. Mitochondrial ROS generation and the degree of fatty acid unsaturation are the only two known factors linking oxidative stress with longevity in vertebrates. However, it is unknown whether 40% MetR, the relevant methionine restriction degree to clarify the mechanisms of action of standard (40%) DR can reproduce these effects in mitochondria from vital tissues of strong relevance for aging. Here we study the effect of 40% MetR on ROS production and oxidative stress in rat brain and kidney mitochondria. Male Wistar rats were fed during 7 weeks semipurified diets differing only in their methionine content: control or 40% MetR diets. It was found that 40% MetR decreases mitochondrial ROS production and percent free radical leak (by 62–71%) at complex I during forward (but not during reverse) electron flow in both brain and kidney mitochondria, increases the oxidative phosphorylation capacity of brain mitochondria, lowers oxidative damage to kidney mitochondrial DNA, and decreases specific markers of mitochondrial protein oxidation, lipoxidation, and glycoxidation in both tissues. Forty percent MetR also decreased the amount of respiratory complexes I, III, and IV and apoptosis-inducing factor (AIF) in brain mitochondria and complex IV in kidney mitochondria, without changing the degree of mitochondrial membrane fatty acid unsaturation. Forty percent MetR, differing from 80% MetR, did not inhibit the increase in rat body weight. These changes are very similar to the ones previously found during dietary and protein restriction in rats. We conclude that methionine is the only dietary factor responsible for the decrease in mitochondrial ROS production and oxidative stress, and likely for part of the longevity extension effect, occurring in DR.

## Introduction

THE MODERN VERSION OF THE MITOCHONDRIAL free radical theory of aging is one of the most widely considered theories. In agreement with this theory, interspecies comparisons have already identified two main factors that can contribute to explain the slow aging rate of long-lived animals: A low rate of mitochondrial reactive oxygen species (mitROS) generation<sup>1–3</sup> and a low degree of membrane fatty acid unsaturation.<sup>4,5</sup> Many investigations have also shown

the validity of that theory also within single species. Thus, dietary restriction (DR) increases maximum longevity in practically all of the different kinds of animal species surveyed, including rodents,<sup>6</sup> and very recent studies indicate that this also occurs in rhesus monkeys.<sup>7</sup> DR also consistently lowers the rate of mitROS generation and the steady-state level of oxidative damage to tissue macromolecules like mitochondrial DNA (mtDNA) and proteins in rats and mice,<sup>8</sup> but the particular dietary factors responsible for these beneficial effects have not been identified.

<sup>1</sup>Department of Animal Physiology II, Faculty of Biological Sciences, Complutense University of Madrid (UCM), Madrid, Spain.

<sup>2</sup>Department of Experimental Medicine, Faculty of Medicine, University of Lleida-IRBLLEIDA, Lleida, Spain.

Classically, the antiaging effects of DR have been exclusively attributed to the lower ingestion of calories themselves. However, recent studies question this classical consensus in part because changes in some of the dietary components also modulate longevity in insects<sup>9,10</sup> and rodents.<sup>11–13</sup> The scarce studies available do not support the notion that carbohydrate or lipid restriction increase rodent longevity.<sup>14–16</sup> However, reevaluation of the abundant studies concerning protein restriction indicates that this dietary manipulation indeed causes an increase in maximum longevity in rats and mice because it has been observed in 16 out of 18 long-term survival studies.<sup>12,13</sup> Those increases suggest that up to 50% of the life extension effect of DR can be due to the decrease in protein intake, the remaining percentage increase being due to unknown factors or to calories themselves.<sup>12,13</sup> In relation to this, it is interesting that 40% protein restriction in the diet lowers mitROS generation and mtDNA oxidative damage in a way that is quantitatively and qualitatively similar to that induced by 40% DR,<sup>17</sup> whereas neither 40% lipid restriction<sup>18</sup> nor 40% carbohydrate restriction<sup>19</sup> modifies these parameters.

In an attempt to find out which amino acid from dietary proteins is responsible for these changes during protein restriction and DR, we focused on methionine because several kinds of studies had previously related methionine to rodent longevity. Eighty percent methionine restriction (MetR) without caloric restriction increases mean and maximum longevity in rats,<sup>20–22</sup> mice,<sup>23,24</sup> and insects.<sup>25</sup> MetR also replicates many of the beneficial changes induced by DR during rodent aging including: (1) Decreases in visceral fat, blood levels of triglycerides, cholesterol, glucose, insulin, or insulin-like growth factor-1 (IGF-1)<sup>26</sup>; (2) slowing of cataract development<sup>23</sup>; (3) protection against detrimental changes in the immune system<sup>23</sup>; and (4) reductions in the incidence of cancer.<sup>27</sup> Restriction of methionine dietary intake also decreases mitROS generation and oxidative stress at 80% MetR in rat heart<sup>11</sup> and at 40% and 80% in rat liver.<sup>11,28</sup> The same happens in the liver of rats subjected to protein restriction,<sup>17</sup> and in DR in rodent organs in general.<sup>8</sup> In contrast, DR of all the other amino acids is needed in the diet, but methionine does not alter mitROS generation and oxidative stress.<sup>29</sup>

In the present investigation, we tested whether 40% MetR during 7 weeks induces a decrease in mitROS production and oxidative stress in male Wistar rat brain and kidney mitochondria. The main reasons for this experimental design are the following: (1) Previous studies on the effect of DR, MetR, or protein restriction in rats have successfully lowered mitROS generation when applied during 7 weeks of dietary intervention. (2) The brain plays a principal role in the aging process due to its regulatory actions on the other vital organs, and its functions are strongly dependent on postmitotic cells, the large majority of brain neurons, but the effect of MetR on mitROS generation has not been studied in brain mitochondria from any animal. (3) Kidney mitROS generation and oxidative stress have never been studied at any level of MetR, whereas renal failure is the main spontaneous cause of death in rats. (4) All previous studies about the effect of MetR on specific markers of oxidative stress and damage have focused in whole organs instead of in mitochondria,<sup>11,28,30</sup> whereas it is currently believed that mitochondria not only can be a main source of the aging problem but could also be a main target. (5) The previous studies on the effect of

MetR on mitROS production and oxidative stress have been performed at 80% MetR,<sup>11,30</sup> not at 40% MetR, with the single exception of rat liver,<sup>28</sup> an organ that conserves mitotic capacity. To be able to attribute the effects of standard DR (40%) to dietary methionine, the level of implementation of MetR must be also 40%, not 80%. Furthermore, using 40% MetR instead of 80% MetR could perhaps avoid the strong decreases in growth rate, maturation, and final body size that also occur in (40%) DR. Those decreases could complicate the interpretation of the results obtained as well as the future possible applicability of MetR to children to best optimize the health-enhancing effects of adequate limitation of methionine dietary intake, while minimizing the occurrence of undesirable side effects.

At the end of the 7 weeks of dietary experimentation, functional kidney and brain rat mitochondria were isolated and their mitochondrial oxygen consumption rates in both the resting (state 4) and phosphorylating state (state 3), basal and maximum mitROS production rates with different substrates, and the percentage free radical leak (%FRL) in the respiratory chain were measured. Oxidative damage to mtDNA (estimated as 8-oxodG [8-oxo-7,8-dihydro-2'-deoxyguanosine]), and five markers of oxidative, lipoxidative, and glicoxidative damage to proteins measured by highly specific gas chromatography/mass spectrometry (GC/MS) techniques were also estimated. Because the degree of protein lipoxidation is secondarily influenced by the sensitivity of the membranes to lipid peroxidation, which strongly depends on their fatty acid unsaturation degree, the full fatty acid composition from kidney and brain mitochondrial membranes was also measured. The amounts of the respiratory complexes I–IV were also measured because they can modify the total capacity for electron flow in the respiratory chain as well as the total number of ROS generation sites, and therefore they can affect the rates of mitROS generation. The apoptosis-inducing factor (AIF) was also studied because it is currently considered a two-edged sword.<sup>31,32</sup> It can stimulate apoptosis, but it is also required for the assembly/maintenance of complex I, the respiratory complex at which mitROS generation is lowered in long-lived animals,<sup>2,33,34</sup> as well as in rats subjected to DR,<sup>35,36</sup> protein restriction,<sup>17</sup> and MetR.<sup>11,28</sup>

## Materials and Methods

### *Animals and diets*

A total of 16 male Wistar rats 7 weeks old were randomly divided into two groups: Control and MetR. All animals were caged individually and maintained in a 12:12 (light: dark) cycle at  $22 \pm 2^\circ\text{C}$  and  $50 \pm 10\%$  relative humidity in the pathogen free (high-efficiency particulate air [HEPA]-filtered) facilities of the Complutense University of Madrid (UCM) animal house. After 7 weeks of dietary treatment, rats were killed by decapitation, and brain and kidney samples were immediately processed to isolate functional kidney and brain mitochondria by appropriate specific methods, which were subsequently used to measure rates of oxygen consumption and ROS production under different conditions. The mitochondria remaining after these assays were quickly frozen in liquid nitrogen and maintained at  $-80^\circ\text{C}$  for later assays.

Semipurified diets prepared by MP Biochemicals (Irvine, CA) and imported to Spain by MP Biolink (Barcelona, Spain) were used in this investigation. The diets contained a mixture of amino acids instead of protein, and their precise composition is shown in Table 1. In the MetR diet, the amount of methionine was reduced by 40% in relation to the Control American Institute of Nutrition AIN93G diet, which contains 0.86% methionine. The absolute decrease in the amount of methionine in the restricted group (0.34%) was compensated for by increasing all the other dietary components in proportion to their presence in the diet. With this procedure, the amounts of all the dietary components, except methionine, were almost exactly the same in both experimental diets. Both diets had the same caloric content. The control group received the same amount of food every day that the MetR rats had eaten *ad libitum* as a mean the previous week (pair feeding). Therefore, the two groups ingested the same final amount of calories.

#### *Mitochondria isolation*

Rat brain and kidney functional mitochondria were obtained in parallel from fresh tissue from each rat by differential centrifugation, which, in the case of brain mitochondria, used discontinuous Ficoll gradients. One control and one MetR animal were processed simultaneously in parallel each day that measurements were performed. The isolated mitochondria were used within the next 2 h, during which they maintain their capacity for oxidative phosphorylation, to assay in parallel their rates of ROS generation and oxygen consumption, as well as to calculate the %FRL. This last parameter represents the efficiency of mitochondria to avoid univalent leak of electrons from the respiratory chain to generate ROS; the lower the %FRL, the higher such efficiency.

TABLE 1. DETAILED COMPOSITION OF THE SEMIPURIFIED DIETS USED IN THIS INVESTIGATION: CONTROL AND 40% METHIONINE RESTRICTED

Component	Control (g/100g)	MetR (g/100g)
L-Arginine	1.12	1.124
L-Lysine	1.44	1.445
L-Histidine	0.33	0.331
L-Leucine	1.11	1.114
L-Isoleucine	0.82	0.823
L-Valine	0.82	0.823
L-Threonine	0.82	0.823
L-Tryptophan	0.18	0.181
L-Methionine	0.86	0.516
L-glutamic acid	2.70	2.709
L-Phenylalanine	1.16	1.164
L-Glycine	2.33	2.338
Dextrine	5.00	5.017
Corn starch	31.80	31.93
Sucrose	31.80	31.93
Cellulose	5.0	5.017
Choline bitartrate	0.2	0.201
Vitamin mix AIN 93G	1.0	1.003
Mineral mix AIN 93G	3.5	3.512
Corn oil	8.0	8.28
Total	100	100

The brain was rinsed several times, chopped, and homogenized manually with a fitting glass pestle in 35 mL of isolation buffer (250 mM sucrose, 0.5 mM EDTA, 10 mM Tris-HCl, pH 7.4). The homogenate was centrifuged at 2000×g during 3 min in a Sorvall RC5C centrifuge; the pellet was discarded and the centrifugation protocol was repeated. The new supernatant was centrifuged at 12,500×g for 8 min to obtain the crude mitochondrial pellet. Nonsynaptic free mitochondria were obtained from this pellet by centrifugation in discontinuous Ficoll gradients according to the procedure of Lai and Clark.<sup>37</sup> The kidney was rinsed and homogenized in 30 mL of isolation buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EDTA, pH 7.35). Nuclei and cell debris were removed by centrifugation at 1000×g for 10 min in a Haeraeus Multifugue 1S-R centrifuge. The supernatant was centrifuged at 10,000×g for 10 min, and the resulting supernatant was eliminated. The pellet was resuspended in 20 mL of isolation buffer without EDTA and centrifuged at 1000×g for 5 min. Mitochondria were obtained after centrifugation of the supernatant at 10,000×g for 10 min. After each centrifugation step, any overlaying layer of fat was eliminated. The mitochondrial pellets were resuspended at high concentration in 1 mL of isolation buffer without EDTA. All of the above procedures were performed at 5°C. All of the homogenizations and resuspensions were performed inside a cold chamber ( $5 \pm 1^\circ\text{C}$ ). Mitochondrial protein concentration was measured by the Biuret method.<sup>38</sup> The final mitochondrial suspensions were maintained over ice and were immediately used for oxygen consumption and H<sub>2</sub>O<sub>2</sub> production measurements within 2 h after isolation.

#### *Mitochondrial oxygen consumption*

The rate of oxygen consumption from brain and kidney mitochondria was measured at 37°C in a water-thermostatized incubation chamber with a computer-controlled Clark-type O<sub>2</sub> electrode (Oxygraph, Hansatech, UK) in 0.5 mL of the same incubation buffer used for the H<sub>2</sub>O<sub>2</sub> measurements (see the next section). The substrates used in the assays were complex I-linked (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 adenosine diphosphate (ADP). The respiratory control ratio (RCR = state 3/state 4 oxygen consumption) with succinate + rotenone was 2.5±0.1 in control and 2.5±0.0 in MetR kidney mitochondria, and 1.5±0.1 in control and 1.6±0.1 in MetR nonsynaptic brain mitochondria. These RCR values indicated that the isolated mitochondria were functional (they had capacity to synthesize adenosine triphosphate [ATP] by oxidative phosphorylation).

#### *Mitochondrial hydrogen peroxide generation*

The rate of mitochondrial hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) 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<sub>2</sub>O<sub>2</sub> in the presence of horseradish peroxidase<sup>39</sup> with some modifications.<sup>40</sup> Reaction conditions were 0.25 mg of mitochondrial protein per mL, 6 U/mL of horseradish peroxidase, 0.1 mM homovanillic

acid, 50 Units/mL of superoxide dismutase, and 2.5 mM pyruvate/2.5 mM malate, 2.5 mM glutamate/2.5 mM malate, or 5 mM succinate with or without 2  $\mu$ M rotenone as substrates, added at the end (to start the reaction) to the incubation buffer (145 mM KCl, 30 mM HEPES, 5 mM KH<sub>2</sub>PO<sub>4</sub>, 3 mM MgCl<sub>2</sub>, 0.1 mM EGTA, 0.1% albumin, pH 7.4) at 37°C, in a total volume of 1.5 mL. Additional experiments with pyruvate/malate + 2  $\mu$ M rotenone, and with succinate + rotenone + 2  $\mu$ M antimycin A, were performed to assay maximum rates of complex I or complex III H<sub>2</sub>O<sub>2</sub> generation, respectively. 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, 2.2 M NaOH, 50 mM EDTA, pH 12),<sup>40</sup> and the fluorescence was read in a LS50B Perkin-Elmer fluorometer. Known amounts of H<sub>2</sub>O<sub>2</sub> generated in parallel by glucose oxidase with glucose as substrate were used as standards. Because the superoxide dismutase added in excess converts the superoxide radical (O<sub>2</sub><sup>•-</sup>) excreted by mitochondria (if any) to H<sub>2</sub>O<sub>2</sub>, the measurements represent the total (O<sub>2</sub><sup>•-</sup>) plus H<sub>2</sub>O<sub>2</sub> rate of mitROS production.

#### *Mitochondrial free radical leak*

The rates of H<sub>2</sub>O<sub>2</sub> production and O<sub>2</sub> consumption from brain and kidney mitochondria were measured in parallel in the same samples under similar experimental conditions. This allowed the calculation of the percentage of electrons out of sequence, which reduces O<sub>2</sub> to ROS at the respiratory chain (%FRL) instead of reaching cytochrome oxidase to reduce O<sub>2</sub> to water. Because two electrons are needed to reduce 1 mol of O<sub>2</sub> to H<sub>2</sub>O<sub>2</sub>, whereas four electrons are transferred in the reduction of 1 mol of O<sub>2</sub> to water, the %FRL was calculated as the rate of H<sub>2</sub>O<sub>2</sub> production divided by twice the rate of O<sub>2</sub> consumption, and the result was multiplied by 100.

#### *Oxidative damage to mitochondrial DNA*

Isolation of mtDNA was performed by the method of Latorre et al.<sup>41</sup> as adapted to mammals.<sup>42</sup> The isolated mtDNA was digested to deoxynucleoside level by incubation at 37°C with 5 Units of nuclease P1 (in 20  $\mu$ L of 20 mM sodium acetate, 10 mM ZnCl<sub>2</sub>, 15% glycerol, pH 4.8) for 30 min and 4 Units of alkaline phosphatase (in 20  $\mu$ L of 1 M Tris-HCl, pH 8.0) for 1 h.<sup>43</sup> Steady-state oxidative damage to mtDNA was estimated by measuring the level of 8-oxodG referred to that of the nonoxidized base (deoxyguanosine, dG). 8-OxodG and dG were analyzed by high-performance liquid chromatography (HPLC) with on-line electrochemical and ultraviolet detection, respectively. The nucleoside mixture was injected into a reverse-phase Teknokroma SEA18 column (5  $\mu$ m, 4.6 mm  $\times$  25 cm), and was eluted with a mobile phase containing 6% acetonitrile and 50 mM phosphate buffer, pH 5.0. A Gilson 305 pump at 0.9 mL/min was used. 8-OxodG was detected with an ESA Coulchem II electrochemical coulometric detector (ESA, Inc. Bedford, MA) with a 5011A analytical cell run in the oxidative mode (275 mV/20 nA), and dG was detected with a Biorad model 1806 UV detector at 254 nm. For quantification, peak areas of dG and pure 8-oxodG standards (Sigma) were analyzed during each HPLC run. 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 chromatography.

#### *Oxidative, glycoxidative, and lipoxidative damage to mitochondrial proteins*

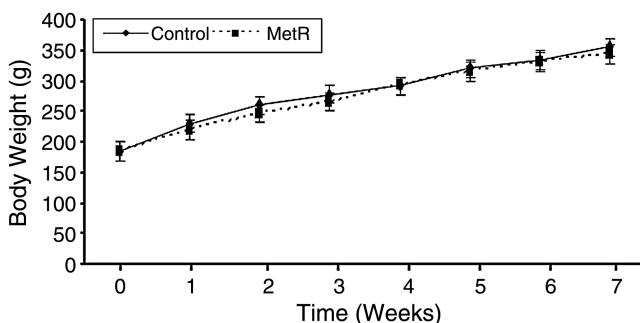
The markers of protein oxidation—the protein carbonyl glutamic (GSA) and amino adipic (AASA) semialdehydes, glycoxidation (carboxyethyl lysine [CEL] and carboxymethyl lysine [CML]), and lipoxidation (malondialdehyde lysine [MDAL] and CML) were determined by GC/MS. The trifluoroacetic acid methyl esters derivatives of these five markers were measured in acid hydrolyzed, delipidated, and reduced mitochondrial protein samples using an isotope dilution method as previously described<sup>44</sup> with a HP6890 Series II gas chromatograph (Agilent, Barcelona, Spain), a MSD5973A Series and a 7683 Series automatic injector, a HP-5MS column (30-m  $\times$  0.25-mm  $\times$  0.25-  $\mu$ m), and the described temperature program.<sup>44</sup> 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 [<sup>2</sup>H<sub>8</sub>]lysine, *m/z* 180 and 187, respectively; 5-hydroxy-2-aminovaleric acid and [<sup>2</sup>H<sub>5</sub>]5-hydroxy-2-aminovaleric acid (stable derivatives of GSA), *m/z* 280 and 285, respectively; 6-hydroxy-2-aminocaproic acid and [<sup>2</sup>H<sub>4</sub>]6-hydroxy-2-aminocaproic acid (stable derivatives of AASA), *m/z* 294 and 298, respectively; CML and [<sup>2</sup>H<sub>4</sub>]CML, *m/z* 392 and 396, respectively; CEL and [<sup>2</sup>H<sub>4</sub>]CEL, *m/z* 379 and 383, respectively; and MDAL and [<sup>2</sup>H<sub>8</sub>]MDAL, *m/z* 474 and 482, respectively. The amounts of product were expressed as the  $\mu$ molar ratio of GSA, AASA, CML, CEL, or MDAL/mol lysine.

#### *Measurement of mitochondrial protein electron transfer chain (ETC) complexes and AIF*

The protein contents of brain and kidney mitochondrial respiratory chain complexes, and AIF were estimated using western blot analyses, as previously described.<sup>44</sup> Because complexes I<sup>33,34,45</sup> and III<sup>46</sup> are the respiratory chain components responsible for mitROS generation, two different subunits of these complexes were used to better corroborate their possible changes in abundance. Immunodetection was performed using monoclonal specific antibodies for the 39-kDa (NDUFA9) and 30-kDa (NDUFS3) subunit of complex I (1:1,000 in both cases), 70-kDa subunit (Flavoprotein) of complex II (1:500), 48-kDa (CORE 2) and 29-kDa (Rieske iron-sulfur protein) subunits of complex III (1:1,000 in both cases), and COXI subunit of complex IV (1:1,000) (Molecular Probes, Invitrogen Ltd., UK) and a polyclonal antibody for the carboxyl terminus (amino acids 593–613) of AIF anti-AIF (1:1,000, Sigma, Madrid, Spain). An antibody to porin (1:15,000, Molecular Probes) was also used to refer the proportion of those proteins to total mitochondrial mass. Appropriate peroxidase-coupled secondary antibodies and chemiluminescence horse-radish peroxidase substrate (Millipore, MA) were used for primary antibody detection. Signal quantification and recording was performed with a ChemiDoc equipment (Bio-Rad Laboratories, Inc., Barcelona, Spain). Protein concentration was determined by the Bradford method.

#### *Fatty acid analyses*

Fatty acyl groups from brain and kidney mitochondrial lipids were analyzed as methyl esters derivatives by GC/MS,



**FIG. 1.** Total body weight of rats during the 7 weeks of dietary treatment in the control and (40%) methionine restriction (MetR) groups. No significant differences between dietary groups were found at any of the 7 weeks of experimentation. Values are means  $\pm$  standard error of the mean (SEM) from 6–8 animals per group.

as previously described.<sup>44</sup> Separation was performed in a SP2330 capillary column ( $30\text{ m}\times 0.25\text{ mm}\times 0.20\text{ }\mu\text{m}$ ) in a GC Hewlett Packard 6890 Series II gas chromatograph (Agilent, Barcelona, Spain). A Hewlett Packard 5973A mass spectrometer was used as detector in the electron-impact mode. 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 indexes 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 (PUFA*n*-3 and PUFA*n*-6); average chain length (ACL) =  $[(\Sigma\%\text{Total}14\times 14) + (\Sigma\%\text{Total}16\times 16) + (\Sigma\%\text{Total}18\times 18) + (\Sigma\%\text{Total}20\times 20) + (\Sigma\%\text{Total}22\times 22)]/100$ ; double bond index (DBI) =  $[(1\times \Sigma\%\text{monoenoic}) + (2\times \Sigma\%\text{dioenoic}) + (3\times \Sigma\%\text{trienoic}) + (4\times \Sigma\%\text{tetraenoic}) + (5\times \Sigma\%\text{pentaenoic}) + (6\times \Sigma\%\text{hexaenoic})]$ , and peroxidizability index (PI) =  $[(0.025\times \Sigma\%\text{monoenoic}) + (1\times \Sigma\%\text{dioenoic}) + (2\times \Sigma\%\text{trienoic}) + (4\times \Sigma\%\text{tetraenoic}) + (6\times \Sigma\%\text{pentaenoic}) + (8\times \Sigma\%\text{hexaenoic})]$ .

#### Statistical analyses

All values were expressed as means  $\pm$  standard error of the mean (SEM). Comparisons between control and MetR animals were analyzed statistically with Student *t*-tests in

mitochondria from each organ, brain, or kidney. The minimum level of statistical significance was set at  $p < 0.05$  in all of the analyses.

#### Results

At the start of the experiment, the mean body weight was identical in both dietary groups. MetR did not affect the gain in body weight nor the final body weight after the 7 weeks of experimentation (Fig. 1). The weight of the liver, heart, spleen, kidney, or brain was not significantly modified by MetR either (results not shown).

The rate of oxygen consumption from kidney and non-synaptic brain mitochondria was measured in the absence (state 4) and in the presence (state 3) of  $500\text{ }\mu\text{M}$  ADP, with complex I-linked (pyruvate/malate for brain and glutamate/malate for kidney mitochondria, respectively) and complex II-linked (succinate + rotenone) substrates. In brain mitochondria, MetR induced a significant increase in the oxygen consumption in state 3 both with complex I- and complex II-linked substrates, whereas state 4 values did not change (Table 2). No significant changes in oxygen consumption were observed in kidney mitochondria.

MetR significantly decreased the basal rates of  $\text{H}_2\text{O}_2$  production (Fig. 2) from kidney (62% decrease) and brain (67% decrease) mitochondria with complex I-linked substrates (pyruvate/malate for brain and glutamate/malate for kidney mitochondria) compared to the control group, but did not significantly change them with the complex II-linked substrate (succinate + rotenone). When the assays with succinate as substrate were performed in the absence of rotenone, which allows the occurrence of reverse flow of electrons from succinate-complex II to complex I, the absolute levels of  $\text{H}_2\text{O}_2$  production were 2.1- to 2.7-fold higher (brain) and 1.7-fold higher (kidney) than in the assays performed with succinate + rotenone, but there were no significant differences between the MetR and control groups in any kind of mitochondria (Fig. 2).

Mitochondrial  $\text{H}_2\text{O}_2$  production was also assayed in the presence of the respiratory chain inhibitors rotenone or antimycin A to estimate the maximum rates of mitROS generation when the ROS-generating respiratory complex I (pyruvate/malate + rotenone or glutamate/malate + rotenone) or complex III (succinate + rotenone + antimycin A) are fully reduced. No significant differences in maximum

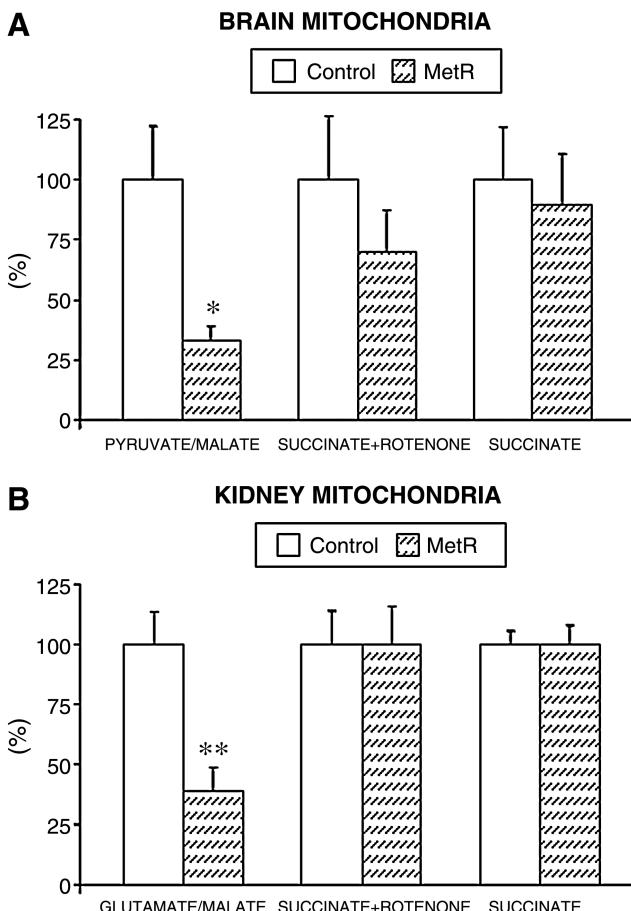
**TABLE 2. RATES OF OXYGEN CONSUMPTION OF BRAIN AND KIDNEY MITOCHONDRIA FROM CONTROL AND 40% METHIONINE RESTRICTED RATS**

Substrate added	State	Brain mitochondria		Kidney mitochondria	
		Control	MetR	Control	MetR
Complex I-linked substrate	4	$12.0 \pm 1.2$	$13.0 \pm 1.4$	$24.9 \pm 2.5$	$29.2 \pm 2.0$
	3	$22.7 \pm 1.5$	$28.2 \pm 2.3^a$	$80.5 \pm 7.0$	$80.8 \pm 10.0$
Complex II-linked substrate (succinate + rotenone)	4	$15.5 \pm 1.7$	$17.6 \pm 0.9$	$76.7 \pm 3.9$	$76.8 \pm 5.3$
	3	$20.3 \pm 0.8$	$28.0 \pm 2.9^a$	$166.2 \pm 21.4$	$190.6 \pm 13.7$

Values are mean  $\pm$  SEM (nmoles  $\text{O}_2$  /min  $\times$  mg protein) from 8 different animals. Complex I-linked substrate: Pyruvate-malate for brain mitochondria and glutamate-malate for kidney mitochondria. State 4: Oxygen consumption in the absence of ADP. State 3: Oxygen consumption in the presence of  $500\text{ }\mu\text{M}$  adenosine triphosphate (ADP).

<sup>a</sup> $P < 0.05$ : Significant differences between Control and MetR rats.

Abbreviations: MetR, Methionine restriction; SEM, standard error of the mean.

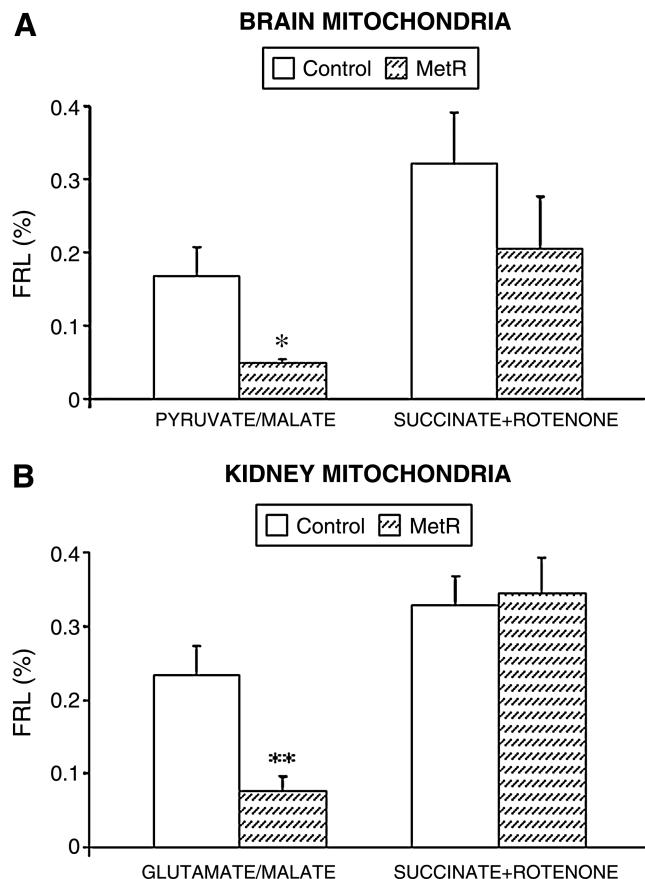


**FIG. 2.** Rates of reactive oxygen species (ROS) production of brain (A) and kidney (B) mitochondria from control and (40%) methionine restriction (MetR) rats. Values are means  $\pm$  standard error of the mean (SEM) from 6–8 animals different animals and are expressed as percentage of those in the controls (100%) for each substrate and tissue. Control values in brain mitochondria (nmoles H<sub>2</sub>O<sub>2</sub>/ min  $\times$  mg protein): 0.036  $\pm$  0.008 (pyruvate/malate); 0.095  $\pm$  0.025 (succinate + rotenone); 0.202  $\pm$  0.045 (succinate). Control values in kidney mitochondria: 0.105  $\pm$  0.014 (glutamate-malate); 0.499  $\pm$  0.07 (succinate + rotenone); 0.841  $\pm$  0.047 (succinate). Asterisks represent significant differences between control and MetR rats: (\*)  $p < 0.05$ , (\*\*)  $p < 0.01$ .

ROS production between dietary groups were found for both kinds of mitochondria supplemented with the two types of substrates (results not shown).

Methionine restriction induced a statistically significant decrease in the percentage of total electron flow in the respiratory chain directed to ROS generation (%FRL) in rat brain and kidney mitochondria with complex I-linked substrates but not with succinate + rotenone (Fig. 3). These decreases reached 71% in brain and 68% in kidney mitochondria, respectively.

Restriction of dietary methionine decreased the amounts of the peptides belonging to the mitochondrial respiratory complex I (peptide subunits NDUFS3 and NDUFA9), complex III (Rieske iron-sulfur and CORE II subunits) and complex IV (COX-I), and AIF, in brain mitochondria, whereas the trend to decrease (by 28.2%) observed in the case of complex



**FIG. 3.** Free radical leak (FRL%) of brain (A) and kidney (B) mitochondria from control and (40%) methionine restriction (MetR) rats in the presence of pyruvate/malate or succinate + rotenone as substrates. The FRL% is the percentage of electrons out of sequence which reduce O<sub>2</sub> to reactive oxygen species (ROS) at the respiratory chain instead of reaching cytochrome oxidase to reduce O<sub>2</sub> to water. It represents the efficiency of the mitochondria in avoiding the univalent lateral leak of electrons out of the respiratory chain that generates ROS. The lower the FRL%, the higher such efficiency. Values are means  $\pm$  standard error of the mean (SEM) from 6–8 animals (different animals). Asterisks represent significant differences between control and MetR rats: (\*)  $p < 0.05$ , (\*\*)  $p < 0.01$ .

II (flavoprotein subunit) did not reach statistical significance ( $p < 0.078$ ; Table 3). In kidney mitochondria, however, complex IV (COX-I subunit) significantly decreased in the MetR group, whereas the analyzed subunits corresponding to complexes I, II, and III, and AIF did not significantly differ between the Control and MetR groups (Table 3).

The steady-state level of the oxidative damage marker 8-oxodG in mtDNA was significantly lower (18% decrease) in the kidney from the MetR animals than in the Controls (Fig. 4). In the brain, however, the trend to decreased (by 14%) 8-oxodG levels in the MetR group did not reach statistical significance ( $p < 0.101$ ; Fig. 4).

Concerning the three general kinds of oxidative modification of mitochondrial proteins, the levels of all the five different oxidative (the specific protein carbonyls GSA and AASA, glycoxidative (CEL and CML), and lipoxidative

TABLE 3. AMOUNTS OF RESPIRATORY CHAIN COMPLEXES AND AIF IN BRAIN AND KIDNEY MITOCHONDRIA FROM CONTROL AND (40%) METR RATS

	Brain mitochondria		Kidney mitochondria	
	Control	MetR	Control	MetR
Complex I (30-kDa subunit, NDUFS3)	100 ± 8.56	75.11 ± 4.75*	100 ± 15.63	90.82 ± 4.44
Complex I (39-kDa subunit, NDUFA9)	100 ± 5.93	60.99 ± 4.34***	100 ± 11.36	95.96 ± 15.97
Complex II (70-kDa subunit, Flavoprotein)	100 ± 11.56	71.82 ± 4.44	100 ± 11.05	104.06 ± 11.51
Complex III (29.6-kDa subunit, Rieske iron-sulfur protein)	100 ± 6.15	79.87 ± 2.59**	100 ± 2.91	96.56 ± 5.68
Complex III (48.5-kDa, CORE II)	100 ± 7.80	80.98 ± 3.48*	100 ± 15.48	94.96 ± 8.06
Complex IV (57-kDa subunit, COXI)	100 ± 13.60	59.07 ± 14.31*	100 ± 6.88	50.71 ± 5.39***
AIF	100 ± 10.19	66.76 ± 2.23**	100 ± 5.67	93.42 ± 6.66

Values are means ± SEM from  $n=5$ -7 different animals. Units: Ratio complex I, II, III, IV, and AIF/porin in arbitrary units. Significant differences between control and MetR rats: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

Abbreviations: AIF, Apoptosis-inducing factor; MetR, methionine restriction; SEM, Standard error of the mean.

(CML and MDAL) markers analyzed were significantly decreased by methionine restriction in both brain (Fig. 5A) and kidney mitochondria (Fig. 5B). The mean magnitude of all these decreases taken together was 34.0% in brain and 34.6% in kidney mitochondria.

The full fatty acid composition from kidney and brain mitochondrial lipids was also measured (results not shown). MetR did not change the amount of any individual fatty or any general class of fatty acids in kidney mitochondria. In brain mitochondria, only 16:1n-7 showed a significant increase (0.20% in absolute terms;  $p < 0.05$ ) in the MetR group. Neither the total amount of saturated, monounsaturated, or polyunsaturated fatty acids, nor their average chain length, were significantly affected by the MetR treatment. The total number of double bonds (DBI, double bond index) and the peroxidizability index (PI) of all the fatty acids taken together were not significantly affected by the MetR dietary regime in

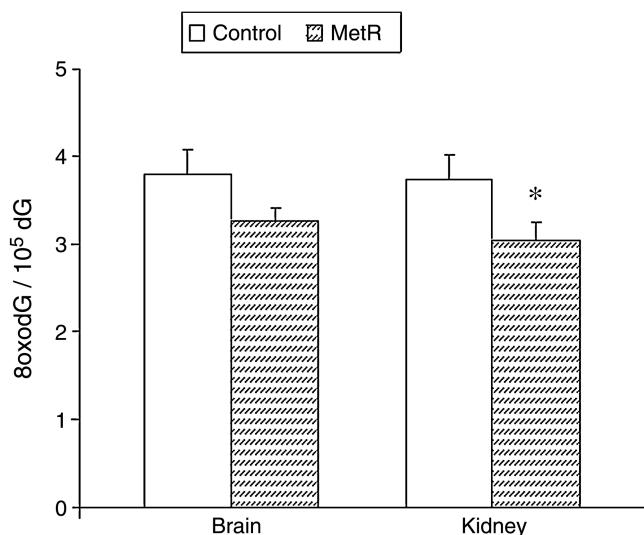


FIG. 4. Oxidative damage to mitochondrial DNA (8-oxodG/ $10^5$  deoxyguanosine [dG]) in brain and kidney from control and (40%) methionine restriction (MetR) rats. Values are means ± standard error of the mean (SEM) from 6-8 animals (different animals). Asterisks represent significant differences between control and MetR rats: (\*)  $p < 0.05$ .

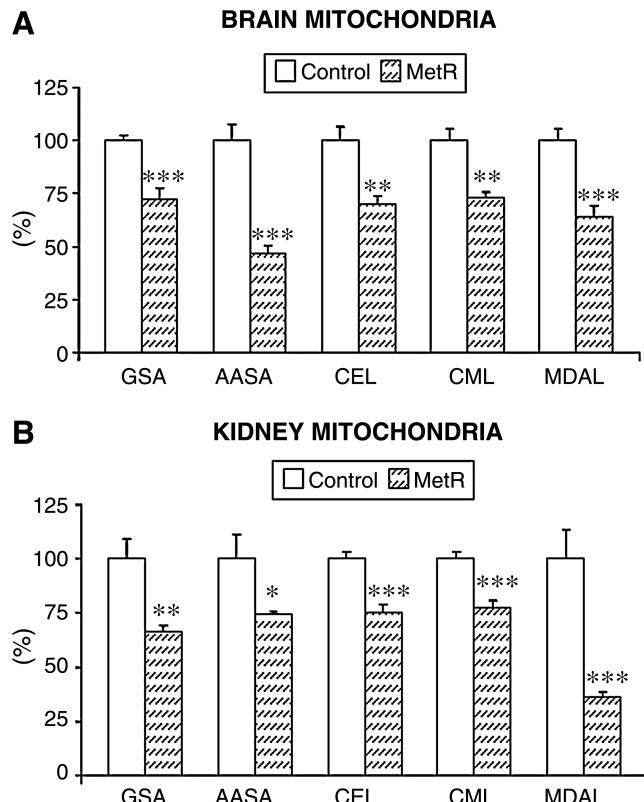


FIG. 5. Protein modification markers in brain (A) and kidney (B) mitochondria from control and (40%) methionine restriction (MetR) rats. Values are means ± standard error of the mean (SEM) from  $n=8$  different animals and are expressed as percentage of those in the controls (100%) for each parameter. Control values in brain mitochondria: 9933.62 ± 238.67 (glutamic semialdehyde, GSA); 277.11 ± 20.47 (aminoacidic semialdehyde, AASA); 596.12 ± 38.74 (carboxyethyl lysine, CEL); 670.22 ± 37.95 (carboxymethyl lysine, CML); 571.75 ± 30.98 (malondialdehyde lysine, MDAL). Control values in kidney mitochondria: 5603.40 ± 509.03 (GSA); 301.19 ± 32.94 (AASA); 531.25 ± 18.44 (CEL); 744.52 ± 25.84 (CML); 450.26 ± 60.42 (MDAL). Units:  $\mu\text{mol/mol}$  lysine. Asterisks represent significant differences between control and MetR rats: (\*)  $p < 0.05$ , (\*\*)  $p < 0.01$ , (\*\*\*)  $p < 0.001$ .

rat kidney or brain mitochondria. The DBI values were  $154.83 \pm 2.01$  in control and  $158.67 \pm 1.83$  in MetR brain mitochondria, and  $181.44 \pm 4.82$  in control and  $176.17 \pm 6.20$  in MetR kidney mitochondria. The PI values were  $150.30 \pm 3.11$  in control and  $155.52 \pm 2.68$  in MetR brain mitochondria, and  $159.10 \pm 4.22$  in control and  $153.21 \pm 5.77$  in MetR kidney mitochondria.

## Discussion

This investigation reports for the first time that 7 weeks of 40% methionine DR decreases mitROS generation and %FRL at complex I during forward electron flow and lowers oxidative damage to mtDNA and proteins in rat kidney and brain nonsynaptic mitochondria without changing the degree of unsaturation of their membrane fatty acids.

Methionine restriction without calorie restriction increases maximum longevity in rats<sup>20–23</sup> and mice,<sup>23,24</sup> analogously to protein restriction in rodents (reviewed in refs. 12 and 13) and insects,<sup>9,10</sup> and to DR in all kinds of species tested so far, including yeast, nematodes, insects, spiders, fishes, or rodents (reviewed in ref. 6). Recent results obtained after 20 years of experimentation in the Wisconsin primate study strongly suggest that moderate (30%) DR most likely increases maximum longevity also in a nonhuman primate, *Macacus rhesus*.<sup>7</sup> In that study moderate DR increased monkey survival from 50% to 85%, delayed the onset of cancer, cardiovascular diseases, and grey matter loss in the brain, and totally suppressed type II diabetes at the most recent time point available.<sup>7</sup> Although largely overlooked for a long time, recent reevaluation<sup>12,13</sup> of the 18 independent long-life longevity experiments available in rats and mice, which include the full survival curves, indicates that protein restriction in rodents also extends life span and the increases in maximum longevity are up to 50% of those exerted by DR.

Restriction of methionine, an essential amino acid, in the diet, also decreases or delays many detrimental changes related to aging and degenerative diseases in rodents. Thus, 80% MetR without calorie restriction induces a strong decrease in visceral fat mass (46% in relative and 72% in absolute terms), prevents partially (by 73%) or totally the age-related increases in blood cholesterol or triglycerides, respectively, lowers the levels of plasma glucose (by 14%), insulin (by 86%), and IGF-I (by 66%), and improves insulin response to an oral glucose challenge (by 70%) in Fisher 344 rats.<sup>26</sup> Eighty percent MetR without calorie restriction in BALB/cJ × C57BL/6J F1 mice lowers plasma glucose, insulin, the thyroid hormone T<sub>4</sub>, and IGF-1, slows down cataract development, minimizes age-related changes in T cells, increases the liver mRNA of the macrophage inhibition factor (which is elevated in other long-lived mice), increases resistance to acetaminophen-induced liver damage, and increases maximum life span.<sup>23</sup> MetR also inhibits tumor development in azoxymethane-induced colon cancers in rats.<sup>27</sup>

In the present investigation, 40% MetR did not affect the body weight gain or the final body and organ weights of the animals. This agrees with a previous study focused on liver tissue,<sup>28</sup> whereas 80% MetR in two different studies strongly decreased the gain in body weight of the growing rats and their final body size after the same period of dietary intervention used here.<sup>11,28</sup> This can be relevant for possible future extrapolation of the MetR regimen to humans because

the beneficial decreases in mitROS generation and oxidative damage to mitochondrial DNA proteins observed in our study are already obtained with 40% MetR, which does not affect body weight gain and the final size of the animals. Therefore, it is not necessary to use the more strongly restrictive 80% MetR or 40% DR regimes, which deeply inhibit animal growth and maturation, to obtain beneficial decreases in mitROS production and mitochondrial oxidative stress. If the same results occur in humans, 40% MetR could generate the same benefits in children without affecting their final adult height, in contrast with the decrease in adult stature, delayed maturation, and diminished fecundity expected from the hypothetical application of standard (40%) DR to growing human beings.

The presence of beneficial effects already at 40% MetR, while for some parameters like body growth 80% MetR can be detrimental, also seems to apply to the mitochondrial respiration rate. In the present investigation 40% MetR increased brain mitochondrial oxygen consumption in state 3, but not in state 4, both with complex I-linked (24% increase) and with complex II-linked (38% increase) substrates. In kidney mitochondria only a nonsignificant trend to increase (by 15%) was observed with succinate + rotenone in state 3. This suggests that the brain mitochondria of the 40% MetR animals have an increased capacity for ATP production during oxidative phosphorylation (in state 3 respiration), without increasing state 4 oxygen consumption, which would lower their efficiency of respiratory coupling and biological energy generation through oxidative phosphorylation. In contrast, in a previous investigation we found that 80% MetR (substituting methionine for glutamate in the diet) significantly increases both state 3 and 4 respiration with complex I- and complex II-linked substrates in rat liver and heart mitochondria, although the increases with succinate were only marginally significant in the case of rat heart.<sup>11</sup> The idea that those increases in state 4 respiration could reflect some degree of mitochondrial uncoupling is reinforced by the finding that 80% MetR causes an ~85% increase in the amount of the uncoupling protein UCP4 in total rat brain tissue.<sup>30</sup> However, in a previous study in rat liver, no significant changes were detected for state 3 or 4 respiration with any substrate in either 40% or 80% MetR.<sup>28</sup> In this last study, as in the present investigation, methionine was the only dietary factor that significantly varied between the control and the experimental diets, whereas in our first 80% MetR study in liver and heart, methionine was substituted for glutamate in the diet.<sup>11</sup> In summary, the evidence available suggests that 40% MetR causes improvements in brain mitochondrial oxidative phosphorylation capacity whereas, in contrast, 80% MetR (substituting methionine for glutamate in the diet) can cause cardiac and hepatic mitochondrial uncoupling, although this cannot be generalized to other organs until more information is obtained.

Many previous investigations performed during the last two decades have shown that the rate of mitROS generation is the main parameter known to link longevity with oxidative stress, both in comparative studies among homeothermic vertebrates with different longevities<sup>1,2</sup> and in DR rodent models.<sup>8</sup> In the present investigation, we show that 40% MetR is sufficient to strongly decrease the rate of mitROS production (62% decrease in kidney mitochondria and 67% decrease in brain mitochondria) and the %FRL (68% decrease

in kidney mitochondria and 71% decrease in brain mitochondria). Therefore, it is not necessary to reach the much higher level of 80% MetR used in our previous investigations in rat brain,<sup>30</sup> heart, and liver<sup>11</sup> to obtain those beneficial changes, at least in the case of rat kidney or brain mitochondria (this investigation) or liver mitochondria.<sup>28</sup> The decrease in mitROS generation occurred in mitochondria from both tissues exclusively at complex I, because it was observed with complex I-linked substrates (pyruvate/malate or glutamate/malate) but not with the complex II-linked one (succinate + rotenone). Under this last condition, electrons flow from succinate complex II to complex III, but not back to complex I due to the presence of the rotenone block between the ubiquinone pool and this last complex. In mitochondria respiring with complex I-linked substrates, basal ROS generation comes from both complex I and complex III, whereas with succinate + rotenone only complex III produces ROS. Because the decrease in mitROS induced by MetR occurred only with complex I-linked substrates but not with succinate + rotenone, ROS production must come exclusively from complex I under state 4 respiration. Therefore it can be concluded that among the two well-known respiratory complexes responsible for mitROS production, complex I<sup>33,34</sup> and complex III,<sup>46,47</sup> the decrease induced by MetR occurs exclusively at complex I.

Results from a previous investigation indicated that the electrons follow two different paths when they flow within complex I<sup>48</sup>: (1) They follow one pathway when they flow in the more physiological forward direction (from complex I-linked substrates to ubiquinone); and (2) they follow another different pathway during reverse electron flow (when they flow back to complex I from succinate-complex II). Our results fit with that possibility because mitROS generation decreased in MetR during forward electron flow through complex I (with complex I-linked substrates alone), it did not change in MetR when the substrate used was succinate in the absence of rotenone. In this last condition, part of the electrons coming from complex II flow back to complex I, with the rest of them proceeding forward to complex III. Therefore, our results indicate that the decrease in complex I mitROS generation induced by 40% MetR in rat brain mitochondria occurs at a site localized in the forward but not in the reverse electron path within complex I.

Interestingly, the decrease in the rate of mitROS generation induced by MetR and its main sites and mechanisms of occurrence are extremely similar to the previously observed ones concerning 40% protein restriction and 40% DR in rats, as well as, in many cases, to the differences observed between long-lived and short-lived mammals and birds.<sup>1,2,33,34,49</sup> Decreases in mitROS generation exclusively at complex I and lowering of %FRL have been found in DR in many different rat organs,<sup>8</sup> in 40% protein restriction in rat liver,<sup>17</sup> and in 80% MetR in rat liver and heart,<sup>11,28</sup> and in 40% MetR in rat liver.<sup>28</sup> On the other hand, 40% restriction of all the different amino acids needed in the diet (except methionine) did not change the rate of ROS generation of rat liver mitochondria.<sup>29</sup> All of this taken together demonstrates that methionine is the single dietary factor responsible for the decrease in mitROS generation and %FRL at complex I observed during protein restriction and DR in rat liver, and likely during DR in kidney, brain, and possibly the heart and other rat organs.

In addition to the decrease in mitROS production, the mitochondrial electron transport chain of MetR rats was much more efficient than that of the controls in avoiding the univalent electron leak that generates oxygen radicals, because the %FRL was strongly decreased at complex I in both kidney and brain MetR mitochondria. The same was found in DR<sup>35,36</sup> and in protein-restricted rats<sup>17</sup> as well as in long-lived mammals or birds when compared to shorter-lived ones.<sup>1,2,33,34</sup> Therefore, the rate of mitROS generation per unit oxygen consumed is low in all of these different kinds of animals showing high longevity. Lowering the FRL% is a very convenient mechanism because it can increase longevity without the need to limit mitochondrial oxygen consumption and then the metabolic rate and the general level of activity.

It is worth emphasizing that the basal conditions in which the decrease in mitROS generation induced by MetR was observed in this investigation are more physiological than the measurements performed with substrate plus inhibitor combinations. These last conditions strongly stimulate mitROS production by blocking the respiratory chain at specific sites. It is also interesting that, differing from the basal rates, the maximum rates of mitROS generation coming from fully electronically reduced complex I (assayed with complex I-linked substrates plus rotenone) or complex III (assayed with succinate + rotenone + antimycin A) were not modified by MetR. This indicates that the decreases in mitROS generation cannot be simply due to decreases in the amount of the respiratory complexes containing ROS generation sites and that qualitative changes in complex I affecting the final rate of ROS production must be involved. Estimating the amount of the different respiratory multiprotein complexes was more directly addressed by measuring the amounts of particular peptides of each complex. We found significant decreases in the amount of complex I, complex III, and complex IV in brain mitochondria of MetR rats, whereas only complex IV was decreased in kidney mitochondria.

Previously, we have found that pigeons, which have a maximum longevity 8- to 9-fold higher than rats in spite of their similar metabolic rates and body weights, also have a lower rate of mitROS generation at complex I in different vital organs when compared to those rodents, and the same occurs when comparing canaries or parakeet to mice<sup>2,33,34,49-51</sup> (maximum longevity is 6- to 7-fold higher in these two birds than in the mouse). This has been attributed to the presence of smaller amounts of complex I protein in the pigeon than in the rat,<sup>52</sup> whereas canaries and parakeets also have lower amounts of complex I than mice.<sup>53</sup> On the other hand, the FRL% at complex I is also lower in parakeet than in mouse heart mitochondria<sup>51</sup> and in pigeon than in rat lung, heart, and brain mitochondria.<sup>33,34,50</sup> This means that the quantitatively smaller amounts of complex I can not fully explain the lower rates of complex I mitROS generation of these birds and qualitative differences of bird mitochondria must be also involved. A lower amount of various respiratory complexes, always including complex I, has been found also in DR in rat liver mitochondria,<sup>54</sup> in every other day feeding in mouse liver mitochondria,<sup>44</sup> in protein restriction in rat liver mitochondria,<sup>55</sup> in MetR in rat heart and liver mitochondria and brain tissue,<sup>11,28,30</sup> and in MetR in kidney and brain mitochondria (this investigation). Such a decrease,

although it cannot be the only causal factor, can contribute to explaining the lower complex I ROS generation of these animal models.

However, our results do not allow us to conclude that the decrease in the amount of respiratory complexes is a main general factor responsible for the decreases observed in ROS production in MetR mitochondria in our investigation, because in kidney mitochondria the amounts of the two complex I subunits measured did not change, whereas mitROS production also decreased in this organ. In addition, in brain mitochondria, we observed a significant decrease in the amount of complex III, but ROS production from this complex (with succinate + rotenone) did not show significant changes. When the sites of ROS production are fully reduced, it is expected that mitROS generation will be mainly dependent on the amount of respiratory complexes containing ROS generation sites. However, in spite of the decreases in the amount of complexes I and III in brain mitochondria, the maximum rates of mitROS generation at complex I (measured with complex I-linked substrates + rotenone) and at complex III (measured with succinate + rotenone + antimycin A) did not change in MetR. Nevertheless, although it cannot be the only factor, the decrease in the amount of complex I in the brain mitochondria of MetR animals could have contributed to lower the basal rate of mitROS generation of this complex, although not the %FRL as discussed below.

In relation to the decrease in complex I, it is interesting that the AIF was lowered by 33% in brain mitochondria from MetR rats in the present investigation. AIF is a mitochondrial flavoprotein involved in the assembly/maintenance of complex I. AIF, in addition to apoptotic functions, is also required for mitochondrial oxidative phosphorylation.<sup>32</sup> In particular, AIF-deficient cells and mice show lower amounts of complex I,<sup>31</sup> which indicates a role for AIF in the biogenesis or maintenance of this complex. Therefore, it is possible that the decrease observed in the amount of AIF in brain mitochondria could have contributed to lower their amount of complex I. Also in agreement with the existence of a relationship between AIF and complex I levels, MetR did not change either the amount of complex I or AIF levels in kidney mitochondria. Simultaneous decreases in the amounts of complex I and AIF have been previously observed in 40% and 80% MetR in rat liver<sup>28</sup> and in total brain tissue from 80% MetR rats.<sup>30</sup> In the case of total brain tissue, the trend to decreased AIF levels did not reach statistical significance,<sup>30</sup> but the degree of MetR implemented was much more intense (80%) than that used here, and the experimental design substituted methionine for glutamate in the diet, whereas in the present investigation methionine was the only dietary component significantly differing (by 40%) between the control and the MetR diets.

The %FRL was lowered in the MetR groups only with complex I-linked substrates in both kidney and brain mitochondria, not with succinate + rotenone. This means that the decrease in %FRL, like that in ROS generation, occurs only at complex I. Such a decrease in %FRL indicates that the mitochondria isolated from MetR animals are qualitatively different. They avoid ROS generation more efficiently because they produce less oxygen free radicals per unit of electron flow. Those qualitative changes induced by MetR must be involved in the decrease in complex I ROS production observed. The decrease in the amount of complex I protein can

contribute to lower total mitROS production but can not explain the lower %FRL.

What is the nature of those qualitative changes in complex I? The results of this investigation indicate that a lowering of the degree of electronic reduction of complex I can be involved because the decrease in mitROS generation occurred with complex I-linked substrates alone, when complex I is partially reduced, but not with complex I-linked substrates in the presence of rotenone. In this last condition, complex I is fully reduced by the electrons accumulated inside the multiprotein due to the rotenone block near the site of exit of the electrons from the complex. Therefore, qualitative changes in MetR mitochondria could decrease the degree of electronic reduction of the complex I ROS generator during state 4 respiration.

What is the mechanism responsible for that qualitative mitochondrial change? One possibility is that MetR also modifies mitochondria indirectly through changes in DNA methylation *in vivo* that are known to modify the degree of expression of many different genes. Many microarray studies have shown that DR (which necessarily includes MetR) modifies the expression of a very large number of genes<sup>56</sup> and some of them can be involved in the qualitative changes observed at mitochondrial level in this investigation. This possibility would be consistent with the recent observation that MetR and DR decrease p-JNK and p-AKT signaling, whereas DR but not MetR decreases m-TOR, p-4EBP1b, p-P38, and p-ERK1/2 signaling in CB6F1 mice.<sup>24</sup> Changes in the expression of some complex I regulatory subunits, or in the levels of metabolites finally modifying the subunits of this complex, could increase the midpoint redox potential of the complex I generator sites, thus decreasing its tendency to donate electrons univalently to oxygen. This will not only lower complex I ROS production but could also decrease the FRL%.

Further research, including the measurement of changes in global DNA methylation and wide-scale microarray mRNA studies in rats subjected to 40% MetR, are necessary to clarify these possibilities. Two main changes could finally decrease mitROS generation in MetR: (1) Qualitative changes in complex I likely due to variations in the expression of many specific genes during chronic MetR; (2) quicker changes in cellular/mitochondrial concentrations of methionine, homocysteine, or other related metabolites secondary to the lower methionine intake. Both kinds of changes could act additively or even synergically *in vivo* during the chronic MetR treatment, in such a way that the final decrease in complex I mitROS production *in vivo* could be even stronger than the one observed in this investigation.

Previous investigations have shown that the lowering of mitROS production induced by long-term DR,<sup>36,57</sup> protein restriction,<sup>17</sup> or MetR<sup>11,28,30</sup>, is always accompanied by decreases in the steady-state level of 8-oxodG in mtDNA in rat tissues, whereas when mitROS production does not change (e.g., during short or medium term DR in rat heart) neither does 8-oxodG.<sup>36,58</sup> The reason why mtDNA oxidative damage correlates better with mitROS generation than oxidative damage to other cellular macromolecules like proteins has been attributed<sup>59</sup> to the fact that mtDNA is situated close to or even in contact with<sup>60</sup> the inner mitochondrial membrane, very near to where mitROS are formed. This contact could help to explain why antioxidants are not able to decrease local ROS levels at those critical sites and therefore do not

increase maximum longevity.<sup>2</sup> In agreement with previous studies of 80% MetR in rat heart, brain and liver,<sup>11,30</sup> as well as with those of 40% MetR in rat liver,<sup>28</sup> 40% MetR decreased 8-oxodG in rat kidney (by 18%) and rat brain (by 14%) mtDNA in this investigation, although in the brain case the trend to decrease did not reach statistical significance ( $p < 0.10$ ). These results, together with previous ones,<sup>17,29</sup> are consistent with the hypothesis that the decreased ingestion of methionine is responsible for the decrease in mitROS generation and oxidative damage to mtDNA that occurs during DR and protein restriction. Because ROS can produce DNA single- and double-strand breaks and 8-oxodG is mutagenic,<sup>61</sup> MetR could contribute to extend life span by helping to decrease the formation rate of mtDNA mutations that occurs during rodent and human aging,<sup>1,62</sup> and perhaps also lowering the age-related accumulation in nuclear DNA of inserted mtDNA fragments coming from mitochondria during the life span of the rat.<sup>63,64</sup>

An overall decrease in the oxidative, lipoxidative, and glycoxidative damage to brain and kidney mitochondrial proteins (by 22–64%) was also found in MetR rats in our study. This also agrees with the lower rate of mitROS generation observed. Similar decreases in these markers of protein modification have been observed in various rat organs during DR.<sup>13</sup> However, in medium-term DR (4 months) investigations in rat heart, all of these markers of protein oxidative damage decreased whereas mitROS generation was not changed.<sup>65</sup> This suggests that other factors varying in DR, like an increase in protein catabolism,<sup>66</sup> or other proposed hypothetical mechanisms related to protein turnover<sup>67</sup> must be also involved in the decreases in protein oxidative modifications. In any case, the decrease in mitochondrial protein oxidative damage in MetR animals can have important beneficial effects.

The stronger relative decreases were observed for MDAL in kidney mitochondria (64% decrease) and for AASA in brain mitochondria (53% decrease), while the largest decrease in absolute levels corresponded to GSA in both cases due to the much higher abundance of this protein oxidation marker. The decreases in the highly specific protein carbonyls GSA and AASA observed in our study show that oxidative damage to kidney and brain mitochondrial proteins is lowered in rats subjected to 40% MetR. On the other hand, the decrease in CML and MDAL indirectly indicate that decreases in kidney and brain mitochondrial lipid peroxidation, perhaps caused by the lowered rates of mitROS production, also occur during 40% MetR. This is most important because lipid peroxidation is the oxidative process occurring with highest intensity in normal aerobic cells, and its abundant toxic products can secondarily bind to and chemically modify other kinds of mitochondrial molecules, including proteins and most likely DNA. Those decreases in CML and MDAL were not due to increases in the degree of unsaturation and thus in the sensitivity to oxidative damage of kidney or brain mitochondrial membrane fatty acids, because neither the number of double bonds (DBI) nor the PI were modified by 40% MetR. On the other hand, the decrease observed in CEL suggests that kidney and brain mitochondrial protein glycoxidation processes are also lowered during 40% MetR, perhaps also secondarily due to the decrease in mitROS production. Increases in the catabolism of modified proteins during MetR could also explain, like in

DR,<sup>66</sup> the decreases in the five markers of oxidative modification measured in our study.

Whereas in homeothermic vertebrates high longevity is always associated with low levels of both mitROS generation<sup>1,2</sup> and with a low degree of fatty acid unsaturation (low DBI),<sup>4,68,69</sup> only mitROS generation decreased in the present 40% MetR study. In many previous investigations, 40% DR did not change the tissue DBI either, or the changes were too small to decrease the sensitivity to lipid peroxidation. We have found decreases in DBI in 80% MetR in rat liver and heart,<sup>11</sup> rat brain,<sup>30</sup> and rat liver.<sup>28</sup> But when 40% and 80% levels of MetR were studied simultaneously in rat liver,<sup>28</sup> the decrease in the DBI and PI only occurred in 80% MetR, not in 40% MetR. In the present study of 40% MetR, only nonsignificant trends to decreased DBI and PI values were observed in kidney but not in brain mitochondria. This lack of change in DBI at 40% MetR also fits with the commonly found lack of effect of 40% DR on the global degree of fatty acid unsaturation. Therefore, among the two main oxidative stress-related traits of long-lived animals, a low rate of mitROS production and a low degree of fatty acid unsaturation, only the first would be involved in the life extension effect of 40% MetR and 40% DR in rats.

In conclusion, the results from the present study, together with many previous ones concerning MetR, restriction of dietary amino acids except methionine, protein restriction, and DR, strongly indicate that the decrease in methionine intake is the single dietary factor responsible for the decrease in mitROS production and oxidative damage to mitochondrial DNA and proteins that occurs during DR, at least in rat liver, heart, kidney, and brain mitochondria. This, together with various previous studies showing the many beneficial effects of MetR on many other parameters relevant for aging, like the decreased incidence and delayed onset of degenerative diseases and the lowering of their associated risk factors, and especially the increase in maximum longevity,<sup>21,23,24,26</sup> suggests that 40% MetR can be responsible for a substantial part (up to 50%) of the life extension effect of 40% DR.

These results can be important for possible future extrapolations to humans because 40% MetR can circumvent many of the problems that make difficult the implementation of DR to humans: (1) The feeling of hunger leading to low observance of the dietary regime; (2) the risk of malnutrition and its detrimental consequences specially in children and old people; (3) the slowing of body growth rate and the decrease in final adult height; and (4) the decrease in fecundity. All of these problems will be much less important or absent in 40% MetR compared to 40% DR.

Forty percent MetR in humans could be easily obtained by decreasing the excessive consumption of animal proteins like meat, typically present in Western diets at levels 2- to 3-fold higher than the current recommended daily allowance (RDA) or the protein dietary requirement, emphasizing their substitution by sources of vegetal proteins rich in essential amino acids like pulses. Pulses were among the main components of the Mediterranean diets widely used 50 years ago in Greece and other Mediterranean countries, whereas meat consumption was typically low in those diets strongly beneficial for human health. The low methionine content of pulses, classically considered their main limitation for human nutrition, can ironically now turn out to be an advantage, taking into account the discovery of the health benefits of methionine

restriction without methionine deficiency in mammals. Another possibility is to partially substitute animal proteins by soybean protein, which has around half the methionine content of animal proteins like casein and is currently considered to have also positive effects for human health. In this context, it is also interesting that methionine supplementation eliminates the decreased fat deposition induced by diets using soy as source of protein instead of animal proteins.<sup>70</sup>

Recommending life-long methionine restriction to humans, however, should wait until more protein restriction or MetR investigations are performed in mammals substantially longer-lived than rats. Such investigations are urgently needed, although the results of the already available short-term<sup>71–74</sup> or medium-term (3–15 years long)<sup>75</sup> studies of DR, or short-term<sup>76,77</sup> or medium-term 39% protein restriction (2–10 years long)<sup>78</sup> have already shown their benefits for health and for reduction of risk factors of degenerative diseases in human beings. An important role of protein or methionine restriction in the life extension effect of DR in mammals perhaps including nonhuman primates<sup>7</sup> would be consistent with the fact that whenever DR is implemented, it automatically produces protein and methionine restriction.

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Address correspondence to:

Prof. Dr. G. Barja

Departamento de Fisiología Animal II

Facultad de Ciencias Biológicas

Universidad Complutense

c/ Jose Antonio Novais 2

Madrid 28040

Spain

E-mail: gbarja@bio.ucm.es

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