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RESEARCH ARTICLE

Effects of aging and methionine restriction applied at old age on ROS generation and oxidative damage in rat liver mitochondria

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Abstract It is known that a global decrease in food ingestion (dietary restriction, DR) lowers mitochondrial ROS generation (mitROS) and oxidative stress in young immature rats. This seems to be caused by the decreased methionine ingestion of DR animals. This is interesting since isocaloric methionine restriction in the diet (MetR) also increases, like DR, rodent maximum longevity. However, it is not known if old rats maintain the capacity to lower mitROS generation and oxidative stress in response to MetR similarly to young immature animals, and whether MetR implemented at old age can reverse aging-related variations in oxidative stress. In this investigation the effects of aging and 7 weeks of MetR were investigated in liver mitochondria of Wistar rats. MetR implemented at old age decreased mitROS generation, percent free radical leak at the respiratory chain and mtDNA oxidative damage without changing oxygen consumption. Protein oxidation, lipoxidation and glycoxidation increased with age, and MetR in old rats partially or totally reversed these age-related increases. Aging increased the amount of SIRT1, and MetR decreased SIRT1 and TFAM and increased complex IV. No changes were observed in the protein amounts of PGC1, Nrf2, MnSOD, AIF, complexes I, II and III, and in the extent of genomic DNA methylation. In conclusion, treating old rats with isocaloric short-term MetR lowers mitROS production and free radical leak and oxidative damage to mtDNA, and reverses aging-related increases in protein modification. Aged rats maintain the capacity to lower mitochondrial ROS generation and oxidative stress in response to a short-term exposure to restriction of a single dietary substance: methionine.

Keywords Calorie restriction · Methionine restriction · Diet · Mitochondria · Reactive oxygen species

Abbreviations

AASA	Aminoadipic semialdehyde
CEL	Carboxyethyl-lysine
CML	Carboxymethyl-lysine
DR	Dietary restriction
FRL	Percent free radical leak at the respiratory chain
GSA	Glutamic semialdehyde
MetR	Methionine restriction
mitROS	Mitochondrial ROS
mtDNA	Mitochondrial DNA
Nrf2	Nuclear factor erythroid 2-related factor 2

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8-oxodG	8-Oxo-7,8-dihydro-2′deoxyguanosine
PGC1	Peroxisome proliferator-activated receptor γ coactivator 1
ROS	Reactive oxygen species
SIRT1	Sirtuin 1
MnSOD	Mitochondrial manganese superoxide dismutase
TFAM	Mitochondrial transcription factor A

Introduction

The results of many investigations including those concerning age-related changes, comparisons between short- and long-lived animals, and the effects of global decreases in food ingestion (dietary restriction, DR) in relation to longevity, are generally consistent with the mitochondrial free radical theory of aging (Harman 1972; Barja 2004a). Damage to mitochondrial DNA (mtDNA) has been linked to aging and longevity in many studies performed in laboratory mammals, as well as in rhesus monkeys (Castro et al. 2012). Previous studies have identified two characteristics of long-lived animals linked to oxidative stress. Long-lived mammals and birds show a low rate of reactive oxygen species (ROS) generation at mitochondria (Barja 2004a, b), and have cellular membranes with a low degree of fatty acid unsaturation (Pamplona et al. 2002; Pamplona and Barja 2011). Their low ROS production decreases the damage to key molecules like mtDNA, and the low fatty acid unsaturation diminishes the intensity of lipid peroxidation (Pamplona 2011). Among those two factors, DR lowers the first one, the rate of mitochondrial ROS (mitROS) production (Gredilla et al. 2001; Gredilla and Barja 2005).

The best characterized experimental intervention that increases longevity in animals including mammals is DR. Although the exact mechanisms by which DR increases longevity are unknown, many previous investigations have shown that DR lowers oxidative stress in mammals. In particular, DR lowers the rate of mitROS production (Gredilla and Barja 2005) and the steady-state level of oxidative damage to mtDNA in rats and mice. Previous studies from our laboratory have clarified that the cause of these decreases is the lowered ingestion of dietary protein of DR animals (Sanz et al. 2004), whereas the lowered ingestion of dietary carbohydrates (Sanz et al. 2006a), lipids (Sanz et al. 2006b), or the calories themselves are not involved. Further studies have identified the amino acid methionine as the dietary factor responsible for these beneficial changes in protein restricted and DR animals. Thus, isocaloric methionine restriction (MetR) reproduces the decreases in mitROS generation and oxidative damage to mtDNA observed in protein restricted and DR rodents (Caro et al. 2008; López-Torres and Barja 2008). This is interesting, taking into account that long-life experiments have shown that isocaloric MetR increases maximum longevity in rats and mice (Richie et al. 1994; Miller et al. 2005; Sun et al. 2009) and recent studies link essential amino acids and especially methionine with the positive effect of DR on longevity also in insects and yeast (Grandison et al. 2009; Min and Tatar 2006; Kabil et al. 2011; Petti et al. 2011; Piper et al. 2011).

However, all the previous studies showing that MetR lowers mitochondrial oxidative stress have been performed in young immature rats. It is currently unknown if MetR can also decrease mitochondrial ROS generation and oxidative stress in old animals and whether it can reverse putative increases in oxidative damage in aged individuals. Furthermore, it is not known if a short period of MetR is enough to bring about these beneficial changes in animals that have already experienced the long-term deleterious effects of aging. There is hope that scarcely stressful life extending manipulations in animals could be extrapolated to humans in the future. Since a large part of the human population is already mature or old, interventions capable of ameliorating the health state in mature adult or old individuals are desirable. These interventions, ideally, should show efficacy even when they are started late in the life span, when the individuals have lived most of their previous life span without being exposed to them. In particular, it is important to know if a short period of MetR applied at old age is beneficial in animals that have been feeding ad libitum during all their previous lifespan.

Trying to overcome those limitations, we have studied the effect of short-term (7 weeks) MetR in 24 months old Wistar rats. Three groups of animals were used, Young mature controls, Old controls, and Old MetR. In liver mitochondria from these animals, mitochondrial ROS generation, free radical leak (FRL), oxygen consumption, oxidative damage to mtDNA, oxidative, glycoxidative and lipoxidative protein modification, the amount of mitochondrial

respiratory complexes (I–IV), and the apoptosis-inducing factor (AIF) were measured. Since dietary methionine is essential for synthesis of S-adenosyl-methionine that provides methyl groups required for DNA methylation, an important modification mechanism of gene expression, the global methylation of liver genomic DNA was also measured. In order to look for possible factors and mechanisms involved in the effects of MetR, various specific proteins, mitochondrial biogenesis and antioxidant-related factors that seem to be involved in the longevity effects of DR were also studied. Thus, Sirtuin 1 (SIRT1), Peroxisome Proliferator-Activated Receptor γ Coactivator 1 (PGC1), Mitochondrial Transcription Factor A (TFAM), Nuclear Factor Erythroid 2-related factor 2 (Nrf2), and mitochondrial manganese superoxide dismutase (MnSOD) were also measured.

Materials and methods

Animals and diets

Male Wistar rats of 6 months (Young mature adult controls) and 24 months (Old controls and Old MetR animals) of age were obtained from Charles River, were caged individually, and were maintained in a 12:12 (light–dark) cycle, 22 ± 2 °C and 50 ± 10 % relative humidity during the experimental period of dietary methionine restriction (7 weeks). Semipurified diets were specifically prepared after our request by MP biochemicals (Irvine, CA) and were imported to Spain by Leti (Barcelona, Spain). Young controls and Old controls received the control diet and the Old-MetR group received the 40 % MetR diet. The composition of the control diet (in g/100 g of diet) was: L-arginine 1.12, L-lysine 1.44, L-histidine 0.33, L-leucine 1.11, L-isoleucine 0.82, L-valine 0.82, L-threonine 0.82, L-tryptophan 0.18, L-methionine 0.86, L-glutamic acid 2.70, L-phenylalanine 1.16, L-glycine 2.33, Dextrine 5.0, Corn starch 31.82, sucrose 31.79. cellulose 5.0, choline bitartrate 0.20, MP vitamin diet fortification mixture 1.0, mineral mix (AIN) 3.50 and corn oil 8.0. The composition of the 40 % MetR diet was similar to that of the control diet except that L-methionine was present at 0.516 %, which corresponds to an amount of this amino acid 40 % lower than in the control diet (0.86 %). This 0.34 % decrease in L-methionine in the 40 % MetR diet was compensated by increasing all the rest of the dietary components in proportion to their presence in the diet. Since the decrease in L-methionine was very small, with this procedure the percent presence of all the rest of the dietary components was almost the same in the two experimental diets. After 7 weeks of dietary treatment the animals were sacrificed by decapitation. The liver was then processed to isolate mitochondria, which were immediately used to measure mitochondrial respiration and H_2O_2 generation, and liver mitochondrial samples were stored at −80 °C for the assay of the rest of the measurements.

Isolation of mitochondria

Liver was rinsed and fat was removed before homogenization in 60 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. Supernatants were centrifuged at 10,000×g for 10 min and the resulting supernatants were eliminated. 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 every centrifugation step any overlaying layer of fat was eliminated. 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 were immediately used for H_2O_2 production and O_2 consumption measurements.

Mitochondrial ROS generation

The rate of mitochondrial ROS production (O_2^- + H_2O_2) was assayed by measuring the increase in fluorescence as a function of time (excitation at 312 nm, emission at 420 nm) due to oxidation of homovanillic acid by H_2O_2 in the presence of horseradish peroxidase, as previously described (Barja 2002; Sanz and Barja 2006). Reaction conditions were 0.25 mg of mitochondrial protein per ml, 6 U/ml of horseradish peroxidase, 0.1 mM homovanillic acid, 50 U/ml of superoxide dismutase, and 2.5 mM glutamate/2.5 mM malate, or 5 mM succinate in 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 volume of 1.5 ml. In some assays 2 μM rotenone or 2 μM antimycin A were also added. 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), and the fluorescence was read in a LS50B Perkin-Elmer fluorometer. Known amounts of H_2O_2 generated in parallel by glucose oxidase with glucose as substrate were used as standards. Since the superoxide dismutase added in excess converts all $O_2^{\cdot -}$ excreted by mitochondria (if any) to H_2O_2, the measurements represent the total rate of mitochondrial ROS production ($O_2^{\cdot -}$ plus H_2O_2).

Mitochondrial oxygen consumption

The rate of oxygen consumption of liver 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 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). The substrates used were complex I-linked (2.5 mM glutamate/2.5 mM malate) or complex II-linked (5 mM succinate + rotenone). The assays were performed in the absence (State 4-resting) and in the presence (State 3-phosphorylating) of 500 μM ADP.

Free radical leak

The rates of H_2O_2 production and O_2 consumption of rat liver 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 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 FRL 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 (FRL).

Oxidative damage to mitochondrial DNA

The isolation of mtDNA was performed by the method of Latorre et al. (1986) adapted to mammals (Asunción et al. 1996). The isolated mitochondrial DNA was 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. 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 level of 8-oxo-7,8-dihydro-2′deoxyguanosine (8-oxodG) referred to that of the non-oxidized dG (deoxyguanosine) and the results were expressed as 8-oxodG/10^5 dG. 8-oxodG and dG were analyzed by HPLC with on line electrochemical and ultraviolet detection respectively. The nucleoside mixture was injected into a reverse-phase Mediterranea Sea 18 column (5 μm, 4.6 mm × 25 cm; Teknokroma, Barcelona, Spain), and was eluted with a mobile phase containing 6.5 % acetonitrile and 50 mM phosphate buffer pH 5.0. The volume of sample injected in the column was 100 μl. A Gilson 305 pump with nanometric module 805 at 0.9 ml/min was used. 8-oxodG was detected with an ESA Coulochem II electrochemical coulometric detector (ESA, Inc. Bedford, MA) with a 5011A analytical cell run in the oxidative mode (350 mV/20 nA), and dG was detected with a Biorad model 1806 UV detector at 254 nm. For quantification, peak areas of dG standards and of three level calibration 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.

Protein oxidation, glycoxidation and lipoxidation

Markers of protein oxidation—the protein carbonyls glutamic (GSA) and aminoadipic (AASA) semialdehydes, glycoxidation (carboxyethyl-lysine [CEL] and carboxymethyl-lysine [CML]), and lipoxidation (CML)—were determined as trifluoroacetic acid methyl esters derivatives in acid hydrolyzed delipidated and reduced heart mitochondrial protein samples by GC/MS (Ayala et al. 2007) using a HP6890 Series II gas chromatograph (Agilent, Barcelona, Spain) with a MSD5973A Series and a 7683 Series automatic injector, a Rtx-5MS Restek column (30-m × 0.25-mm × 0.25-μm), and the described

temperature program (Ayala et al. 2007). Quantification was performed by internal and external standardization using standard curves constructed from mixtures of deuterated and non-deuterated 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; and CEL and [$^2H_4$]CEL, *m/z* 379 and 383, respectively. The amounts of product were expressed as the μmolar ratio of GSA, AASA, CML, or CEL per mol of lysine.

Mitochondrial complexes (I, II, III and IV), AIF, SIRT1, PGC1, TFAM, Nrf2, MnSOD

The protein contents of liver mitochondrial respiratory chain complexes (Complex I-Complex IV), AIF, SIRT1, PGC1, TFAM, Nrf2, and MnSOD were estimated using western blot analyses. Mitochondrial protein concentration was measured using the Bradford method (Bio-Rad Protein Assay 500-0006) and mitochondrial proteins were separated by one-dimensional SDS-PAGE. The protease inhibitor mix (80-6501-23, Amersham Biosciences) was added to the samples. The samples were mixed with sample buffer (62.5 mM Tris–HCl pH 6.8, 2 % SDS, 10 % glycerol, 20 % 2-β-mercaptoethanol and 0.02 % bromophenol blue) and were heated for 5 min at 95 °C. Proteins (10 μg for respiratory chain complexes, AIF and MnSOD; and 80 μg for SIRT1, PGC1, TFAM, Nrf2) were subjected to electrophoresis on 10 % SDS-polyacrylamide minigels. For immunodetection, proteins were transferred using a Mini Trans-Blot Transfer Cell (Bio-Rad) in a buffer containing 25 mM Tris, 192 mM Glycine and 20 % methanol, to polyvinylidene difluoride membranes (Immobilon-P Millipore, Bedford, MA). The membranes were immersed in blocking solution (0.2 % I-Block Tropix AI300, 0.1 % Tween in PBS) for 1 h at room temperature. After blocking, the membrane was washed two times using 0.05 % TBS-T buffer. Afterwards, the membrane was incubated in primary solution using specific antibodies for the 39 kDa (NDUFA9) and 30 kDa (NDUFS3) subunit of complex I (1:1000 in both cases), 70 kDa subunit (Flavoprotein) of complex II (1:500), 48.5 kDa (CORE 2) and 29.6 kDa (Rieske iron-sulfur protein) subunits of complex III (1:1000 in both cases), and COXI subunit of complex IV with apparent molecular weight of 57 kDa (1:1000) (ref. A21344, A21343, A11142, A11143, A21346 and A6403, respectively; Molecular Probes), anti-AIF (1:1000, ref. A7549, Sigma), anti-SIRT1 (1:500, ref. Ab28170, Abcam), anti-PGC1 (1:100, ref.101707, Cayman), anti-TFAM (1:500, ref.3885-100, Biovision), anti-Nrf2 (1:200, ref. SC722, Santa Cruz Biotechnology) and anti-MnSOD (1:2000, ref. Ab16956, Abcam). An antibody to porin (1:15000, ref. A31855, Molecular Probes) and to actin (1:10000, ref. A5441, Sigma) was also used in order to determine the proportion of protein levels referred to total mitochondrial mass or total mass, respectively. The primary antibody was incubated 1 h at room temperature, except in the case of SIRT1, PGC1, TFAM and Nrf2 which were incubated 16 h at 4 °C. The membrane was washed three times in 0.05 % TBS-T buffer and was incubated 1 h at room temperature with the appropriate secondary antibodies: ECL Anti-mouse IgG, horseradish peroxidase linked whole antibody (1:5000; ref. NA93IV, GE Healthcare), and ImmunoPure Goat Anti-Rabbit IgG, (H + L), peroxidase conjugated (1:10000; ref. 31460, Pierce Biotechnology). After five washes with 0.05 % TBS-T buffer, bands were visualized by using an enhanced chemiluminescence HRP substrate (Millipore, MA, USA). Signal quantification and recording was performed with a ChemiDoc equipment (Bio-Rad Laboratories, Inc., Barcelona, Spain).

Genomic DNA methylation

Genomic DNA was isolated with the GenElute Mammalian Genomic DNA miniprep Kit (G1N70 Sigma). The degree of methylation of this genomic DNA was quantified with a Methylamp Global DNA Methylation Quantification Kit (P-1014 Epigentek). In this assay, the methylated fraction of DNA (expressed as percentage of the total genomic DNA) is recognized by a 5-methylcytosine antibody and quantified through an ELISA-like reaction.

Statistics

Values were expressed as means ± standard error of the mean (SEM). Comparisons between groups were

analyzed by ANOVA followed by DMS tests for paired groups. The minimum level of statistical significance was set at $P < 0.05$ in all the analyses.

Results

Methionine restriction did not change the total body weight and the fresh weight of the main vital organs (heart, brain, liver, kidney, spleen, and visceral adipose tissue) in Old-MetR compared to Old controls (results not shown).

Methionine restriction significantly decreased the rate of ROS production of liver mitochondria with glutamate/malate as substrates (in Old-MetR rats compared to Old controls; Fig. 1A). The Old-MetR values were also significantly lower than in Young controls (Fig. 1A). In the presence of glutamate/malate + rotenone, however, no significant differences were present among the three groups of animals (Table 1). No significant differences in ROS production between groups were observed either with succinate, succinate + rotenone, or succinate + rotenone + antimycin A (Table 1).

Table 2 shows the rates of oxygen consumption of rat liver mitochondria with different combinations of substrates and inhibitors. High values were observed for the respiratory control index (RCI), around 4 with succinate plus rotenone and around 8 with glutamate/malate. Old controls showed lower state 4 oxygen consumption than Young control animals both with glutamate/malate and with succinate + rotenone as substrates. Old-MetR rats also showed lower values than Young controls with succinate + rotenone in state 4. The rest of the comparisons did not show significant differences in oxygen consumption.

Similarly to what was observed for mitROS production, the percent free radical leak (FRL) of rat liver mitochondria was significantly lower in Old-MetR rats than in both Old and Young controls with glutamate/malate (Fig. 1B) whereas it did not change with succinate + rotenone (results not shown). Methionine restriction also significantly decreased the steady-state level of oxidative damage of liver mtDNA (8-oxodG) in Old-MetR animals below that of Old controls (Fig. 2).

Old controls showed significantly higher levels of protein oxidation (GSA and AASA), glycoxidation (CEL and CML) and lipoxidation (CML) than Young controls (Fig. 3, a*). All these four different markers

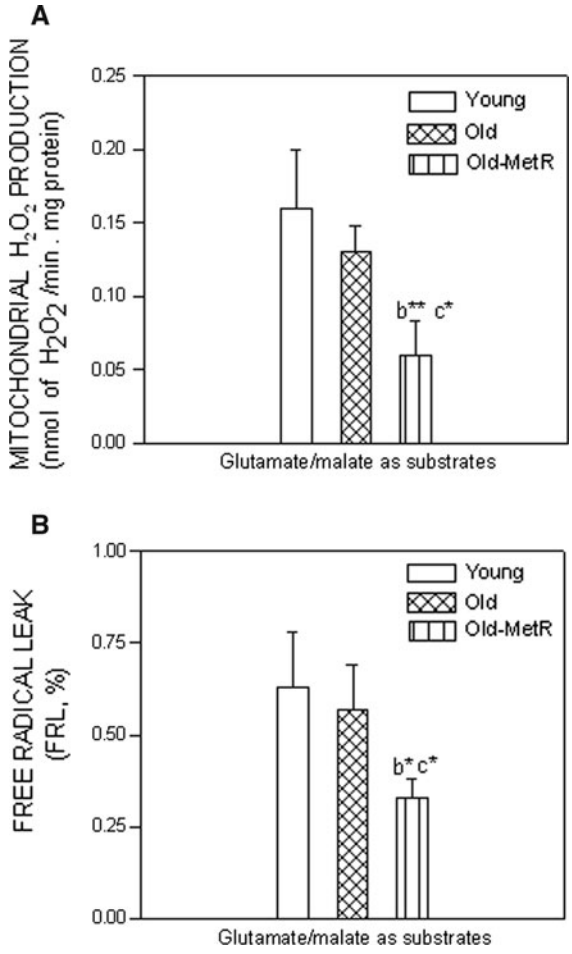

Fig. 1 Rates of ROS production (**A**) and Free Radical Leak (**B**) of liver mitochondria from Young control, Old control and Old MetR rats with glutamate/malate as substrates. Values are means ± SEM from 7 to 8 different animals. *b** Significant difference between Old control and Old-MetR, *c** significant difference between Young control and Old-MetR, $*P < 0.05$; $**P < 0.01$

of protein modification were significantly lower in Old-MetR than in Old Controls (Fig. 3, b*). CEL was higher in Old-MetR than in Young controls (Fig. 3, c*), whereas GSA, AASA and CML did not show differences between these two groups.

The amounts of the respiratory protein complex I (measured both as the 39 and 30 kDa subunits), complex II (70 kDa) and complex III (48.5 and 29.6 kDa subunits) as well as the AIF did not show significant differences among the three experimental groups (Table 3). Only complex IV of Old-MetR animals showed higher levels than those of Young controls (Table 3).

Table 1 Rates of reactive oxygen species (ROS) production of liver mitochondria from Young control, Old control and Old MetR rats under different assay conditions

	Young	Old	Old-MetR
Glutamate/malate + rotenone	0.57 ± 0.06	0.44 ± 0.05	0.36 ± 0.08
Succinate	0.68 ± 0.11	0.45 ± 0.10	0.45 ± 0.08
Succinate + rotenone	0.25 ± 0.03	0.24 ± 0.03	0.24 ± 0.02
Succinate + rotenone + AA	1.01 ± 0.18	0.97 ± 0.16	0.87 ± 0.09

Values are means ± SEM (nmoles of H_2O_2/min mg protein) from 7 to 8 different animals

AA Antimycin A

Table 2 Rates of oxygen consumption and respiratory control index of liver mitochondria from Young control, Old control and Old MetR rats

	Young	Old	Old-MetR
Glutamate/malate (State 4)	13.3 ± 1.1	10.9 ± 0.8[a*]	10.8 ± 1.2
Glutamate/malate (State 3)	96.2 ± 3.7	88.8 ± 3.5	89.0 ± 3.3
Glutamate/malate (RCI)	7.5 ± 0.6	8.4 ± 0.5	8.7 ± 0.7
Succinate + rotenone (State 4)	34.4 ± 2.3	27.2 ± 1.7[a*]	27.8 ± 2.5[c*]
Succinate + rotenone (State3)	124.5 ± 7.7	109.8 ± 7.3	117.2 ± 3.5
Succinate + rotenone (RCI)	3.8 ± 0.1	4.1 ± 0.3	4.4 ± 0.4

Values are means ± SEM (nmoles of O_2/min mg protein) from 8 different animals. *State 4* oxygen consumption in the absence of ADP. *State 3* oxygen consumption in the presence of 500 μM ADP. *RCI* Respiratory control index. a* Significant difference between Young and Old control rats, c* significant difference between Young control and Old-MetR, * $P < 0.05$

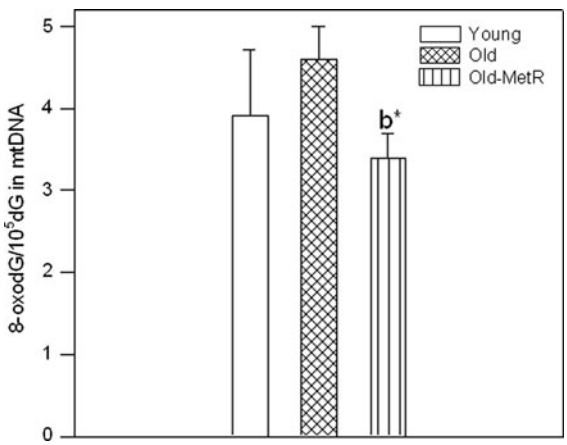

Fig. 2 Oxidative damage to mtDNA in liver mitochondria from Young control, Old control and Old MetR rats. Values are means ± SEM from 6 to 7 different animals. *b** Significant difference between Old control and Old-MetR, *$P < 0.05$

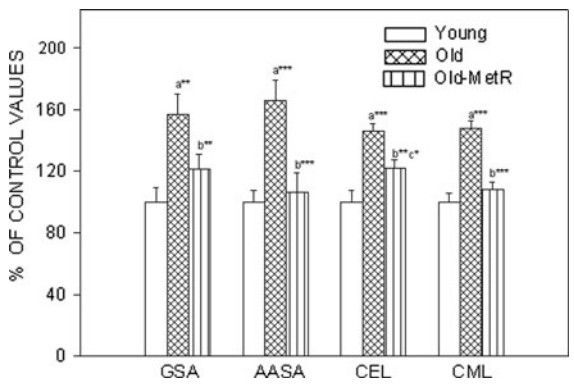

Fig. 3 Protein oxidation, glycoxidation and lipoxidation indicators in liver mitochondria from Young control, Old control and Old MetR rats. Values are means ± SEM from 8 different animals and are expressed as percentage of those in the controls for each protein modification marker. Control values: 3,637.91 ± 325.25 (glutamic semialdehyde, GSA); 108.03 ± 7.52 (AASA, aminoadipic semialdehyde, AASA); 225.35 ± 16.26 (carboxyethyl-lysine, CEL); 949.07 ± 54.37 (carboxymethyl-lysine, CML). Units: μmol/mol lysine. a* Significant difference between Young and Old control rats, b* significant difference between Old control and Old-MetR, c* significant difference between Young control and Old-MetR, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Representative immunoblots of the mitochondrial biogenesis and antioxidant factors measured are shown in Fig. 4. SIRT1 was higher in Old controls than in Young controls, and lower in Old-MetR than in

Table 3 Amounts of respiratory complexes and AIF in liver mitochondria from Young control, Old control and Old MetR rats

	Young	Old	Old-MetR
Complex I (39 kDa subunit, NDUFA9)	100 ± 2.35	95.1 ± 5.41	97.8 ± 16.7
Complex I (30 kDa subunit, NDUFS3)	100 ± 0.11	99.7 ± 0.18	100.1 ± 0.30
Complex II (70 kDa subunit, Flavoprotein)	100 ± 6.23	138.9 ± 16.85	140.7 ± 14.10
Complex III (48.5 kDa, CORE II)	100 ± 8.58	95.6 ± 2.85	110.7 ± 2.40
Complex III (29.6 kDa subunit, Rieske iron-sulfur protein)	100 ± 10.83	132.2 ± 12.86	102.0 ± 13.50
Complex IV (57 kDa subunit, COXI)	100 ± 13.22	143.9 ± 21.11	164.3 ± 4.80^{c*}
AIF (Apoptosis-inducing factor)	100 ± 9.55	91.29 ± 8.77	107.7 ± 8.50

Values are means ± SEM from 7 to 8 different animals. Units: ratio of complex I, II, III or IV and AIF/porin in arbitrary units. c* Significant difference between Young control and Old-MetR rats, * $P < 0.05$

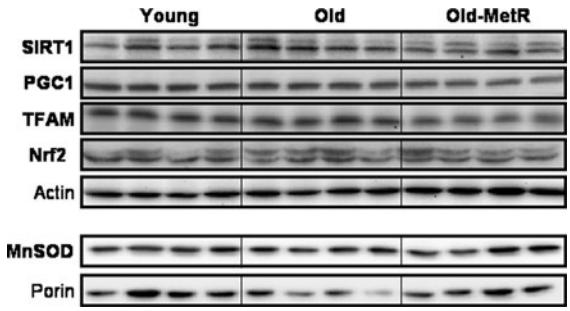

Fig. 4 Representative immunoblots showing relative levels of the mitochondrial biogenesis and antioxidant factors PGC1, TFAM, Nrf2 and MnSOD, as well as SIRT1, and actin and porin (used for reference) in liver from Young control, Old control and Old MetR rats

Old controls (Table 4). TFAM was significantly lower in Old-MetR than in Old controls. PGC1, Nrf2, MnSOD and genomic DNA methylation did not show significant differences between groups (Table 4).

Discussion

In this investigation it is shown for the first time that old rats maintain the capacity to lower the rate of mitochondrial ROS production and percent free radical leak at complex I as well as the oxidative damage to mitochondrial DNA and proteins in the liver in response to a short period of isocaloric 40 % methionine restriction in the diet, similarly to what occurs during 40 % caloric restriction (Gredilla and Barja 2005), and during 40 % MetR in various tissues (including liver) of young immature animals (Caro et al. 2008, 2009a).

It has been described that among components of the mitochondrial respiratory chain, complex III and complex I (Barja and Herrero 1998; Treberg et al. 2011) produce ROS, but the decrease in mitROS production induced by DR has been localized at complex I (Barja 2004b; Gredilla et al. 2001). The decrease in the basal rate of mitROS production induced by 7 weeks of MetR in old rats in the present

Table 4 Mitochondrial biogenesis and antioxidant factors, SIRT1, and genomic DNA methylation, in liver from Young control, Old control and Old MetR rats

	Young	Old	Old-MetR
SIRT1	100 ± 8.29	128.57 ± 8.29^{a*}	80.00 ± 7.14^{b**}
PGC1	100 ± 0.09	100.07 ± 0.04	100.05 ± 0.07
TFAM	100 ± 5.07	109.26 ± 7.99	83.45 ± 4.89^{b*}
Nrf2	100 ± 34.54	111.29 ± 12.33	89.84 ± 20.63
MnSOD	100 ± 19.23	148.31 ± 16.37	117.07 ± 12.59
% DNA methylation	0.74 ± 0.008	0.60 ± 0.086	0.70 ± 0.113

Values are means ± SEM from 4 to 8 different animals. Units: ratio protein content/actin except for MnSOD (protein content/porin) and DNA methylation (methylated genomic DNA as percentage of total genomic DNA). a* Significant difference between Young and Old control rats, b* significant difference between Old control and Old-MetR, * $P < 0.05$, ** $P < 0.01$

investigation took place also at complex I because it was observed with glutamate/malate but not with succinate + rotenone as substrates. Electrons flow through complex I in the first but not during the second of these conditions. Thus the notion that the lowering of complex I ROS generation during DR is due to decreased ingestion of methionine and not to other dietary components or to the calories themselves (Caro et al. 2009b; López-Torres and Barja 2008) is reinforced by the present data and it is extended to aged animals. Such decrease, in agreement with other lines of evidence (Pérez-Campo et al. 1998; Barja 2004a, b), does not seem to be secondary to an increase in enzymatic superoxide radical scavenging since the mitochondrial form of superoxide dismutase (MnSOD) was not changed by the MetR treatment, although since what we measured was the amount of MnSOD protein, changes in SOD activity can not be totally ruled out. The decrease in mitROS generation was not a by-product of variations in oxygen consumption either, since this parameter did not change in MetR with glutamate/malate as substrate in State 4 (the condition in which mitROS production decreased). On the other hand, the decreases in mitochondrial oxygen consumption in old animals compared to young ones do not imply a lowered bioenergetic capacity during aging since they were observed only in state 4 but not in the ATP producing state 3. Another possible mechanism to lower complex I ROS generation is a simple decrease in amount of the complex I protein, as it has been described in the long-lived pigeon compared to the much more short-lived rat species (St-Pierre et al. 2002). But this does not seem to be the case in the present model either, because no changes in the amounts of complex I, II and III were observed in MetR. The absence of changes in AIF in MetR also agrees with the lack of changes in the amount of complex I because AIF, in addition to apoptotic functions, is also required for mitochondrial oxidative phosphorylation and has a specific role in the biogenesis and maintenance of complex I (Vahsen et al. 2004; Porter and Urbano 2006). Only the increase in the amount of complex IV detected in the present investigation in Old MetR animals could indirectly contribute to lower mitROS generation. The higher availability of complex IV could in principle facilitate the rate of electron transport and therefore would tend to decrease the accumulation of electrons upwards in the respiratory chain, lowering the degree of electronic reduction of complex I and thus its tendency to generate ROS. However, against this possibility are the following facts: (a) complex IV is usually in large excess and therefore is usually not limiting for the rate of electron flow; (b) the rate of oxygen consumption in state 4 was not increased in Old MetR rats; (c) the increase in complex IV was significant only when compared to Young but not to Old control animals. On the other hand, the lack of changes in maximum mitROS production (which was measured with glutamate/malate + rotenone and with succinate + rotenone + AA for complexes I and III respectively) also agrees with the idea that the lowering of mitROS generation in MetR is not secondary to changes in the amounts of the mitochondrial ROS generators. Instead, what was decreased by MetR was the FRL, the percentage of total electron flow in the respiratory chain directed to ROS production. Thus, the liver mitochondria from Old MetR animals are more efficient than those of Old controls in avoiding ROS generation. They leak less radicals per unit of electron flow in the respiratory chain, similarly to what has been found in long-lived compared to short-lived animals (Barja 2004a, b). This increased efficiency in avoiding free radical leak was localized at complex I since the decrease in FRL in MetR was observed with glutamate/malate as substrates but not with succinate + rotenone.

In agreement with the decrease in mitROS generation, the mtDNA of Old MetR animals showed a significantly lower level of steady-state oxidative damage (measured as 8-oxodG) than that of Old controls. And the mtDNA 8-oxodG level, like mitROS production, did not show significant differences between Young and Old control animals. Previous results have shown the same kind of results in DR (Gredilla et al. 2001; López-Torres et al. 2002), and in MetR models (Caro et al. 2008) in young animals. This close correspondence between variations or lack of changes between these two parameters suggests that the decrease in 8-oxodG in the mtDNA of Old MetR animals is due, at least impart, to their lowered mitROS production, whereas the mtDNA oxidative damage did not change as a function of age because there were no changes in mitROS generation between Young and Old controls either.

In our study, the lowering of oxidative damage was not limited to mtDNA since it took place also in liver mitochondrial proteins. All the four protein modification markers measured showed MetR-induced

decreases in Old animals, similarly to what has been observed in various rat organs during DR, and during MetR in young animals (López-Torres and Barja 2008). Thus, protein oxidation, lipoxidation and glycoxidation were lower in Old MetR than in Old control animals, similarly to what has been found in liver tissue, and in kidney and brain mitochondria of young immature animals subjected to MetR (Caro et al. 2008, 2009a). On the other hand, in the present investigation Old controls showed higher levels of all the protein modification markers (GSA, AASA, CEL, AND CML) than the Young controls. Therefore, MetR was able to partially (CEL) or totally (GSA, AASA and CML) reverse the aging-related increases in protein modification. The lowering of aging-related increases in protein damage is one of the most prominent beneficial effects of MetR among those found in the present investigation. While the decrease in mitROS generation in MetR could contribute to those reversals in protein damage it is not necessarily the only factor controlling protein oxidative modification since Old controls had a higher level of the four protein markers than Young controls whereas their rates of mitROS production were similar. Another factor that could be involved is an increased catabolism of modified proteins in Old MetR animals. Aging is known to increase protein oxidation in association with a functional decline of proteasome activity (Kastle and Grune 2011) whereas decreases in protein oxidation and increases in the catabolism of modified proteins have been described in DR (Dhabi et al. 2001).

Although not specifically measured in the present study, the decreases in mitochondrial ROS production and oxidative damage to mtDNA and mitochondrial proteins induced by MetR likely lead to beneficial effects on health and functionality of the animals. This relates to many previously published studies. Thus, it has been shown that MetR decreases visceral fat mass, improves glucose tolerance, strongly decreases plasma insulin and IGF-1 and lowers blood cholesterol and triglycerides in rats, independently of calorie restriction (Malloy et al. 2006). Diets low in methionine decrease the risk of obesity (Newby et al. 2005) and diabetes (Vang et al. 2008) in humans, and MetR enhances metabolic flexibility and increases uncoupling respiration (Hasek et al. 2010). In addition, a recent study showed that expressing the yeast Ndi1 complex I analogue in *Drosophila* decreased mitochondrial ROS production and specific protein oxidation markers and increased mean and maximum lifespan of the animals (Sanz et al. 2010). Most importantly, it has been repeatedly found that MetR increases mean and maximum longevity in both rats and mice (Richie et al. 1994; Miller et al. 2005; Sun et al. 2009). Maximum longevity integrates all the different functional age-related changes and its increase is an excellent indicator of decreases in aging rate. Increases in maximum longevity only occur when the many different age-related decreases in molecular, tissue and organism functionalities and the incidence of age-related diseases are coordinately minimized or delayed.

What are the signalling mechanisms possibly involved in the beneficial changes induced by MetR detected in this investigation? Many signalling pathways have been linked to the aging process not only in invertebrate model animals but also in mammals (Taguchi and White 2008; Mair and Dillin 2008), and many microarray studies have shown that DR (which necessarily includes MetR) modifies the expression of many different genes (Park and Prolla 2005). On the other hand, partly separated although finally convergent pathways are being delineated for the actions of DR (the insulin/IRS pathway) on the one hand, and of diet-derived amino-acids on the other (the amino acid response pathway) (Mair and Dillin 2008; Taguchi and White 2008; Liu and Qian 2011). This last pathway could be specifically implicated in the mechanism of action of MetR. On the other hand, sirtuins have been implicated in the control of life span during DR, although this concept is currently under debate (Baur et al. 2010). Other possible mechanisms involved in the beneficial effect of DR on aging are an induction of mitochondrial biogenesis (Nisoli et al. 2005; López-Lluch et al. 2006; Hock and Kralli 2009), or decreased methylation of DNA which can change gene expression (Robert et al. 2010; Passarino et al. 2010; Madrigano et al. 2012). Dietary methionine is essential for the synthesis of S-adenosymethionine that provides methyl groups required for DNA methylation. Increases in transmethylation of methionine and S-adenosylmethionine/S-adenosylhomocysteine ratio in the liver of protein restricted rats (Kalhan et al. 2011) and increases in S-adenosylmethionine in the heart of dietary methionine-supplemented rats (Gómez et al. 2009) have been recently observed. Therefore, this last result indicates that a lower level of DNA methylation could be expected in MetR.

In order to look generally at those different signalling mechanisms, in this investigation we have measured some proteins that could be involved in those pathways. Among them, only an increase in the amount of SIRT1 with age, and decreases in SIRT1 and TFAM with MetR were detected, whereas PGC1, Nrf2, MnSOD and percent DNA methylation did not change in either situation. Among sirtuins, SIRT1 is the better one characterized in mammals. SIRT1 seems to be induced by DR and it has been proposed that this decreases mitROS generation through the increased expression of antioxidant-related genes induced by the forkhead/FOXO family of transcription factors (Hajnoczky and Hoek 2007). But our results suggest that MetR does not decrease mitROS generation and oxidative stress through this pathway in our model because a decrease (instead of an increase) in SIRT1 back to young levels was observed, and MnSOD was not changed. Perhaps the mechanisms of action of short-term MetR, which are only a fraction of those of DR, do not involve SIRT1 induction at least in old rat liver. The decrease in TFAM and the lack of changes in its upstream factor PGC1 do not suggest that increased mitochondrial biogenesis is involved in the lowering of oxidative stress by MetR in our model either. The lack of changes in most of the multiprotein complexes of the respiratory chain observed in our study also point in the same direction. We have previously observed an increase in the amount of PGC1α in the brain of young rats with the same MetR protocol used in the present investigation (Naudí et al. 2007). Therefore, the effects of MetR on mitochondrial biogenesis could be tissue- or age-specific. Concerning Nrf2, it is currently referred as a "master regulator" of the antioxidant response. It up-regulates a battery of about 100 antioxidant response element-driven genes including the familiar antioxidant enzymes as well as many others related to immunity and inflammation (Kensler et al. 2007; Hayes et al. 2010). Nrf2 induces the expression of a number of genes involved in protecting cells against free radicals, electrophiles, and oxidative stress, including those coding for superoxide dismutases. The lack of changes in Nrf2 and MnSOD in the present study, however, suggests that the decrease in mitROS generation induced by MetR is not antioxidant-dependent, which would be consistent with a large body of previous evidence concerning DR as well as comparisons among animal species widely differing in longevity (Pérez-Campo et al. 1998; Sohal et al. 1994; Barja 2004a). However, like in the case of MnSOD, since what we measured was the amount of the Nrf2 protein, the possibility that Nrf2 could be activated through nuclear translocation can not be discarded. Concerning DNA methylation, we have previously found that the same protocol of MetR used here led to a small but statistically significant decrease in global DNA methylation in the liver of young immature rats (Sanchez-Roman et al. 2011), whereas in the present study performed in old rats the decrease in this parameter did not reach statistical significance. Since the difference between both studies was the age of the animals, this could mean that the capacity of MetR to decrease global DNA methylation is lost during aging in rat liver.

In summary, the results of the present investigation demonstrate for the first time that old rats maintain the capacity to respond to methionine restriction with decreases in mitROS production and oxidative damage to mtDNA and mitochondrial proteins. Importantly, these beneficial changes, which are well described in DR rats, can be obtained after subjection of old animals to only 7 weeks of methionine restriction in the diet without any decrease in the ingestion of calories. Further studies are needed to delineate what are the exact molecular mechanisms by which MetR, a hardly stressful and quick intervention, exerts these benefits in aged individuals.

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