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Forty percent methionine restriction lowers DNA methylation, complex I ROS generation, and oxidative damage to mtDNA and mitochondrial proteins in rat heart

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Abstract Methionine dietary restriction (MetR), like dietary restriction (DR), increases rodent maximum longevity. However, the mechanism responsible for the retardation of aging with MetR is still not entirely known. As DR decreases oxidative damage and mitochondrial free radical production, it is plausible to hypothesize that a decrease in oxidative stress is the mechanism for longevity extension with MetR. In the present investigation male Wistar rats were subjected to isocaloric 40% MetR during 7 weeks. It was found that 40% MetR decreases heart mitochondrial ROS production at complex I during forward electron flow, lowers oxidative damage to mitochondrial DNA and proteins, and decreases the degree of methylation of genomic DNA. No significant changes occurred for mitochondrial oxygen consumption, the amounts of the four respiratory complexes (I to IV), and the mitochondrial protein apoptosis-inducing factor (AIF). These results indicate that methionine can be the dietary factor responsible for the decrease in mitochondrial ROS generation and oxidative stress, and likely for part of the increase in longevity, that takes place during DR. They also highlight some of the mechanisms involved in the generation of these beneficial effects.

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Introduction

Dietary restriction (DR) is the better described experimental manipulation that extends longevity in animals including mammals. Although the classical consensus proposed that this effect is due to the decreased intake of calories themselves, a role for particular dietary components is becoming apparent. Studies in insects suggest a role for aminoacids (Mair et al. 2005; Min and Tatar 2006; Grandison et al. 2009) and investigations in rodents indicate a role for dietary proteins. Review of the subject shows that in 16 out of 18 long-term survival studies in rats and mice protein restriction increased maximum longevity (Pamplona and Barja 2006) whereas the available studies do not indicate that carbohydrate or lipid restriction change rodent longevity (Iwasaki et al. 1988; Khorakova et al. 1990; Shimokawa et al. 1996). The magnitude of the longevity extension effect of protein restriction in rodents is up to 50% that of DR. Concerning particular amino acids, methionine restriction (MetR) without restriction of calories increases maximum longevity in rats and mice (Richie et al. 1994; Miller et al. 2005; Sun et al. 2009). The beneficial effects of MetR in rodents also include decreases in visceral fat, triglycerides, cholesterol, glucose, insulin and IGF1 (Malloy et al. 2006), slowing of cataract development, protection against age-related changes in immunity (Miller et al. 2005), improvements in colon tight junction barrier function (Ramaligan et al. 2010), enhanced metabolic flexibility (Hasek et al. 2010), and lowering of the incidence of cancer (Komninou et al. 2006). It has been

also shown that overexpression of methionine sulfoxide reductase increases animal longevity (Chung et al. 2010). On the other hand, excessive intake of dietary methionine has toxic effects including increases in oxidative stress (Park et al. 2008; Yalcinkaya et al. 2009; Song et al. 2009; Gomez et al. 2009).

Concerning the possible mechanisms involved, previous studies have shown that DR in rodents invariably decreases mitochondrial reactive oxygen species (mitROS) generation and oxidative damage to mitochondrial DNA (mtDNA) and proteins (reviewed in Gredilla and Barja 2005). This is interesting since long-lived mammals have lower mitROS production and fatty acid unsaturation than short-lived ones (see Pamplona and Barja 2007 for review). Previous studies have shown that protein but not carbohydrate or lipid restriction also decrease mitROS production and oxidative stress (Lopez-Torres and Barja 2008) and the same occurs in the heart after restriction of a single amino acid, methionine (Sanz et al. 2006). Furthermore, when all the other dietary aminoacids except methionine are restricted the benefits in oxidative stress are no longer present (Caro et al. 2009a). All this suggests that methionine is the dietary factor responsible for the decrease in mitROS generation and oxidative stress in DR. However, the previous studies performed in rat heart (Sanz et al. 2006) were performed at 80% MetR whereas classic DR studies are performed at a level of 40% food restriction. To be able to attribute to dietary methionine the beneficial effects of standard DR (40%) on oxidative stress, the level of implementation of MetR must be also 40%, not 80%. Furthermore, the use of 40% MetR also avoids the decrease in growth rate, maturation, and final body size that occurs in 80% MetR and in 40% DR. Those decreases can complicate the interpretation of the results obtained.

In the present study the effect of 40% MetR during 7 weeks on mitROS generation and oxidative stress is investigated in the heart of male Wistar rats. This time of experimentation was selected since it successfully decreased mitROS production and oxidative stress in previous studies of DR, protein restriction, and 80% MetR. The heart was selected because it is a vital tissue and is mainly composed of postmitotic cells, making it ideal for aging-related studies. Measurements include various markers of oxidative damage in mitochondria since many MetR studies of this kind have focused in whole organs instead of in mitochondria (Sanz et al. 2006; Naudi et al. 2007; Caro et al. 2008). The parameters measured include mitochondrial oxygen consumption in both resting (state 4) and phosphorylating state (state 3), the rate of mitROS production and location of the free radical source, oxidative damage to mitochondrial DNA (8-oxodG; 8-oxo-7,8,dihydro-2'-deoxyguanosine), and five markers of oxidative, glycoxidative, and lipoxidative damage to proteins measured by gas chromatography/mass spectrometry techniques. Since fatty acid unsaturation is negatively correlated with animal longevity (Pamplona and Barja 2007) and 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 of heart lipids was also measured. The amounts of respiratory complexes I-IV were measured because they can modify the total electron flow in the respiratory chain and the total number of ROS generation sites, and therefore they can potentially affect the rates of mitROS generation. The amount of the mitochondrial enzyme MnSOD which scavenges superoxide anion was also measure to ascertain to what extent changes in mitochondrial superoxide radical release are due to modifications in the rate of superoxide radical production or elimination. The apoptosis-inducing factor was studied because it is considered a two-edged sword (Porter and Urbano 2006). It can stimulate apoptosis, but it is also required for the assembly/maintenance of complex I, the respiratory complex where mitROS generation is lowered in long-lived animals and in DR and MetR (Barja 2004; López-Torres and Barja 2008). Since sirtuins are considered important proteins in relation to longevity (Imai and Guarente 2010), the amount of the mitochondrial sirtuin SIRT5 was also measured. Finally, since dietary methionine is essential for synthesis of S-adenosylmethionine that provides methyl groups required for DNA methylation, an important mechanism of modification of gene expression, the global methylation of heart genomic DNA was also measured.

Materials and methods

Animals and diets

Male Wistar rats of 250–300 g of body weight were caged individually and maintained in a 12:12 (light-dark) cycle, 22 °C±2 °C and 50%±10% relative humidity. Semipurified diets prepared by MP biochemicals (Irvine, CA) and imported to Spain by Leti (Barcelona, Spain) were used. The detailed composition of the diet is shown in Table 1. 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%). The 0.34% absolute decrease in L-methionine in the 40% MetR diet was compensated by increasing all the other dietary components in proportion to their presence in the diet. Since the % absolute decrease in L-methionine was small, with this procedure the % presence of all the other dietary components was almost the same in the two experimental diets. After 7 weeks of dietary treatment the animals were

Table 1 Detailed composition of the semi purified diets used in this study (control and 40% methionine restricted)

Component	Control (g/100 g)	40%MetR (g/100 g)
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	5.017
Corn starch	31.80	31.92
Sucrose	31.79	31.92
Cellulose	5	5.017
Choline bitartrate	0.2	0.201
MP Vitamin diet fortification mixture	1.02	1.023
Mineral mix (AIN)	3.5	3.512
Corn oil	8	8.028
Total (% weight)	100	100

sacrificed by decapitation. The heart was then processed to isolate mitochondria, which were immediately used to measure mitochondrial respiration and H_2O_2 generation, and mitochondrial and heart samples were stored at −80 °C for the assay of the rest of the biochemical parameters.

Heart mitochondria isolation

Heart mitochondria were obtained from fresh tissue by the procedure of Mela and Seitz (1979) with modifications. The heart was chopped into small pieces and was homogenized with a loose-fitting glass-glass pestle in 10 ml of isolation buffer (220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) containing 5 mg of subtilisin A and 25 mg of fatty acid-free albumin. After the sample stood for 1 min, 25 ml of additional isolation buffer containing 25 mg of albumin were added, and gentle homogenization was performed again with a tighter fitting pestle. The nuclei and cell debris were removed by centrifugation at 700×*g* for 10 min. Supernatants were centrifuged at 8,000×*g* for 10 min and the resulting supernatants were eliminated. The pellets were resuspended in 25 ml of isolation buffer and centrifuged at 8,000×*g* for 10 min. The mitochondrial pellets were resuspended in 1 ml of isolation buffer. All the above procedures were performed at 4 °C. Mitochondrial protein was measured by the Biuret method. The final mitochondrial suspensions were maintained at high concentration of mitochondrial protein over ice and were immediately used for the oxygen consumption and H_2O_2 production measurements during the next two hours.

Mitochondrial H_2O_2 generation

The rate of mitochondrial ROS production ($O_2^{\cdot-}$ + 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, essentially as 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 pyruvate/2.5 mM malate, or 5 mM succinate as substrates in the presence or absence of 2 μM rotenone or 2 μM antimycin A 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. 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 ($O_2^{\cdot-}$ plus H_2O_2) rate of mitochondrial ROS production.

Mitochondrial oxygen consumption

The rate of oxygen consumption of heart mitochondria was measured at 37 °C in a water-thermostatized incubation chamber with a computer-controlled Clark-type O_2 electrode (Oxygraph, Hansatech, UK) in 0.5 ml of 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 pyruvate/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.

DNA analyses

For the measurement of oxidative damage to mtDNA, 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 base (deoxyguanosine, 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.

For the measurement of 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 % of the total genomic DNA) is recognized by a 5-methylcytosine antibody and quantified through an ELISA-like reaction.

Mitochondrial complexes (I, II, III and IV), AIF, MnSOD and SIRT5

The protein contents of heart mitochondrial respiratory chain complexes, AIF, MnSOD and SIRT5 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 70 μg for SIRT5) 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-MnSOD and anti-SIRT5 (1:2000 and 1:500, respectively, ref. ab16956 and ab13697, Abcam). An antibody to porin (1:15000, ref. A31855, Molecular Probes) was also used in order to determine the proportion of protein levels referred to total mitochondrial mass. The primary antibody was incubated 1 h at room temperature, except in the case of MnSOD and SIRT5 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).

Oxidation-derived protein damage markers

Markers of protein oxidation – the protein carbonyl glutamic (GSA) and aminoadipic (AASA) semialdehydes-, glycoxidation (carboxyethyl lysine [CEL] and carboxymethyl lysine [CML]), and lipoxidation (malondialdhyde lysine [MDAL] and CML) were determined as trifluoroacetic acid methyl esters derivatives in acid hydrolyzed delipidated and reduced heart mitochondrial protein samples by GC/MS (Pamplona et al. 1999) 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 (Pamplona et al. 1999). 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; CEL and [$^2H_4$]CEL, *m/z* 379 and 383, respectively; and MDAL and [$^2H_8$]MDAL, *m/z* 474 and 482, respectively. The amounts of product were expressed as the μmolar ratio of GSA, AASA, CML, CEL or MDAL per mol of lysine.

Fatty acid analyses and global fatty acid unsaturation indexes

Fatty acid groups of mitochondrial heart lipids were analyzed as methyl esters derivatives by GC/MS as previously described (Pamplona et al. 1999). Separation was performed by a DB-WAX capillary column (30 m×0.25 mm×0.20 μm) in a GC System 7890A with a Series Injector 7683B and a FID detector (Agilent Technologies, Barcelona, Spain). Identification of fatty acid methyl esters was made by comparison with authentic standards (Larodan Fine Chemicals, Malmö, Sweden). Results are expressed as mol%. The following fatty acid indexes were calculated: saturated fatty acids (SFA); unsaturated fatty acids (UFA); monounsaturated fatty acids (MUFA); polyunsaturated fatty acids from n-3 and n-6 series (PUFAn-3 and PUFAn-6); average chain length (ACL)=[(Σ%Total$_{14}$ x 14) + (Σ%Total$_{16}$ × 16) + (Σ%Total$_{18}$ × 18) + (Σ%Total$_{20}$ × 20) + (Σ%Total$_{22}$ × 22) + (Σ%Total$_{24}$ × 24)]/100]; double bond index (DBI)=[(1 × Σmol% monoenoic) + (2 × Σmol% dienoic) + (3 × Σmol% trienoic) + (4 × Σmol% tetraenoic) + (5 × Σmol% pentaenoic) + (6 × Σmol% hexaenoic)]; and peroxidizability index (PI)=[(0.025 × Σmol% monoenoic) + (1 × Σmol% dienoic) + (2 × Σmol% trienoic) + (4 × Σmol% tetraenoic) + (6 × Σmol% pentaenoic) + (8 × Σmol% hexaenoic)].

Statistics

Values were expressed as means ± standard error of the mean (SEM). Comparisons between control and MetR groups were analyzed by Student t-tests. The minimum level of statistical significance was set at $P<0.05$ in all the analyses.

Results

The mean body weight of the animals did not show significant differences between the two experimental groups at the start of the experiment (234±2.65 g in the control and 232±2.40 g in the MetR group) and no significant differences in body weight were observed after the 7 weeks of treatment (363±8.28 g in controls and 370±4.78 g in MetR). The weight of liver, heart, spleen, kidney, or brain and the food intake was not significantly modified by dietary treatment (results not shown).

The rate of oxygen consumption from heart mitochondria was measured in the absence (state 4) and in the presence (state 3) of 500 μM ADP, with complex I-linked (pyruvate/malate) and complex II-linked (succinate + rotenone) substrates (Table 2). MetR significantly increased state 4 oxygen consumption with succinate + rotenone but not with pyruvate/malate, whereas state 3 values did not change with any substrate.

The mitochondrial rate of ROS production was significantly decreased by MetR in heart mitochondria respiring with pyruvate/malate + rotenone (Table 3). However, when complex II-linked substrate (succinate) was used, no significant differences were observed between dietary groups either with succinate alone, with succinate + rotenone or with succinate + antimycin A.

Oxidative damage to mtDNA was estimated by measuring the marker 8-oxodG. The steady-state level of 8-oxodG in rat heart mtDNA was significantly decreased by MetR (Fig. 1). MetR also significantly decreased the global methylation of genomic DNA in the rat heart (Fig. 2).

Three general kinds of oxidative modification of rat heart mitochondrial proteins were measured, oxidative (the specific protein carbonyls GSA and AASA), glycoxidative (CEL and CML) and lipoxidative (CML and MDAL) markers (Fig. 3). All these five markers were significantly decreased by MetR. The marker showing the strongest

Table 2 Rates of oxygen consumption of heart mitochondria from control and 40% methionine restricted (MetR) rats

	Control	MetR
Pyruvate/malate (State 4)	57.5±4.9	46.3±3.0
Pyruvate/malate (State 3)	223.2±23.1	213.7±13.0
Pyruvate/malate (RCI)	4.1±0.3	4.7±0.2
Succinate + rotenone (State 4)	111.6±6.8	133.7±6.5 *
Succinate + rotenone (State3)	297.2±16.1	317.0±17.8
Succinate + rotenone (RCI)	2.5±0.1	2.3±0.1

Values are means ± SEM (nmoles of O_2/ min. mg protein) from 6–7 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.*$P<0.05$ Significant differences between Control and MetR rats

Table 3 Rates of reactive oxygen species (ROS) production of heart mitochondria from control and 40% methionine restricted (MetR) rats

	Control	MetR
Pyruvate/malate + rotenone	3.56±0.19	3.03±0.14*
Succinate	0.38±0.05	0.40±0.05
Succinate + rotenone	0.42±0.04	0.45±0.04
Succinate + antimycin A	5.37±0.27	5.72±0.21

Values are means ± SEM (nmoles of H_2O_2/min · mg protein) from 5–7 different animals. *P<0.05 Significant differences between Control and MetR rats

decrease after MetR was MDAL (58% decrease) whereas the other four markers showed 19–35% decreases.

Methionine restriction did not change the amounts of the peptides of heart mitochondrial respiratory complex I peptides (NDUFA9 and NDUFS3), complex II (flavoprotein), complex III (CORE II and Rieske iron-sulfur protein) and complex IV (COX I) (Table 4). The amounts of MnSOD, SIRT5 and AIF were not significantly changed by MetR either (Table 4).

Table 5 shows the full fatty acid composition of heart fatty acids in control and MetR rats. MetR significantly decreased 18:0 and 22:5n-3 and increased 16:1n-7. No significant differences in acyl chain length, general types of fatty acids (SFA, UFA, MUFA or PUFA), and global unsaturation indexes (DBI and PI) were observed between control and MetR rats.

Discussion

In this investigation it is shown for the first time that 7 weeks of 40% MetR significantly lowers mitROS production at complex I during forward electron flow, oxidative damage to mitochondrial DNA, oxidation, glycoxidation and lipoxidation

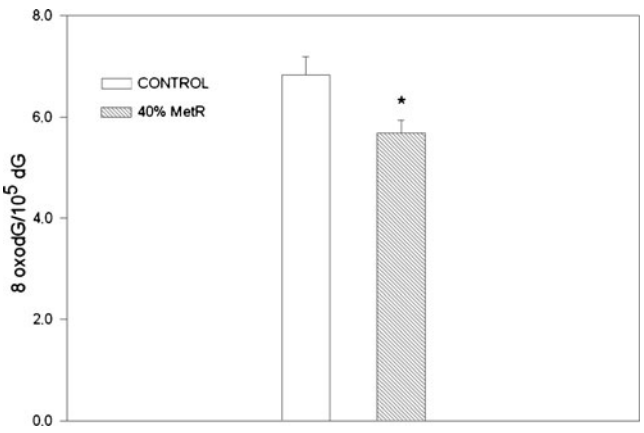

Fig. 1 Oxidative damage in heart mitochondrial DNA from control and 40% methionine restricted rats. Values are means ± SEM from 5 different animals. Asterisks represent significant differences between control and MetR rats (*) P<0.05

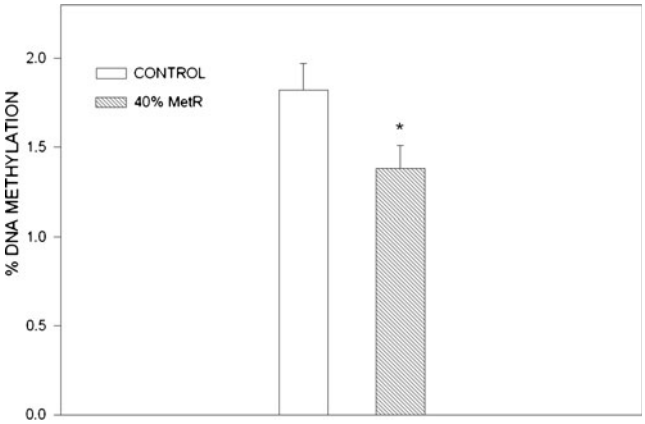

Fig. 2 Genomic DNA methylation in heart from control and 40% methionine restricted rats. Values are means ± SEM from 8 different animals. Asterisks represent significant differences between control and MetR rats (*) P<0.05

of mitochondrial proteins as well as the methylation level of genomic DNA in the rat heart.

Previous studies have shown that the rate of mitROS generation is lowered at complex I both in long-lived species and in rodents subjected to 40% DR (Barja 2004; Gredilla and Barja 2005) as well as in 80% MtR in rat heart (Sanz et al. 2006). In this investigation it is shown that 40% MetR is enough to decrease mitROS production in rat heart. Therefore, MetR can be responsible for the decrease in mitROS production and ensuing oxidative damage that occurs in the heart of rats subjected to (40%) DR which can be involved in their longevity extension. The decrease in mitROS generation after 40% MetR observed in our study

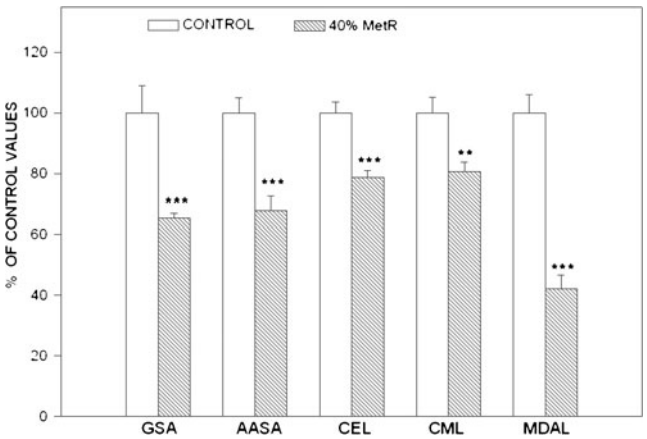

Fig. 3 Protein oxidation, glycoxidation and lipoxidation markers in heart mitochondria from control and 40% methionine restricted rats. Values are means ± SEM from 7 different animals and are expressed as percentage of those in the controls for each parameter. Control values: 5,496.01±493.55 (glutamic semialdehyde, GSA); 216.96±10.74 (AASA, aminoadipic semialdehyde, AASA); 675.19±24.54 (carboxyethyl-lysine, CEL); 1,932.61±99.90 (carboxymethyl-lysine, CML); 581.20±35.52 (malondialdehyde-lysine, MDAL). Units: μmol/mol lysine. Asterisks represent significant differences between control and MetR rats: **P<0.01, ***P<0.001

Table 4 Amounts of respiratory chain complexes, SODMn, SIRT5 and AIF in heart mitochondria from control and 40% methionine restricted (MetR) rats

	Control	MetR
Complex I (39 KDa subunit, NDUFA9)	100±5.01	100.58±5.73
Complex I (30 KD subunit, NDUFS3)	100±15.47	117.56±12.88
Complex II (70 KDa subunit, Flavoprotein)	100±5.83	111.09±4.31
Complex III (48.5 KDa, CORE II)	100±4.70	110.21±5.62
Complex III (29.6 KDa subunit, Rieske iron–sulfur protein)	100±3.29	113.16±13.67
Complex IV (57 KDa subunit, COX I)	100±17.40	115.33±21.34
MnSOD	100±9.34	127.32±12.64
SIRT5	100±7.42	80.12±5.31
AIF	100±3.27	95.89±4.77

Values are means±SEM from 6–7 different animals. Units: Ratio complex I, II, III, IV, MnSOD, SIRT5, and AIF/porin in Arbitrary Units (AUs). Abbreviations: MnSOD, manganese superoxide dismutase, AIF, Apotosis-inducing factor

took place exclusively at complex I since it occurred only with pyruvate/malate + rotenone, a condition in which the free radical source is confined to complex I. This conclusion is also consistent with the lack of significant changes in mitROS generation in the different assays performed with succinate (a complex II-linked substrate). The lack of changes with succinate alone indicates that the decrease in mitROS production does not occur during reverse electron flow from complex II to complex I. Instead, it must occur at a free radical generation site working during forward electron flow and located within complex I between the one receiving electrons from NADH and the rotenone inhibition site. This kind of result is consistent with a previous study in other rat tissues (Caro et al. 2009b).

Concerning the mechanism responsible for the decrease in mitROS production, a general decrease in electron flow is not involved since mitochondrial oxygen consumption only showed changes with succinate + rotenone, a situation in which electrons cannot flow back to complex I, but did not change with pyruvate/malate. Another possibility would be a decrease in the total amount of complex I which would decrease the total amount of superoxide radical generation. However, neither of the two subunits of complex I studied (the 39 and 30 KDa subunits NDUFA9 and NDUFS3) showed changes in their amount, and the same was true for the subunits measured from the other complexes (complex II, III and IV). Therefore, qualitative rather than quantitative changes in complex I seem to be involved in the decrease in mitROS generation in 40% MetR. As previously suggested for DR models (Gredilla et al. 2001) this qualitative change can be due to a decrease in the steady-state degree of electronic reduction of the complex I

Table 5 Fatty acid composition (mol %) of total heart lipids from control and methionine restricted (MetR) rats

	Control	MetR
14:0	1.17±0.03	1.24±0.14
16:0	22.68±0.60	24.06±1.14
16:1n-7	0.91±0.04	1.12±0.07*
18:0	26.49±0.34	24.94±0.11***
18:1n-9	13.23±0.39	13.43±0.21
18:2n-6	9.27±0.42	10.34±0.63
18:3n-3	0.38±0.01	0.42±0.02
18:4n-6	0.11±0.01	0.11±0.01
20:0	0.20±0.01	0.19±0.01
20:1n-9	0.19±0.02	0.20±0.02
20:2n-6	0.97±0.14	0.96±0.04
20:3n-6	0.79±0.06	0.78±0.04
20:4n-6	15.69±0.45	15.09±0.77
20:5n-3	0.15±0.01	0.10±0.01
22:0	0.21±0.02	0.16±0.01
22:4n-6	0.73±0.06	0.69±0.06
22:5n-6	1.40±0.22	1.14±0.06
22:5n-3	0.84±0.04	0.71±0.04*
22:6n-3	4.28±0.26	4.07±0.37
24:0	0.25±0.04	0.19±0.01
ACL	18.15±0.03	18.07±0.06
SFA	51.02±0.71	50.80±1.24
UFA	48.97±0.71	49.19±1.24
MUFA	14.34±0.44	14.76±0.26
PUFA	34.63±0.94	34.43±1.45
PUFAn-6	28.97±0.92	29.12±1.13
PUFAn-3	5.66±0.25	5.31±0.33
DBI	142.16±3.51	138.8±5.74
PI	127.71±4.16	122.01±6.47

Values are means ± SEM from 8 different animals. For abbreviations see the "Matherials and methods" section. Asterisks describe significant differences between Control and MetR rats: *$P<0.05$; ***$P<0.001$

generator which would decrease its tendency to univalently reduce oxygen to the superoxide radical. On the other hand, we observed that the amount of the superoxide dismutase mitochondrial form (MnSOD), the enzyme that scavenges superoxide radical, is not changed by 40% MetR in the rat heart. This is consistent with the idea that the decrease in superoxide radical release from the mitochondria observed in this investigation is due to a true decrease in superoxide radical production and not to an increase in its elimination by MnSOD.

Concerning the cellular mechanism responsible for the qualitative change leading to mitochondria with a lower rate of mitROS generation in MetR, a logical possibility is that changes in DNA methylation are involved. Many

microarray studies have shown that the longevity extension induced by dietary restriction in rodents is dependent on medium- or long-term changes in gene expression (Park and Prolla 2005). This has not been extensively studied in MetR, although a recent study found changes in various messenger RNAs and some protein signaling molecules in the liver of mice subjected to this dietary manipulation (Sun et al. 2009). Dietary methionine is essential for synthesis of S-adenosylmethionine that provides methyl groups required for DNA methylation, an important mechanism of gene expression modification. In fact, we have recently showed that lower levels of methionine supplementation in the diet lead to a lower concentration of S-adenosylmethionine (SAM) in the heart of Wistar rats (Gomez et al. 2009). Therefore, a lower level of global methylation of heart genomic DNA was expected in MetR as it was found indeed in this investigation. Thus, it is possible that the qualitative change leading to a lower mitROS production in DR is secondary to changes in gene expression induced by the decrease in DNA methylation observed in the heart genomic DNA, although this does not eliminate the possible additional involvement of other molecular mechanisms.

Another prolongevity mechanism seems to involve sirtuin proteins. Classic studies in invertebrate models have related the sirtuin SIR2 to longevity. In mammals, seven sirtuins (SIRT1-7) have been described (Nagakawa et al. 2009; Verdin et al. 2010) and they are emerging as important proteins in aging, stress resistance and metabolic regulation. Three mammalian sirtuins, SIRT 3, 4 and 5 are located within the mitochondrial matrix and SIRT3 and SIRT5 are NAD(+)-dependent acetylases that remove acetyl groups from acetyllysine-modified proteins. Recent findings reveal that a large fraction of mitochondrial proteins are acetylated and that mitochondrial protein acetylation is modulated by nutritional status. Among mitochondrial sirtuins, recent studies have highlighted a role for SIRT3 in DR. It has been found that DR decrease oxidative DNA damage in mice tissues and prevents age-related hearing loss, but these changes do not occur in SIRT3-lacking mice (Someya et al. 2010). In that study it is proposed that in response to DR SIRT3 directly deacetylates and activates isocitrate dehydrogenase 2, leading to increased NADPH and increased reduced-to-oxidized glutathione ratio in mitochondria which would decrease mitochondrial oxidative stress and could be partially responsible for protection against age-related changes. To our knowledge this is the study better relating the action of a sirtuin to DR and oxidative stress. In our study, we measured the amount of SIRT5 in order to clarify if it could be involved in the life-extending MetR paradigm, which to our knowledge has never been previously studied. Our results show that the amount of SIRT5 is not modified by MetR in the rat heart. Therefore, SIRT5 does not seem to be involved in the decreases in mitochondrial ROS generation and oxidative damage observed in this investigation. This would be consistent with a role for SIRT5 during DR different from oxidative stress modulation. It has been observed that SIRT5 deacetylates carbamoyl phosphate synthase 1, the rate limiting enzyme for urea synthesis in the urea cycle, increasing its activity (Huang et al. 2010). The regulation of this enzyme by SIRT5 supports a role for SIRT5 under conditions that require ammonia removal such as fasting, and DR. Under these conditions, SIRT5 is increased, resulting in deacetylation of carbamoyl phosphate synthase 1, increased activity of this enzyme, and subsequent conversion of ammonia into carbamoyl phosphate, to be excreted as urea. Therefore, the known role of SIRT5 seems to be rather related to modifications in catabolic flux. Although SIRT5 is likely to target other different mitochondrial substrates, our results do not support the idea that it is involved in the decreases in mitochondrial oxidative stress in MetR (and DR), although further studies are clearly needed before discarding that possibility.

Among possible mechanisms of aging, apoptosis can potentially decrease the number of cells leading to decrements in tissue functionality. This would be especially deleterious in organs like the heart almost exclusively composed of postmitotic cells if apoptosis occurs to a relevant extent in non-dividing cells. The lack of replacement of cells lost by apoptosis would be limiting during aging. In the present study we measured the amount of AIF. A recent investigation has found age-related increases in cytosolic AIF together with other apoptosis-related factors in rat heart which are associated with mitochondrial dysfunction (Ljubicic et al. 2010). AIF however, in addition to apoptotic functions, is also required for mitochondrial oxidative phosphorylation (Porter and Urbano 2006). AIF is a mitochondrial flavoprotein involved in the assembly/maintenance of complex I. Therefore, it has been considered a double edged sword having both life and death functions in cells (Porter and Urbano 2006). Our results show that the amount of AIF was not changed by 40% MetR in rat heart which is consistent with the lack of differences in the amount of complex I. In previous studies we have observed simultaneous decreases in the amounts of AIF and complex I in MetR in rat liver and brain (Caro et al. 2008, 2009b) whereas in the case of kidney (Caro et al. 2009b) no significant changes in AIF were found. All these data, taken together, indicate that the changes in AIF in MetR are tissue specific. In any case our AIF results do not suggest that changes in apoptosis are involved in the beneficial effects of 40% MetR in rat heart.

The decrease in the rate of mitROS production in the heart of MetR rats was accompanied by beneficial decreases in oxidative damage to important macromolecules. First,

oxidative damage to mtDNA was decreased by 40% MetR. Previous studies had found decreases in mitROS production and 8-oxodG in mtDNA in 80% MetR in rat heart (Sanz et al. 2006). The results obtained here show that 40% restriction of dietary methionine is enough to lower steady-state oxidative damage to mtDNA. This is important since standard DR is usually performed at 40% restriction of food intake. Previous investigations have shown that the lowering of mitROS production induced by 40% DR (Gredilla et al. 2001; Lopez-Torres et al. 2002) and by 40% protein restriction (Sanz et al. 2004) is always accompanied by decreases in the level of 8-oxodG in mtDNA in rat tissues. All these results, together with previous ones (Sanz et al. 2004; Caro et al. 2009a) 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 (40%) DR and protein restriction. Since ROS can produce DNA single- and double-strand breaks in addition to oxidizing the bases, MetR could contribute to extend life-span by helping to decrease the formation of mtDNA mutations that occur during rodent and human aging (Barja 2004; Khrapko and Vijg 2007). In addition to lowering 8-oxodG, in our study a global decrease in oxidative, lipoxidative, and glycoxidative damage to heart mitochondrial proteins was also found. The stronger decrement observed was that of the lipoxidation marker MDAL (58% decrease) whereas the other protein modification markers showed 19%–35% decreases. This higher sensitivity of MDAL compared to the other four protein modification markers has been also observed in various previous studies of DR and MetR, possibly indicating the prominent role of the decrease in MDA-related lipid peroxidation pathways in these anti-aging models. On the other hand, the decrease in the two lipoperoxidation-related markers CML and MDAL was not due to decreases in the degree of unsaturation and thus in the sensitivity to oxidative damage of heart mitochondrial fatty acids, because neither the total number of double bonds (DBI) nor the PI were changed by 40% MetR. Instead, the lower mitROS production of MetR animals seems to directly decrease protein oxidation (lower GSA and AASA) and to also lower glycoxidation and lipoxidation processes finally contributing to a decrease glycoxidative (lower CEL and CML) and lipoxidative (lower CML and MDAL) modification of rat heart mitochondrial proteins.

Finally, it is known that two traits correlate with maximum longevity across animal species. Long-lived mammals and birds have a low rate of mitROS production and a low degree of fatty acid unsaturation (low DBI), which lower their oxidative stress (Pamplona and Barja 2007). In many previous investigations (40%) DR, a longevity-extending manipulation, lowered mitROS production without changing tissue DBI. We have found that 80% MetR decreases rat heart DBI (Sanz et al. 2006), but when we applied MetR at 40% in the present investigation no changes in this parameter were observed. This kind of result has been also observed in other rat organs (Caro et al. 2008, 2009b). Therefore, the lack of change in DBI at 40%MetR fits well with the lack of effect of (40%) DR on the global degree of fatty acid unsaturation. Decreases in DBI in MetR are limited to 80% MetR, a dietary modification not occurring in (40%) DR. Therefore, among the two main oxidative stress-related traits of long-lived animals, only a low rate of mitROS production (and not a low degree of fatty acid unsaturation) seems to be involved in the life extension effect of MetR and DR.

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