

# Effect of methionine dietary supplementation on mitochondrial oxygen radical generation and oxidative DNA damage in rat liver and heart

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**Abstract** Methionine restriction without energy restriction increases, like caloric restriction, maximum longevity in rodents. Previous studies have shown that methionine restriction strongly decreases mitochondrial reactive oxygen species (ROS) production and oxidative damage to mitochondrial DNA, lowers membrane unsaturation, and decreases five different markers of protein oxidation in rat heart and liver mitochondria. It is unknown whether methionine supplementation in the diet can induce opposite changes, which is also interesting because excessive dietary methionine is hepatotoxic and induces cardiovascular alterations. Because the detailed mechanisms of methionine-related hepatotoxicity and cardiovascular toxicity are poorly understood and today many Western human populations consume levels of dietary protein (and thus, methionine) 2–3.3 fold higher than the average adult requirement, in the present experiment we analyze the effect of a methionine supplemented diet on mitochondrial ROS production and oxidative damage in the rat liver and heart mitochondria. In this investigation male Wistar rats were fed either a L-methionine-supplemented (2.5 g/100 g) diet without changing any other dietary components or a control (0.86 g/100 g) diet for 7 weeks. It was found that methionine supplementation increased mitochondrial ROS generation and percent free radical leak in rat liver mitochondria but not

in rat heart. In agreement with these data oxidative damage to mitochondrial DNA increased only in rat liver, but no changes were observed in five different markers of protein oxidation in both organs. The content of mitochondrial respiratory chain complexes and AIF (apoptosis inducing factor) did not change after the dietary supplementation while fatty acid unsaturation decreased. Methionine, S-AdenosylMethionine and S-AdenosylHomocysteine concentration increased in both organs in the supplemented group. These results show that methionine supplementation in the diet specifically increases mitochondrial ROS production and mitochondrial DNA oxidative damage in rat liver mitochondria offering a plausible mechanism for its hepatotoxicity.

**Keywords** Methionine supplementation · Free radicals · Aging · ROS · Oxidative stress · Mitochondria · DNA damage

## Introduction

Both comparative and dietary restriction studies point to the mitochondrial reactive oxygen species (mtROS) generation rate as a main source of oxidative stress contributing to aging (Lopez-Torres and Barja 2008a). Long-lived species show low rates of mitochondrial ROS production together with low levels of oxidative damage to macromolecules including mitochondrial DNA, proteins and lipids (Lopez-Torres and Barja 2008b; Lambert et al. 2007; Robert et al. 2007; Pamplona et al. 2002). Dietary restriction (DR) that correlates with increases of maximum longevity in rodents as well as in a wide range of organisms including yeast, nematodes, and insects, results in the same two kind of changes, low rates of mtROS generation and decreased levels of mitochondrial DNA oxidative damage (Gredilla et al. 2001a). Although the anti-aging effect of DR has been

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classically attributed to the decreased intake of calories themselves, recent studies question this consensus (Mair et al. 2005; Min and Tatar 2006). Furthermore, recent studies confirm that neither lipid (Sanz et al. 2006a) nor carbohydrate restriction (Sanz et al. 2006b) are responsible for those changes in oxidative stress. In contrast, protein restriction reduces mitochondrial ROS production and oxidative stress in the same way as observed in DR, and thus protein must be the dietary factor responsible for the decrease in mtROS production and mitochondrial oxidative damage during DR (Sanz et al. 2004; Gomez et al. 2007). Besides, restriction of protein intake also increases maximum longevity in rats and mice although to a lesser extent than DR, being the increase in magnitude around 30–40% that of DR (Pamplona and Barja 2006). Neither carbohydrate (Khorakova et al. 1990) nor lipid restriction (Iwasaki et al. 1988; Shimokawa et al. 1996) seem to be able to modify rodent longevity but interestingly, several lines of evidence implicate methionine intake and metabolism in modulating life span. It has been observed that restriction of only this amino acid, methionine, increases maximum longevity in rodents (Orentreich et al. 1993; Richie et al. 1994; Zimmerman et al. 2003; Miller et al. 2005), and decreases mtROS generation and mitochondrial oxidative damage (Sanz et al. 2006c) similarly to classical dietary restriction and protein restriction. A capacity of methionine to modify lifespan would be consistent with the exquisite sensitivity of mammals to dietary methionine intake.

Methionine is an essential sulphur amino acid for mammals, important for methylation reactions and as a precursor for cysteine. However excessive intake of methionine is highly toxic and this toxicity far exceeds that produced by any other amino acid (Harper et al. 1970), leading to different vital organs damage and increasing tissue oxidative stress (Mori and Hirayama 2000; Tyagi et al. 2009) with similar negative effects to those observed in rats fed with high protein content diets. Methionine supplementation induces growth retardation (Benevenga et al. 1976), anemia (Yokota et al. 1978), vascular damage (Troen et al. 2003), kidney damage, specifically tubules hypertrophy (Kumagai et al. 2002), iron accumulation and liver dysfunction (Mori and Hirayama 2000), besides other alterations in different organs. Moreover, liver damage has been observed in patients with inborn errors of metabolism associated with hypermethioninemia (Labruno et al. 1990). So it seems apparent that liver plays a central role in methionine metabolism (Stipanuk 2004). Some of these harmful effects have been attributed not to methionine itself but to methionine's product, homocysteine. However, in other cases there is considerable evidence of a direct methionine toxic effect (Harper et al. 1970; Troen et al. 2007).

In order to clarify if oxidative stress is involved in methionine related toxicity, male Wistar rats were fed a

2.5% methionine supplemented diet for seven weeks. The dietary regime changed only dietary methionine content, whereas all the other dietary components or calories were held at similar levels in both dietary groups (control and methionine supplemented). In the liver and heart mitochondria from these rats we have measured mtROS generation rates, mitochondrial oxygen consumption in both resting (state 4) and phosphorylating state (state 3), % free radical leak (FRL) in the respiratory chain, the content of complexes I, II, III and IV of the respiratory chain, apoptosis inducing factor (AIF), the marker of mtDNA oxidative damage 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), and five markers of protein oxidation (the specific protein carbonyls glutamic and aminoadipic semialdehydes, GSA and AASA respectively), glycooxidation (carboxyethyl-lysine, CEL and carboxymethyl-lysine, CML) and lipoxidation (malondialdehydelysine, MDAL). Since it is known that the phospholipid unsaturation degree can affect markers of protein lipoxidation, the full fatty acid composition was measured in liver and heart from control and methionine supplemented animals. Methionine (Met) and the methionine metabolites, S-AdenosylMethionine (SAM) and S-AdenosylHomocysteine (SAH), were also analyzed and the ratio SAM/SAH was calculated as the methylation potential.

## 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 at  $22 \pm 2$  °C and  $50 \pm 10\%$  relative humidity. Semipurified diets prepared by MP Biochemicals (Irvine, CA, USA) were used (detailed composition shown in Table 1). The composition of the methionine supplemented diet was similar to that of the control diet except that L-methionine was present at 2.5% instead of the 0.86% from the control diet (AIN 93G). The % increase in L-methionine in the supplemented diet was compensated with decreases in all the rest of the dietary components in proportion to their presence in the diet. The control group received every day the same amount of food that the 2.5% methionine supplemented animals had eaten as a mean the previous week (pair feeding). After 7 weeks of dietary treatment animals were sacrificed by decapitation. Liver and heart samples were immediately processed or frozen at  $-80$  °C for later assays.

### Liver and heart mitochondria isolation

After decapitation liver and heart were immediately processed to obtain functional mitochondria. Liver was rinsed and fat

**Table 1** Detailed composition of the semipurified diets used in this study (Control and 2.5% Methionine supplemented)

| Component           | Control (g/100g) | Methionine Supplemented (g/100g) |
|---------------------|------------------|----------------------------------|
| L-Arginine          | 1.12             | 1.10                             |
| L-Lysine            | 1.44             | 1.42                             |
| L-Histidine         | 0.33             | 0.33                             |
| L-Leucine           | 1.11             | 1.09                             |
| L-Isoleucine        | 0.82             | 0.81                             |
| L-Valine            | 0.82             | 0.81                             |
| L-Threonine         | 0.82             | 0.81                             |
| L-Tryptophan        | 0.18             | 0.18                             |
| <b>L-Methionine</b> | <b>0.86</b>      | <b>2.50</b>                      |
| L-Glutamic acid     | 2.70             | 2.66                             |
| L-Phenylalanine     | 1.16             | 1.14                             |
| L-Glycine           | 2.33             | 2.29                             |
| Dextrine            | 5.00             | 4.92                             |
| Corn starch         | 43.61            | 42.90                            |
| Sucrose             | 20.00            | 19.67                            |
| Cellulose           | 5.00             | 4.92                             |
| Choline bitartrate  | 0.20             | 0.20                             |
| Vitamin mix (AIN)   | 1.00             | 0.98                             |
| Mineral mix (AIN)   | 3.50             | 3.44                             |
| Corn oil            | 8.00             | 7.87                             |
| Total (% in weight) | 100.00           | 100.00                           |

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  $1,000 \times g$  for 10 min. Supernatants were centrifuged at  $10,000 \times g$  for 10 min and the resulting supernatants were eliminated. Pellets were resuspended in 40 ml of isolation buffer without EDTA and centrifuged at  $1,000 \times g$  for 10 min. Mitochondria were obtained after centrifugation of the supernatants at  $10,000 \times g$  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. Heart mitochondria were obtained by the procedure of Mela and Seitz (1979) with modifications. Briefly, dissected ventricles were chopped into small pieces and were homogenized with a loose-fitting 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 samples stood for 1 min, 25 ml of additional isolation buffer containing 25 mg albumin was added, and gentle homogenization was performed again with a tighter fitting pestle. The nuclei and cell debris were removed by centrifugation at  $1,000 \times g$  for 10 min. Heart mitochondria were obtained by centrifuging the supernatant twice at  $10,000$  for 10 min. The

mitochondrial pellets were resuspended in 1 ml of isolation buffer. All the above procedures were performed at 5 °C. Mitochondrial protein was measured by the Biuret method. The final mitochondrial suspensions were maintained over ice and were immediately used for  $H_2O_2$  production and  $O_2$  consumption measurements.

#### Mitochondrial $H_2O_2$ production

The rate of mitochondrial  $H_2O_2$  production was assayed by measuring the increase in fluorescence (excitation at 312 nm, emission at 420 nm) due to homovanillic acid oxidation by  $H_2O_2$  in the presence of horseradish peroxidase as described in 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, 2.5 mM glutamate/2.5 mM malate, or 5 mM succinate + 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  $KH_2PO_4$ , 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. The assays with succinate as substrate were performed in the presence of rotenone in order to avoid the backward flow of electrons to complex I. In some experiments rotenone (2  $\mu$ M) or antimycin A (2  $\mu$ M) were additionally included in the reaction mixture to assay maximum rates of complex I, or complex III  $H_2O_2$  generation respectively. Duplicated samples were incubated for 15 min at 37 °C. The reaction was stopped 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 liver mitochondria was measured at 37 °C in a water-thermostated incubation chamber with a computer-controlled Clark-type  $O_2$  electrode (Oxygraph, Hansatech, UK) in 0.5 ml of the same incubation buffer used for the  $H_2O_2$  measurements. The substrates used were complex I- (2.5 mM pyruvate/2.5 mM malate or 2.5 mM glutamate/2.5 mM malate) or complex II-linked (5 mM succinate + 2  $\mu$ M rotenone). The assays were performed in the absence (state 4-resting) and in the presence (state 3-phosphorylating) of 500  $\mu$ M ADP.

### Mitochondrial free radical leak (FRL)

H<sub>2</sub>O<sub>2</sub> production and O<sub>2</sub> consumption were measured in parallel in the same mitochondria sample under similar experimental conditions. This allowed the calculation of the fraction of electrons out of sequence which reduces O<sub>2</sub> to ROS at the respiratory chain (the percent free radical leak, FRL) instead of reaching cytochrome oxidase to reduce O<sub>2</sub> to water. Since two electrons are needed to reduce 1 mole of O<sub>2</sub> to H<sub>2</sub>O<sub>2</sub>, whereas four electrons are transferred in the reduction of 1 mole of O<sub>2</sub> to water, the percent free radical leak was calculated as the rate of H<sub>2</sub>O<sub>2</sub> production divided by two times the rate of O<sub>2</sub> consumption, and the result was multiplied by 100. The lower the FRL, the higher is the efficiency of mitochondria in avoiding oxygen radical generation at the respiratory chain.

### Measurement of mitochondrial complexes I, II, III and IV and AIF by western blot

The content of mitochondrial complexes I to IV and AIF (apoptosis-inducing factor) was estimated using western blot analysis as previously described (Caro et al. 2009). Briefly, immunodetection was performed using a monoclonal antibody specific for the NDUFA9 subunit of complex I, the subunit 70 kDa of complex II, the subunit Core II of complex III, and the subunit I of complex IV (1:1000, 1:500, 1:1000 and 1:1000, respectively; Molecular Probes, Invitrogen Ltd., UK), and a polyclonal antibody for C-terminus (amino acids 593–613) of AIF (1:1000; Sigma, Madrid, Spain). An antibody to porin (1:5000, Molecular Probes, Invitrogen Ltd., UK) was also used in order to determine the proportion of complex I, II, III and IV and AIF referred to total mitochondrial mass. Appropriate peroxidase-coupled secondary antibodies and chemiluminescence HRP substrate (Millipore, MA, USA) were used for primary antibody detection. Signal quantification and recording was performed with a ChemiDoc BioRad equipment (Bio-Rad Laboratories, Inc., Barcelona, Spain). Protein concentration was determined by the Bradford method. Data were expressed as arbitrary units.

### Oxidative damage to mtDNA

Isolation of mtDNA was performed by the method of Latorre et al. (1986) adapted to mammals (Asuncion et al. 1996). The isolated mitochondrial DNA was digested to deoxynucleoside level by incubation at 50 °C with 5 U of nuclease P1 (in 20 µl of 20 mM sodium acetate, 10 mM ZnCl<sub>2</sub>, 15% glycerol, pH 4.8) for 30 min and 4 U of alkaline phosphatase (in 20 µl of 1 M Tris-HCl, pH 8.0) for 1 h (Loft and Poulsen 1999). 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 Tecnokroma mediterranea sea18 column (5 µm, 4.6 mm x 25 cm), and was eluted with a mobile phase containing 6.5% acetonitrile and 50 mM phosphate buffer pH 5.0. A Gilson 305 pump with a Gilson 805 manometric module at 1 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 (400 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.

### Oxidation-derived protein damage markers measurements by GC/MS

GSA, AASA, CEL, CLM and MDAL were determined as trifluoroacetic acid methyl esters derivatives in acid hydrolyzed delipidated and reduced mitochondrial protein samples by GC/MS using an isotope dilution method as previously described (Caro et al. 2009) using a 6890 Series II gas chromatograph (Agilent, Barcelona, Spain) with a 5973A Series Mass Selective Detector and a 7683 Series automatic injector, 30 m×0.25 mm × 0.25 µm HP-5MS column, and the described temperature program (Caro et al. 2009). Quantification was performed by external standardization using standard curves constructed from mixtures of deuterated and non-deuterated standards. Analyses were carried out by selected ion-monitoring GC/MS (SIM-GC/MS). The ions used were: lysine and [2H8]lysine, m/z 180 and 187, respectively; 5-hydroxy-2-aminovaleric acid and [2H5]5-hydroxy-2-aminovaleric acid (stable derivatives of GSA), m/z 280 and 285, respectively; 6-hydroxy-2-aminocaproic acid and [2H4]6-hydroxy-2-aminocaproic acid (stable derivatives of AASA), m/z 294 and 298, respectively; CML and [2H4]CML, m/z 392 and 396, respectively; CEL and [2H4]CEL, m/z 379 and 383, respectively; and MDAL and [2H8]MDAL, m/z 474 and 482, respectively. The amounts of product were expressed as micromoles of GSA, AASA, CEL, CLM or MDAL/mol lysine.

### Fatty acid analyses by GC/MS

Fatty acyl groups in liver and heart lipids were analyzed as methyl esters derivatives by GC/MS as previously described



(Caro et al. 2009). Separation was performed in a SP2330 capillary column (30 m×0.25 mm × 0.20 μm) in a Hewlett Packard 6890 Series II gas chromatograph. A Hewlett Packard 5973A mass spectrometer was used as detector in the electron-impact mode. Identification of fatty acyl methyl esters was made by comparison with authentic standards and on the basis of mass spectra. Results are expressed as mol%. The following fatty acyl indices were also calculated: saturated fatty acids (SFA); unsaturated fatty acids (UFA); monounsaturated fatty acids (MUFA); polyunsaturated fatty acids from n-3 and n-6 series (PUFAn-3 and PUFAn-6); average chain length (ACL)=[(Σ%Total14 × 14) + (Σ% Total16×16) + (Σ%Total18×18) + (Σ%Total20×20) + (Σ% Total22×22)]/100; double bond index (DBI)=[(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)].

Methionine, S-Adenosylmethionine and S-Adenosylhomocysteine measurement by LC ESI-QTOF MS

Met, SAM and SAH were detected and quantified by liquid chromatography coupled to quadrupole time-of-flight mass spectrometry operating in positive electrospray ionization mode (LC ESI-QTOF MS). 100 mg of the frozen tissue was powdered under liquid nitrogen in a mortar, transferred into a preweighed vial containing 200 μL of 4 mM ammonium acetate, 0.1% formic acid, 0.1% heptafluorobutyric acid and 440 picomoles of deuterated d3-Met (as internal standard) pH 2.5 and shaken vigorously for 15 min. The solution was heated at 80 °C for 5 min to precipitate endogenous proteins, cooled immediately on ice for 2 min and centrifuged for 15 min at 14 000×g to remove any particulate. Supernatants were recovered and filtered by centrifugal filter device (UFC3LTK00 Ultrafree-MC, Millipore, USA). Samples were analyzed using a LC nano pump 1200 Series G2226A coupled to a QTOF LC-MS G6510A equipment (Agilent Technologies, Barcelona, Spain). An aliquot of 8 μL of extracted samples were applied to a C18 reverse-phase column (Luna 3n pfp(2), 100A, 150×2 mm ID, Phenomenex, CA, USA). The flow rate was 200 μL/min with solvent A composed of water containing 0.1% formic acid and solvent B composed of 95% acetonitrile, 5% water containing 0.1% formic acid. The gradient consisted 0% solvent B for 5 min, followed by a gradient to 100% in 20 min, held at 100% solvent B for 5 min, and re-equilibrated at 100% solvent A for 6 min. Data were collected in positive electrospray mode operated in full-scan mode at 100 to 3000 m/z. The capillary voltage was 3500 V

with a scan rate of 1 scan/s; nitrogen was used as nebulizer gas (gas flow, 5 L/min; gas temperature, 325 °C).

Quantification was performed by external standardization using standard curves constructed from mixtures of standards (Met, SAM and SAH) and deuterated internal standard (d3-met) at the same final concentration than in samples. Linearity was observed over the range of concentrations studied, 0.07–335 μM, 0.25–50 μM and 0.26–130 μM for Met, SAM and SAH, respectively. The Masshunter Data Analysis Software was used to collect the results and the Masshunter Qualitative Analysis Software to do the integration and extraction of peak intensities. The *m/z* values used for quantification were: *m/z* 150.0533 [M+H]<sup>+</sup> for Met, *m/z* 399.14506 [M+H]<sup>+</sup> for SAM, *m/z* 385.12886 [M+H]<sup>+</sup> for SAH and *m/z* 153.07716 [M+H]<sup>+</sup> for d3-Met. The results were calculated to be expressed as nmol·g<sup>-1</sup> of tissue. The ratio SAM/SAH, termed the methylation potencial, was calculated as quantitative parameters of intracellular methylation reactions.

#### Statistical analyses

All values were expressed as means ± SEM. Comparisons between control and methionine supplemented groups were analyzed by Student's *t* tests. The minimum level of statistical significance was set at *p*<0.05 in all the analyses.

#### Results

The dietary treatment did not significantly change animal weight (437.50±8.82 g in control, and 423.63±9.25 g in methionine supplemented animals), and the weight of liver and heart from methionine supplemented animals (11.41±0.78 g and 0.92±0.05 g respectively) did not differ from controls (11.09±0.52 g and 0.98±0.03 g respectively) either.

The rate of oxygen consumption of liver and heart mitochondria was measured without (State 4) and with (State 3) ADP in the presence of complex I- (pyruvate/malate or glutamate/malate) and complex II- (succinate) linked substrates. The addition of ADP strongly increased the rate of oxygen consumption in all cases, indicating tight coupling of mitochondrial preparations (Table 2). Small increases in oxygen consumption in the methionine supplemented group were detected only with succinate in the heart, and with succinate or glutamate/malate (in state 3) in the liver (Table 2).

The rate of mitochondrial ROS generation with pyruvate/malate as substrate was significantly increased in the liver from the methionine supplemented animals compared to controls (Fig. 1). The maximum rates of ROS production were measured using appropriate combinations of substrates and inhibitors of the respiratory chain. Full reduction of

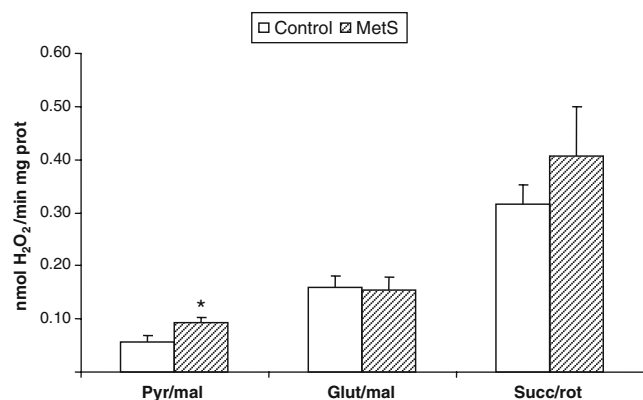
**Table 2** Rates of oxygen consumption (nmoles O<sub>2</sub>/min mg protein) of liver and heart mitochondria from control and methionine supplemented Wistar rats

| LIVER                        | Control   | Methionine Supplemented |
|------------------------------|-----------|-------------------------|
| Pyruvate/malate (state 4)    | 7.8±1.0   | 6.6±0.7                 |
| Pyruvate/malate (state 3)    | 19.5±2.4  | 21.0±2.3                |
| Glutamate/malate (state 4)   | 9.4±0.8   | 10.3±0.8                |
| Glutamate/malate (state 3)   | 72.1±5.3  | 92.3±6.3 <sup>a</sup>   |
| Succinate/rotenone (state 4) | 26.2±2.0  | 29.9±1.6                |
| Succinate/rotenone (state 3) | 100.9±9.1 | 121.7±3.3 <sup>a</sup>  |
| HEART                        | Control   | Methionine Supplemented |
| Pyruvate/malate (state 4)    | 28.4±4.7  | 23.4±1.7                |
| Pyruvate/malate (state 3)    | 72.2±5.4  | 71.0±6.1                |
| Succinate/rotenone (state 4) | 85.8±9.4  | 112.9±9.5 <sup>a</sup>  |
| Succinate/rotenone (state 3) | 112.1±5.3 | 140.3±10.7 <sup>a</sup> |

Values are mean ± SEM from 8 different animals. State 4, oxygen consumption in the absence of ADP. State 3, oxygen consumption in the presence of ADP

<sup>a</sup> significant differences between control and methionine supplemented rats:  $p < 0.05$

complex I was performed adding rotenone to pyruvate/malate or glutamate/malate (complex I-linked substrates) and the maximum rates of ROS production were also increased in the methionine supplemented animals (Table 3). No significant differences were observed using other substrate or substrate plus inhibitor (Fig. 1, Table 3). The free radical leak (FRL), percentage of total electron flow in the respiratory chain directed to ROS generation, was also significantly higher in the liver from the methionine supplemented group only with pyruvate/malate as substrate (Fig. 2). No changes in ROS production or FRL due to the dietary treatment were found in heart tissue (Figs. 3 and 4; Table 3).

**Fig. 1** Rates of H<sub>2</sub>O<sub>2</sub> generation of liver mitochondria from control and methionine supplemented Wistar rats in the presence of pyruvate/malate, glutamate/malate or succinate/rotenone as substrates. Values are means ± SEM from 8 different animals. \*significant differences between control and methionine supplemented rats:  $p < 0.01$ **Table 3** Maximum rates of H<sub>2</sub>O<sub>2</sub> production (nmoles H<sub>2</sub>O<sub>2</sub>/min mg protein) of liver and heart mitochondria in the presence of substrates plus complex I and III specific inhibitors from control and methionine supplemented Wistar rats

| LIVER                       | Control   | Methionine Supplemented |
|-----------------------------|-----------|-------------------------|
| Pyruvate/malate + rotenone  | 0.38±0.04 | 0.58±0.10 <sup>a</sup>  |
| Glutamate/malate + rotenone | 0.46±0.04 | 0.65±0.10 <sup>a</sup>  |
| Succinate + TTFA            | 0.30±0.03 | 0.43±0.07               |
| HEART                       | Control   | Methionine Supplemented |
| Pyruvate/malate + rotenone  | 1.49±0.12 | 1.66±0.26               |
| Glutamate/malate + rotenone | 1.47±0.08 | 2.01±0.29               |
| Succinate + TTFA            | 0.64±0.06 | 0.70±0.06               |

Values are mean ± SEM from 8 different animals

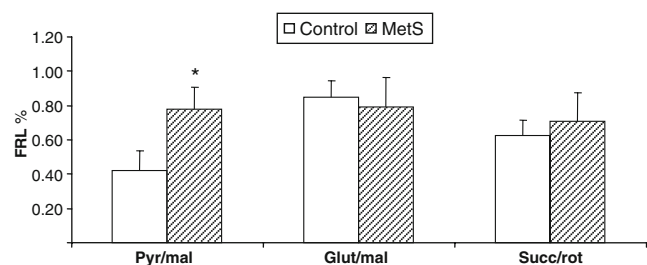
<sup>a</sup> significant differences between control and methionine supplemented rats:  $p < 0.05$  TTFA, thenoyltrifluoroacetone

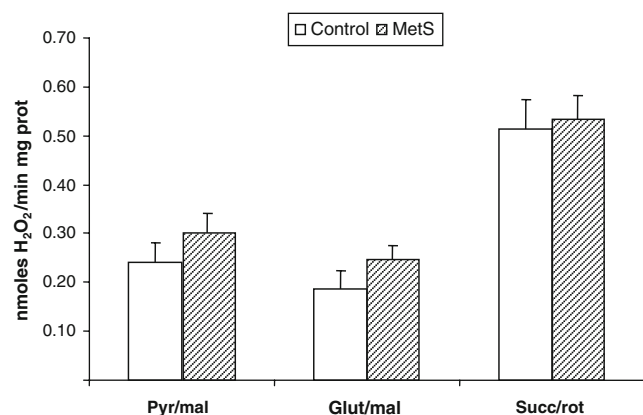
The amount of protein complexes I, II and III of the respiratory chain and AIF did not differ between control and supplemented group in both organs studied (Table 4). Complex IV amount significantly decreased in the methionine supplemented rat liver mitochondria (Table 4).

In correlation to the changes observed in mitochondrial ROS production, mitochondrial DNA oxidative damage increased in the liver but not in the heart from methionine supplemented animals (Figs. 5 and 6).

The fatty acid composition of total lipids from liver is shown in Table 5. Methionine supplementation significantly decreased 16:1n-7, 18:3n-3, 20:3n-6, 20:4n-6, 20:5n-3 and 22:4n-6 and increased 18:1n-9 and 18:2n-6 resulting in a significant decrease in PUFA n-3. Regarding the global fatty acid unsaturation indexes, both the double bond index and the peroxidizability index decreased in the liver of methionine supplemented rats (Table 5). No changes were observed in any fatty acids in the heart tissue (Table 6).

The values of five different markers of protein modification are shown in Table 7. In both organs studied, no

**Fig. 2** Free radical leak (FRL; %) of liver mitochondria from control and methionine supplemented Wistar rats in the presence of pyruvate/malate, glutamate/malate or succinate/rotenone as substrates. Values are means ± SEM from 8 different animals. \*significant differences between control and methionine supplemented rats:  $p < 0.01$



**Fig. 3** Rates of H<sub>2</sub>O<sub>2</sub> generation of heart mitochondria from control and methionine supplemented Wistar rats in the presence of pyruvate/malate, glutamate/malate or succinate/rotenone as substrates. Values are means  $\pm$  SEM from 8 different animals

significant differences for any of them were found after methionine supplementation.

Methionine supplementation in the diet increased the tissue concentration of Met, SAM and SAH in both liver and heart (Table 8). The ratio Met/SAM was higher and the ratio SAM/SAH was lower in methionine supplemented animals in the heart without significant changes in the case of liver (Table 8).

## Discussion

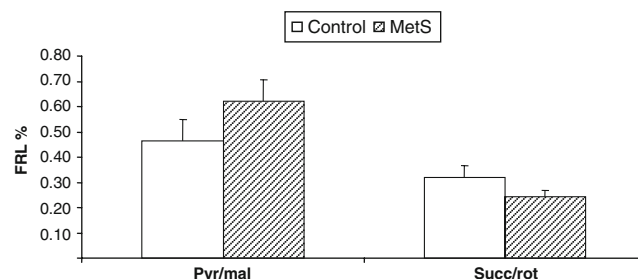
In the present study it is shown for the first time that 7 weeks of 2.5% methionine supplementation in the diet increases mitochondrial ROS production, the percent free radical leak and mitochondrial DNA oxidative damage in the rat liver while these increases do not take place in the rat heart. These results suggest that the increase in mitochondrial ROS production could be one of the mechanisms responsible for methionine-related hepatotoxicity.

Previous studies have shown that methionine restriction, similarly to protein restriction (reviewed in Pamplona and Barja 2006), increases maximum longevity in rats (Richie et al. 1994; Zimmerman et al. 2003) and mice (Miller et al. 2005). Similar results have been reported more recently in *Drosophila melanogaster*, with longevity increases after protein (Mair et al. 2005; Min and Tatar 2006) and methionine restriction (Troen et al. 2007). Besides its effects on maximum longevity, methionine restriction also delays many age-related changes and degenerative diseases in rodents. Among them, MetR in rats strongly decreases visceral fat mass and prevents age-related increases in blood triglycerides and cholesterol (Malloy et al. 2006). In mice, MetR slows cataract development, minimizes age-related changes in T cells, lowers serum glucose, insulin,

IGF-I and thyroid hormone levels, and increases resistance to oxidative liver cell injury (Miller et al. 2005). On the other hand, experiments recently performed in rats demonstrate that protein restriction and specifically methionine restriction are responsible for the decrease in mitochondrial ROS production and mitochondrial DNA oxidative damage that takes place during dietary restriction (Sanz et al. 2006c; Caro et al. 2008), therefore contributing to clarify the relationship between dietary methionine, oxidative stress and life span extension.

In addition, several studies have shown that excessive dietary methionine increases tissue oxidative stress (Toborek et al. 1996; Mori and Hirayama 2000; Park et al. 2008) and is related to the development of atherosclerosis (Troen et al. 2003). Methionine supplementation increases plasma hydroperoxides and LDL-cholesterol (Hidioglou et al. 2004), raises iron and lipid peroxidation, conjugated dienes and cholesterol in rat liver, alters liver antioxidant enzymes and glutathione (Mori and Hirayama 2000; Dever and Elfarra 2008), and decreases vitamin E levels in liver and heart (Hidioglou et al. 2004). It also raises plasma, heart and aortic homocysteine levels leading to angiotoxicity, mitochondrial degeneration in arterial smooth muscle cells and accelerated aging of rat vascular system. Hypertension and coronary disease are also induced by excess methionine, that seems to accelerate brain aging too (Stefanello et al. 2005; Obeid and Herrmann 2006). Moreover, it is known that the high methionine content of western population diets far exceeds the recommended daily dietary allowance (RDA). However the detailed mechanism of methionine toxicity has not been clarified. Our results show that, reversing what we have previously observed after methionine restriction, a decrease in mtROS production, methionine supplementation in the diet leads to an increased mtROS generation in the rat liver.

In mammals, methionine is an essential amino acid usually consumed as component of dietary proteins. Absorption of methionine is highly efficient, and methionine enters the plasma and circulates until it is removed by tissues. Liver plays a central role and removes a substantial proportion of



**Fig. 4** Free radical leak (FRL; %) of heart mitochondria from control and methionine supplemented Wistar rats in the presence of pyruvate/malate or succinate/rotenone as substrates

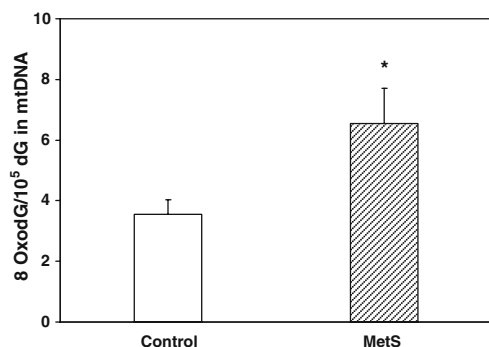
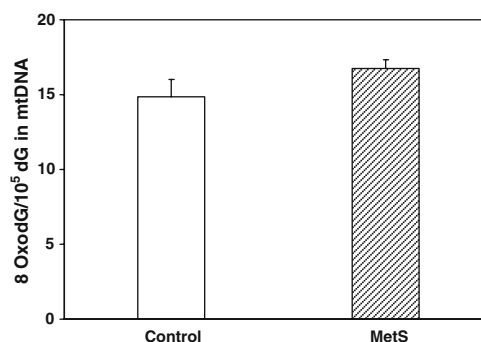
**Table 4** Mitochondrial respiratory complexes and AIF in liver and heart from control and methionine supplemented Wistar ratsValues are mean  $\pm$  SEMUnits: ratio complex I, II, III, IV and AIF/porin in Arbitrary Units from  $n=6-8$  samples. AIF, Apoptosis-inducing factor<sup>a</sup> significant differences between control and methionine supplemented rats:  $p<0.01$ 

| <b>LIVER</b>                              |  | Control         | Methionine Supplemented       |
|---|--|-----------------|-------------------------------|
| Complex I (39 KDa subunit, NDUFA9)        |  | 100 $\pm$ 22.57 | 99.25 $\pm$ 17.08             |
| Complex II (70 KDa subunit, flavoprotein) |  | 100 $\pm$ 22.43 | 68.26 $\pm$ 16.49             |
| Complex III (48.5 KDa, CORE II)           |  | 100 $\pm$ 32.60 | 128.82 $\pm$ 19.74            |
| Complex IV (57 KDa subunit, COXI)         |  | 100 $\pm$ 21.18 | 50.42 $\pm$ 6.38 <sup>a</sup> |
| AIF                                       |  | 100 $\pm$ 9.16  | 77.41 $\pm$ 13.13             |
| <b>HEART</b>                              |  | Control         | Methionine Supplemented       |
| Complex I (39 KDa subunit, NDUFA9)        |  | 100 $\pm$ 7.70  | 92.29 $\pm$ 10.07             |
| Complex II (70 KDa subunit, Flavoprotein) |  | 100 $\pm$ 11.26 | 114.27 $\pm$ 22.33            |
| Complex III ((48.5 KDa, CORE II)          |  | 100 $\pm$ 8.48  | 106.18 $\pm$ 8.82             |
| Complex IV (57 KDa subunit, COXI)         |  | 100 $\pm$ 14.80 | 93.03 $\pm$ 9.24              |
| AIF                                       |  | 100 $\pm$ 11.98 | 88.57 $\pm$ 9.76              |

sulphur containing amino acids from the portal circulation. When we analyzed mitochondrial ROS production in rats supplemented with methionine in the diet, we found that mitochondrial ROS generation is increased in the liver but not in the heart tissue. A greater sensitivity of liver tissue mitochondria to either methionine itself or any of its metabolites could be the reason for this increased mitochondrial ROS production in the rat liver from methionine supplemented animals. Furthermore, excess iron deposition in hepatocytes has been reported after methionine supplementation and would be most likely related to enhancement of oxidative stress and free radical reactions stimulated by iron (Mori and Hirayama 2000).

Among possible mechanisms responsible for the mtROS production increase with pyruvate/malate in liver mitochondria from methionine supplemented rats, in the present study we have analyzed possible variations in the amount of the different electron transport ROS generators discarding an increase in mitochondrial oxygen consumption that remained unchanged with pyruvate/malate. At mitochondria only two of the four multiprotein respiratory complexes, complex I (Barja and Herrero 1998; Genova et al. 2001) and complex III (Boveris et al. 1976) are well known to produce

ROS. In our study, the concentration of either complex I or complex III did not change after 7 weeks of methionine supplementation, thus the increase in ROS generation must be due to a qualitative change rather than a quantitative change in the ROS generator protein complexes. Moreover AIF, besides its apoptotic functions, is known to be also required for mitochondrial oxidative phosphorylation (Porter and Urbano 2006). Specifically, AIF deficient cells exhibit a reduced complex I content (Vahsen et al. 2004) suggesting a role of AIF in the biogenesis or maintenance of this complex. Methionine supplementation did not modify AIF levels, which is consistent with the lack of differences in complex I content. Thus, methionine supplementation must induce qualitative changes of the mitochondrial complexes that increase ROS production, which is consistent with the increased FRL observed in methionine supplemented animals. These changes specifically must take place at complex I, because the increase in ROS production during methionine supplementation occurred with pyruvate plus malate as substrate, but not with succinate plus rotenone. Complex I is also the mitochondrial ROS generator responsible for the lower mitochondrial ROS production in long-lived species or in DR, protein restriction and methionine restriction. It has been observed that the three

**Fig. 5** Oxidative damage to mitochondrial DNA (8-oxodG) in liver from control and methionine supplemented Wistar rats. Values are means  $\pm$  SEM from 8 different animals. \*significant differences between control and methionine supplemented rats:  $p<0.01$ **Fig. 6** Oxidative damage to mitochondrial DNA (8-oxodG) in heart from control and methionine supplemented Wistar rats. Values are means  $\pm$  SEM from 8 different animals



**Table 5** Fatty acid composition (%) of total lipids in liver from control and methionine supplemented Wistar rats

|         | Control     | Methionine Supplemented |
|---------|-------------|-------------------------|
| 14:0    | 0.50±0.06   | 0.40±0.03*              |
| 16:0    | 24.65±0.53  | 23.46±0.91              |
| 16:1n-7 | 1.17±0.09   | 0.70±0.04***            |
| 18:0    | 14.96±0.36  | 16.57±0.63              |
| 18:1n-9 | 9.99±0.32   | 11.78±0.53*             |
| 18:2n-6 | 15.66±0.50  | 19.01±0.69**            |
| 18:3n-3 | 0.69±0.06   | 0.27±0.01***            |
| 20:3n-6 | 0.47±0.03   | 0.25±0.02***            |
| 20:4n-6 | 25.44±0.29  | 22.40±0.66**            |
| 20:5n-3 | 0.58±0.06   | 0.28±0.04**             |
| 22:4n-6 | 0.68±0.05   | 0.38±0.02***            |
| 22:5n-6 | 0.79±0.11   | 0.64±0.01               |
| 22:5n-3 | 1.43±0.17   | 1.26±0.12               |
| 22:6n-3 | 2.82±0.09   | 2.53±0.15               |
| ACL     | 18.21±0.02  | 18.15±0.03              |
| SFA     | 40.21±0.45  | 40.45±0.52              |
| UFA     | 59.78±0.45  | 59.54±0.52              |
| MUFA    | 11.16±0.31  | 12.48±0.54              |
| PUFA    | 48.61±0.55  | 47.05±0.75              |
| PUFAn-6 | 43.06±0.34  | 42.69±0.68              |
| PUFAn-3 | 5.54±0.30   | 4.36±0.24*              |
| DBI     | 181.61±1.60 | 169.43±2.98**           |
| PI      | 162.37±1.82 | 144.98±3.95**           |

Values are mean ± SEM from 8 different animals

\*significant differences between control and methionine supplemented rats;

\* $p<0.05$ ; \*\* $p<0.01$ ; \*\*\* $p<0.001$ . ACL acyl chain length, SFA saturated fatty acids, UFA unsaturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids, DBI double bond index, PI peroxidizability index

dietary protocols, DR, protein restriction and methionine restriction, decrease the amount of complex I and IV (Sanz et al. 2004; Sanz et al. 2006c; Ayala et al. 2007) in rat heart and liver mitochondria. Recently, decreases in complex III have also been reported after methionine restriction in rat brain (Naudí et al. 2007) and liver (Caro et al. 2008). In the present experiment, no changes in the concentration of the respiratory complexes responsible for ROS generation have been reported after methionine supplementation, thus a different mechanism must induce the increase in ROS production. The decrease observed in complex IV concentration after methionine supplementation could result in a greater degree of electronic reduction upstream in the mitochondrial respiratory chain that could explain the increase in ROS production by complex I. An alternative mechanism to regulate complex I ROS production would be

the post-translational modification of complex I subunits. The reversible glutathionylation of specific complex I subunits has been proposed as an alternative mechanism to regulate ROS production by complex I. It has been reported that ROS production by mitochondrial complex I increases in response to oxidation of the mitochondrial glutathione pool. Addition of oxidized glutathione (GSSG) to mitochondrial complex I increases its superoxide radical generation rate, as thiols in complex I subunits react with GSSG to form glutathione-protein mixed disulfides (Taylor et al. 2003).

Methionine supplementation could also be detrimental due to methionine itself or to its in vivo conversion to methionine related metabolites, including SAM, SAH and homocysteine, increasing their concentration (Regina et al. 1993). In our study, methionine supplementation increased methionine, SAM and SAH concentrations both in liver and heart tissue, and it has been reported that diets high in methionine or protein increase plasma homocysteine in

**Table 6** Fatty acid composition (%) of total lipids in heart from control and methionine supplemented Wistar rats

|         | Control     | Methionine Supplemented |
|---------|-------------|-------------------------|
| 14:0    | 0.47±0.14   | 0.45±0.10               |
| 16:0    | 13.98±0.58  | 12.23±0.57              |
| 16:1n-7 | 1.23±0.44   | 0.80±0.20               |
| 18:0    | 22.03±1.49  | 23.19±0.62              |
| 18:1n-9 | 13.21±1.45  | 11.60±0.31              |
| 18:2n-6 | 16.16±1.14  | 18.03±0.37              |
| 18:4n-3 | 1.28±0.12   | 1.61±0.17               |
| 20:0    | 0.39±0.06   | 0.48±0.03               |
| 20:1    | 0.31±0.01   | 0.32±0.02               |
| 20:2n-6 | 0.65±0.09   | 0.79±0.10               |
| 20:3n-6 | 0.35±0.14   | 0.61±0.06               |
| 20:4n-6 | 22.88±1.66  | 22.87±0.78              |
| 22:0    | 2.12±0.16   | 2.40±0.09               |
| 22:6n-3 | 4.43±0.38   | 3.77±0.27               |
| 24:0    | 0.43±0.16   | 0.79±0.08               |
| ACL     | 18.45±0.07  | 18.51±0.04              |
| SFA     | 39.44±1.19  | 39.55±0.74              |
| UFA     | 60.55±1.19  | 60.44±0.74              |
| MUFA    | 14.76±1.84  | 12.74±0.43              |
| PUFA    | 45.78±1.38  | 47.70±0.97              |
| PUFAn-6 | 40.06±1.33  | 42.31±0.71              |
| PUFAn-3 | 5.71±0.45   | 5.38±0.32               |
| DBI     | 172.76±5.15 | 172.82±4.23             |
| PI      | 150.05±8.10 | 148.50±5.14             |

Values are mean ± SEM from 8 different animals

ACL acyl chain length, SFA saturated fatty acids, UFA unsaturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids, DBI double bond index, PI peroxidizability index

**Table 7** Protein markers of oxidative, glycoxidative and lipoxidative damage in liver and heart from control and methionine supplemented Wistar rats

| <b>LIVER</b> | Control        | Methionine Supplemented |
|--------------|----------------|-------------------------|
| GSA          | 3063.61±151.61 | 2638.51±207.33          |
| AASA         | 128.55±11.14   | 147.05±7.52             |
| CEL          | 336.99±31.70   | 313.95±26.44            |
| CML          | 1081.24±47.04  | 1133.96±63.51           |
| MDAL         | 427.41±14.95   | 426.62±16.50            |
| <b>HEART</b> | Control        | Methionine Supplemented |
| GSA          | 2916.46±273.72 | 3012.59±219.47          |
| AASA         | 306.03±32.27   | 324.72±18.56            |
| CEL          | 456.10±26.30   | 488.59±33.27            |
| CML          | 1158.98±88.11  | 1294.28±127.80          |
| MDAL         | 388.14±32.31   | 384.96±27.70            |

Values are mean ± SEM from 8 animals

Units: μmoles/mol lysine

GSA, Glutamic semialdehyde; AASA, aminoadipic semialdehyde; CEL, carboxyethyl-lysine; CML, carboxymethyl-lysine; MDAL, malondialdehyde-lysine

rodents and humans (Velez-Carrasco et al. 2008; Verhoef et al. 2005), although in other studies in mice, the atherogenic effect of excess methionine intake does not seem to be related to increases in homocysteine but to a direct methionine toxic effect (Troen et al. 2003). It has been proposed that the increase in plasma homocysteine induced by dietary methionine supplementation increases ROS production, and this leads to LDL oxidation and atherosclerosis (Hidiroglou et al. 2004). Plasma homocysteine levels increase with age in humans and represent a risk factor for age-related neurodegenerative and free radical-related diseases including vascular diseases, atherosclerosis, cognitive impairment, Parkinson's disease, Alzheimer's disease, dementia, stroke and chronic kidney disease (Guo et al. 2008; Obeid and Herrmann 2006; Ninomiya et al. 2004). Homocysteine has a free thiol group that can be oxidized leading to the generation of protein mixed disulfides or disulfide bridges between different proteins or between subunits of the same protein. Therefore, homocysteine could be responsible of the increase in ROS generation by complex I in the same way as after the previously mentioned GSSG addition. Thus, mitochondrial ROS production, specifically complex I ROS production, could be regulated by thiol agents including homocysteine, offering another plausible molecular mechanism to explain methionine supplementation effects on mitochondrial oxidative stress and tissue oxidative damage. The mentioned effect of GSSG addition is also interesting because it could explain the direct correlation observed between GSSG/GSH ratio and 8-oxodG levels in mtDNA in tissues of aging mice (Asunción et al. 1996). Similarly, some studies

treating rat heart mitochondria with homocysteine in vitro show mitochondrial ROS production increases (Chang et al. 2004). Finally, we cannot exclude the participation of an alternative transamination pathway in the mitochondrial ROS production increase after methionine supplementation. Methanethiol, an end product in the methionine transamination pathway has been shown to exhibit markedly similar toxicological effects to methionine supplementation in mouse hepatocytes (Dever and Elfarra 2008). Moreover, the increase in mitochondrial ROS production and % FRL in the liver of methionine supplemented rats could also be the result of a regulated response instead of a methionine metabolite direct effect. Since methionine is a donor of methyl groups, changes in DNA methylation could also be partially the origin of variations in gene expression described after methionine supplementation (Park et al. 2008; Fukagawa and Galbraith 2004) although the extent to which dietary intake of methionine influences DNA methylation needs further research. In our case the methylation potential, as indicated by the SAM/SAH ratio, was lowered in heart but not in liver tissue.

On the other hand, in our study and in agreement with the increase in mitochondrial ROS production after methionine supplementation, we observe an increased oxidative damage to mitochondrial DNA only in the liver. Methionine supplemented rats have higher 8-oxodG levels in the liver, but no differences were found in five specific markers of protein oxidation, glycoxidation and lipoxidation, GSA, AASA, CML, CEL and MDAL both in liver and in heart tissue. Previous comparative and dietary restriction studies have shown a close correlation between changes in mitochondrial ROS production and 8-oxodG levels in mitochondrial DNA (Lopez-Torres and Barja 2008a). Possibly,

**Table 8** Liver and heart methionine and methionine metabolites from control and methionine supplemented Wistar rats

| <b>LIVER</b>         | Control      | Methionine Supplemented |
|----------------------|--------------|-------------------------|
| Met (nmol/g tissue)  | 343.57±23.73 | 433.09±28.81*           |
| SAM (nmol/g tissue)  | 28.61±5.21   | 61.50±14.35 *           |
| SAH (nmol/g tissue)  | 42.22±3.49   | 119.64±16.77***         |
| Ratio Met/SAM        | 14.38±2.34   | 10.24±2.38              |
| Ratio SAM/SAH#       | 0.67±0.08    | 0.47±0.06               |
| <b>HEART</b>         | Control      | Methionine Supplemented |
| Met (nmol/ g tissue) | 119.31±17.17 | 418.69±55.13***         |
| SAM (nmol/ g tissue) | 12.7±0.74    | 15.94±1.04*             |
| SAH (nmol/g tissue)  | 8.97±0.65    | 15.71±1.42***           |
| Ratio Met/SAM        | 9.31±1.06    | 26.64±3.44***           |
| Ratio SAM/SAH#       | 1.45±0.10    | 1.04±0.06**             |

Values are mean ± SEM from 8 animals

\*significant differences between control and methionine supplemented rats: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$

# Ratio SAM/SAH: Methylation Potential

the great proximity or sometimes even contact between mitochondrial ROS production sites and mitochondrial DNA could be the explanation for this correspondence. Moreover, complex I is responsible for the changes in ROS production reported in all these different studies and this is particularly interesting as complex I ROS production is known to be specifically and completely directed to the mitochondrial matrix (Kudin et al. 2005) where mitochondrial DNA is located. In fact, after reviewing different studies with diverse experimental conditions including, dietary restriction (Gredilla et al. 2001b), protein restriction (Sanz et al. 2004), and methionine restriction (Sanz et al. 2006c) decreases in mitochondrial ROS production and 8-oxodG levels in mitochondrial DNA run in parallel and are of around similar magnitude in rat liver, while carbohydrate (Sanz et al. 2006b) or lipid restriction (Sanz et al. 2006a) do not modify mitochondrial ROS production and they do not decrease 8-oxodG mitochondrial DNA levels either. Furthermore, comparative studies between long-lived and short-lived species (Barja 2004; Pamplona and Barja 2006) report low ROS production and 8-oxodG levels in mtDNA for the former and high for the latter. In heart tissue from methionine supplemented rats, this direct relationship is also observed, no changes in ROS production and consequently no changes in 8-oxodG levels. In relation to protein oxidative modification, in the present study no significant changes in GSA, AASA, CEL, CML and MDAL have been found in either organ and there is no apparent parallelism with the increase in mitochondrial ROS production in the liver case. The increased oxidative stress induced by methionine supplementation, as the one previously reported in other experimental designs or physiological situations (Lopez-Torres et al. 1993; Gomez-Cabrera et al. 2008), eg. moderate exercise (chronic training), can result in the upregulation of endogenous antioxidant enzymes such as superoxide dismutase, glutathione peroxidase or catalase and also non enzymatic antioxidants as reduced glutathione leading to an apparently adaptive response to a chronic high-methionine diet, that could explain the absence of changes in GSA, AASA, CEL, CML and MDAL that have been found in the present study. Different authors have described increases in antioxidant enzymes after methionine supplementation (Toborek et al. 1996; Seneviratne et al. 1999; Mori and Hirayama 2000; Park et al. 2008) although in other study the activities of the antioxidant enzymes did not change (Stefanello et al. 2005). However in this last experiment methionine was added in vitro to rat hippocampus homogenates and no adaptative compensation could take place. In some cases, these adaptative inductions of antioxidant enzymes after methionine supplementation are lost after a long time (6 months) and that correlates with an increase in lipid peroxidation measured as thiobarbituric acid reacting substances (Mori and

Hirayama 2000) that was not observed while the inductions were temporarily taking place. Alternatively, the lack of changes in the markers of protein oxidative modification can be due to compensatory upregulation of catabolic systems involved in the repair of damaged proteins.

Interestingly, when we analyze the fatty acid unsaturation indexes, both DBI and PI, decreases are only found in the case of liver, the tissue where increases in ROS production after methionine supplementation have been described. No changes in heart DBI or PI run in parallel with no increases in ROS production. Among cellular macromolecules, polyunsaturated fatty acids (PUFA) exhibit the highest sensitivity to ROS-induced oxidative damage, and their sensitivity to oxidation exponentially increases as a function of double bond number per fatty acid molecule. Thus, a low fatty acid unsaturation degree would be advantageous by decreasing their sensitivity to lipid peroxidation. In agreement with this, it has been found that long-lived animals have a lower total tissue and mitochondrial fatty acid unsaturation degree than short-lived ones (Pamplona et al. 1996). The low DBI observed in long-lived species is due to changes in the type of unsaturated fatty acid that participates in the membrane composition. So, there is a systematic redistribution between types of PUFAs present from the highly unsaturated arachidonic (20:4n-6, AA) and docosahexaenoic (22:6n-3, DHA) acids in short-lived species to the less unsaturated linoleic acid (18:2n-6, LA) and, in some cases, linolenic acid (18:3n-3, LNA) in the long-lived ones at mitochondrial and tissue levels (Pamplona et al. 2002). In the present study, we have found this type of redistribution in the liver tissue from methionine supplemented animals that, in spite of the ROS production increase observed, would allow a reduction in sensitivity to lipid peroxidation without altering fluidity/microviscosity, a fundamental cellular membrane property. Several studies describe alterations in lipid biosynthesis and metabolism after methionine supplementation; decreases in hepatic triglycerides (Mori and Hirayama 2000; Park et al. 2008), decreased plasma HDL-C levels (Velez-Carrasco et al. 2008), increased hepatic biosynthesis and uptake of cholesterol and triglycerides are attributed to high homocysteine levels through endoplasmic reticulum stress induction (Werstuck et al. 2001). Thus, homocysteine induced endoplasmic reticulum stress leads to dysregulation of the cholesterol and triglyceride biosynthetic pathways activating the expression of lipid biosynthetic genes that may play a role in the association of elevated homocysteine with atherosclerosis and coronary artery disease. In our study, the decrease in the hepatic fatty acid unsaturation degree would be adaptive and would compensate, at least in part, for the increase in ROS production observed in the liver. Alternatively, the decrease in liver fatty acid unsaturation could be a consequence of the increase in ROS

generation, since free radicals cause damage and can lower the amount of unsaturated fatty acids. But this can not be the whole explanation since only some, not all the highly unsaturated fatty acids were decreased by methionine supplementation.

In conclusion, the results from the present study support a mechanism by which excess dietary methionine would result in hepatotoxicity, the increased mitochondrial ROS generation at complex I in the liver and the subsequent mitochondrial DNA damage. Additional studies on the toxicity of methionine and its related products are needed, especially in relation to diseases associated with advancing age.

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