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Methionine and homocysteine modulate the rate of ROS generation of isolated mitochondria in vitro

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Abstract Dietary methionine restriction and supplementation in mammals have beneficial (antiaging) and detrimental effects respectively, which have been related to chronic modifications in the rate of mitochondrial ROS generation. However it is not known if methionine or its metabolites can have, in addition, direct effects on the rate of mitochondrial ROS production. This is studied here for the methionine cycle metabolites S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), homocysteine and methionine itself in isolated rat liver, kidney, heart, and brain mitochondria. The results show that methionine increases ROS production in liver and kidney mitochondria, homocysteine increases it in kidney and decreases it in the other three organs, and SAM and SAH have no effects. The variations in ROS production are localized at complexes I or III. These changes add to previously described chronic effects of methionine restriction and supplementation in vivo.

Keywords Free radicals · Mitochondria · ROS production · Methionine · Homocysteine · Liver · Kidney · Heart · Brain

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Introduction

Two molecular traits correlate with mammalian and bird longevity in the right way, the rate of mitochondrial reactive oxygen species (ROS) generation and the degree of fatty acid unsaturation of cellular membranes (Pamplona and Barja 2007). Long-lived species show low rates of mitochondrial ROS production and low levels of oxidative damage to macromolecules (Pamplona et al. 2002; Lambert et al. 2007; Robert et al. 2007; Lopez-Torres and Barja 2008a). In addition dietary restriction, which increases maximum longevity in rodents, yeast, nematodes, insects and in almost any animal species tested (Bishop and Guarente 2007) and increases survival and delays age-related diseases and detrimental changes in rhesus monkeys (Colman et al. 2009), also lowers the rate of mitochondrial ROS generation and decreases the level of oxidative damage to mitochondrial DNA (Gredilla et al. 2001a; Gredilla and Barja 2005). Classically, the anti-aging effects of DR have been solely attributed to the decreased ingestion of dietary calories. Nevertheless, changes in some of the dietary components, especially protein components, also modulate longevity in insects (Mair et al. 2005; Min and Tatar 2006) and rodents (Pamplona and Barja 2006; Sanz et al. 2006a; Lopez-Torres and Barja 2008b) whereas carbohydrate (Khorakova et al. 1990) and lipid restriction (Shimokawa et al. 1996; Iwasaki et al. 1988) do not seem to change rodent longevity. Revision of the life-long studies available indicates that protein restriction increases maximum longevity in rats and mice and that this longevity increase is around 50% of that found in dietary restriction (Pamplona and Barja 2006). Furthermore, protein restriction reduces mitochondrial ROS production and oxidative stress to an extent similar to that observed in dietary restriction (Sanz et al. 2004; Gomez et al. 2007) whereas lipid (Sanz et al.

2006b) and carbohydrate restriction (Sanz et al. 2006c) do not cause such changes. In addition, restriction of a single 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 methionine restriction (MetR) decreases mitochondrial ROS generation and oxidative damage to a similar extent as protein or dietary restriction in all the rodent organs studied including liver, heart, kidney and brain (Sanz et al. 2006a; Caro et al. 2008, 2009a), whereas this does not occur when all the aminoacids of the diet, except methionine, are restricted (Caro et al. 2009b). Therefore, MetR is responsible for the decrease in mitochondrial ROS generation and oxidative stress that takes place in dietary restriction and possibly for part of its longevity-extension effect. It is then important to clarify the precise mechanisms by which methionine exerts these effects.

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 signalling molecules in the liver of mice subjected to this dietary manipulation (Sun et al. 2009). However, even if this kind of mechanism is operative also in MetR, this does not eliminate the possible existence of additional direct effects of methionine or its immediately derived metabolites on the mitochondria which could also contribute to lower the rate of ROS generation. The decreases in mitochondrial ROS production in MetR are observed in mitochondria isolated from control and MetR animals which are incubated under the same conditions in vitro. The lower ROS generation of the MetR animals measured under those conditions indicates that MetR mitochondria are qualitatively different from those of the controls. However, it is likely that the cellular concentration of methionine or its derived metabolites is modified in MetR animals. In fact, methionine dietary supplementation increases methionine, S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) in the liver and heart of Wistar rats (Gomez et al. 2009). If methionine metabolites directly affect the rate of ROS generation, this would be missed in classic MetR studies in which ROS generation is measured under similar conditions in control and MetR animals (with incubation buffers lacking any of these metabolites). Previous studies have shown that some sulphur-containing compounds can modulate mitochondrial ROS production when they are directly added to isolated mitochondria (Taylor et al. 2003; Chang et al. 2004). If methionine, for instance, directly increases mitochondrial ROS generation, and tissue methionine decreases in MetR, the decreases in ROS production described in MetR animals (Sanz et al. 2006a; Caro et al. 2008, 2009a) would represent an underestimation. The final rate of mitochondrial ROS production of MetR animals in vivo could then be lowered by two kinds of additive effects: indirect chronic effects acting through changes in cellular signalling and gene expression plus direct effects on the mitochondria due to changes in the tissular concentration of methionine itself or its metabolites.

In order to investigate this second possibility in this report we study the direct effect of independently in vitro adding the four metabolites of the methionine cycle to isolated mitochondria. In this pathway, methionine is first activated by ATP to form SAM. SAM serves primarily as a methyl donor in a reaction generating SAH. SAH is then hydrolyzed forming adenosine and homocysteine (HCys). Finally, homocysteine is recycled back to methionine (Stipanuk 2004). Therefore, the direct effect of physiological concentrations of Met, SAM, SAH, and HCys on the rate of ROS production of rat liver, kidney, heart and brain functional mitochondria was systematically studied under conditions allowing to ascertain the sites in the respiratory chain where ROS production is modified and some of the possible mechanisms involved. To our knowledge, this has not been previously studied for methionine, SAM and SAH and only a single report for HCys in rat heart is available (Chang et al. 2004).

Materials and methods

Animal and diets

Male Wistar rats of 250–300 g of body weight were maintained in a 12:12 (light:dark) cycle at 22±2 °C and 50± 10% relative humidity. Animals were fed ad libitum with a standard rodent diet (Panlab, Barcelona, Spain). Rats were sacrificed by decapitation. Liver, heart, brain or kidney samples were immediately processed to obtain functional mitochondria.

Mitochondria isolation

Liver mitochondria were obtained as previously described (Gredilla et al. 2001b). The liver was rinsed, cut in small pieces and homogenized in 60 ml of liver mitochondria 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 × g for 10 min. Supernatants were centrifuged at 10,000 × g for 10 min. The pellets were resuspended in 40 ml of liver mitochondria isolation buffer without EDTA and were centrifuged at 1,000 × g for 10 min. Mitochondria were obtained after centrifugation of the supernatants at 10,000 × g for 10 min. After every centrifugation step any overlaying layer of fat was eliminated. The mitochondrial pellets were finally

resuspended in 1 ml of liver mitochondria isolation buffer without EDTA. Kidney mitochondria were obtained using the same procedure used for liver mitochondria with small modifications. The amount of the mitochondria isolation buffer used in the centrifugation and resuspending of kidney mitochondria was half that used in the case of liver.

Heart mitochondria were obtained by the procedure of Mela and Seitz (1979) with modifications. Dissected ventricles were chopped and homogenized manually with a loose-fitting glass pestle in 10 ml of heart mitochondria isolation buffer (220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4) containing 5 mg of subtisilin A and 25 mg of fatty acid-free albumin. After standing for 1 min, 25 ml of additional heart mitochondria isolation buffer containing 25 mg albumin were added to the samples, and gentle homogenization was performed again with a tighter fitting pestle. The nuclei and cell debris were removed by centrifugation at 1,000 g for 10 min. Heart mitochondria were obtained by centrifuging the supernatant twice at 10,000 for 10 min. The mitochondrial pellets were finally resuspended in 0.5 ml of heart mitochondria isolation buffer.

Brain nonsynaptic free mitochondria were obtained according to the method of Lai and Clark (1979). The brain was rinsed several times, chopped and homogenized manually with a loose-fitting glass pestle in 35 ml of brain mitochondria isolation buffer (250 mM sucrose, 0.5 mM K+-EDTA, 10 mM Tris, pH 7.4). The homogenate was centrifuged at 2,000 g for 3 min and this centrifugation step was repeated in the first supernatant. The second supernatant was centrifuged at 12,000 g for 8 min to obtain the crude mitochondrial pellet. This pellet was resuspended in 6 ml of 3% ficoll buffer (0.24 mM manitol, 60 mM sucrose, 50 μM EDTA, 10 mM Tris, pH 7.4). The sample was underlayered with 25 ml of 6% ficoll buffer. After centrifugation at 11,500 g for 30 min the pellet was resuspended in 5 ml of brain mitochondria isolation buffer. The sample was centrifuged at 11,500 g for 10 min. The resulting pellets were resuspended in 35 ml of brain mitochondria isolation buffer. Brain nonsynaptic free mitochondria were obtained by centrifuging the sample at 11,500 g for 10 min. The final mitochondrial pellet was resuspended in 1 ml of brain mitochondria isolation buffer.

All the above procedures were performed at 5 °C. The final mitochondrial suspensions were maintained over ice and were immediately used for the H_2O_2 production and O_2 consumption measurements. Mitochondrial protein was measured by the Biuret method.

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 (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 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 2 μM rotenone in order to avoid the backward flow of electrons to complex I. 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). 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^- excreted by mitochondria (if any) to H_2O_2, the measurements represent the total (O_2^- plus H_2O_2) rate of mitochondrial ROS production. The assays were performed in the absence and in the presence of two different concentrations of methionine (70 and 100 μM), SAM (250 and 500 μM), SAH (100 and 500 μM) and homocysteine (25 and 100 μM) within the tissue physiological range (Harper 1968; Finkelstein and Martin 1986; Regina et al. 1993; Troen et al. 2003; Devlin et al. 2004; Melse-Boonstra et al. 2005; Verhoef et al. 2005; Velez-Carrasco et al. 2008). In control assays (adding 75 μM H_2O_2 instead of mitochondria to the reaction system) methionine, SAM and SAH did no cause significant changes in the homovanillic acid derived fluorescence, whereas homocysteine caused some decrease in the final fluorescence. Therefore, all the assays performed with mitochondria in the presence of homocysteine were appropriately corrected for this effect.

Mitochondrial oxygen consumption

The rate of mitochondria oxygen consumption was measured at 37 °C in a water-thermostatized incubation chamber with a computer-controlled Clark-type O_2 electrode (Oxygraph, Hansatech, UK) in 0.5 ml of the same incubation buffer used for 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- (5 mM succinate + 2 μM rotenone) linked. The assays were performed in the absence (state 4) and in the presence (state 3) of 500 μM ADP. The effects of 100 μM Met, 500 μM SAM, 500 μM SAH and 25 or 100 μM homocysteine on the rate of mitochondrial oxygen consumption were studied.

Statistics

Comparisons between multiple groups concerning the effect of methionine cycle metabolites was statistically analysed by one way Anova. When only two groups were present Student-t tests were performed. The minimum level of statistical significance was set at $P<0.05$ in all the analyses.

Results

The rate of mitochondria oxygen consumption from liver, kidney, heart and brain 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 + rotenone) linked substrates. The addition of ADP strongly increased the rate of oxygen consumption in all cases, indicating tight coupling of mitochondrial preparations even in the presence of methionine or its metabolites (Tables 1 and 2). Methionine, SAM, SAH and HCys did not change the oxygen consumption from liver or brain mitochondria with any substrate in either state 4 or state 3 (Tables 1 and 2). Methionine, SAM and SAH significantly decreased state 3 oxygen consumption in kidney and heart mitochondria using complex I-linked substrates. SAH also decreased state 4 in heart mitochondria with pyruvate/malate. HCys decreased both state 4 and state 3 in kidney and heart mitochondria with complex I-linked substrates. The metabolites studied did not change oxygen consumption with complex II-linked substrate (succinate + rotenone) in any state (4 or 3) in any of the four organs investigated (Tables 1 and 2).

Table 1 Rates of oxygen consumption (nmoles O_2/min mg protein) from liver and kidney mitochondria in presence of methionine (Met), S-adenosyl methionine (SAM), S-adenosylhomocysteine (SAH) and homocysteine (HCys)

	Liver	Kidney
Glutamate/malate (state 4)	19.6±2.3	23.4±1.3
+ ADP (state 3)	122.2±8.5***b	84.4±9.5***b
+ Met (state 4)	18.1±2.1	21.7±2.4
+ Met+ADP (state 3)	110.4±9.9***b	55.6±2.0*a,***b
+ SAM (state 4)	17.5±2.6	20.1±3.1
+ SAM+ADP (state 3)	106.4±12.7***b	57.0±7.6*a,**b
+ SAH (state 4)	16.7±1.3	19.1±2.2
+ SAH+ADP (state 3)	107.9±10.1***b	45.2±5.1*a,**b
+ HCys (state 4)	17.0±2.5	16.9±2.4*a
+ HCys+ADP (state 3)	105.6±12.7***b	41.3±8.4*a,**b
Succinate+Rotenone (state 4)	41.5±2.1	70.2±3.8
+ ADP (state 3)	172.8±11.4***b	222.9±8.0***b
+ Met (state 4)	36.5±3.0	64.9±3.2
+ Met+ADP (state 3)	158.8 ±12.1***b	196.9±6.3***b
+ HCys (state 4)	38.9±2.1	68.8±1.9
+ HCys+ADP (state 3)	166.2 ±13.1***b	194.3±6.3***b

Values are means ± SEM from 4 to 8 different animals. State 4, oxygen consumption in the absence of ADP. State 3, oxygen consumption in the presence of ADP. Asterisks represent significant differences (*$P<0.05$; **$P<0.01$; $P<0.001$): (a) comparing assays in the presence and absence of metabolite (Met, SAM, SAH or HCys) in the same state. (b) comparing states 4 and 3 (ADP effect) under otherwise similar conditions. The concentrations of metabolites added were: Met (100 μM), SAM (500 μM), SAH (500 μM), HCys (100 μM with glutamate/malate and 25 μM with succinate+rotenone)

Table 2 Rates of oxygen consumption (nmoles O_2/min mg protein) from heart and brain mitochondria in presence of methionine (Met), S-adenosyl methionine (SAM), S-adenosylhomocysteine (SAH) and homocysteine (HCys)

	Heart	Brain
Pyruvate/malate (state 4)	50.2±2.8	10.5±1.5
+ ADP (state 3)	184.9±7.4***b	20.2±2.7**b
+ Met (state 4)	43.7±2.8	9.2±1.3
+ Met+ADP (state 3)	164.1±9.0*a,***b	19.1±2.5**b
+ SAM (state 4)	45.8±2.7	11.3±1.5
+ SAM+ADP (state 3)	159.8±8.9*a,***b	21.6±2.8**b
+ SAH (state 4)	39.8±3.4*a	10.4±1.1
+ SAH+ADP (state 3)	143.4±7.4***a,***b	21.3±1.8***b
+ HCys (state 4)	42.0±3.4*a	9.7±1.2
+ HCys+ADP (state 3)	144.5±10.1**a,***b	17.4±2.4**b
Succinate+Rotenone (state 4)	143.0±4.0	12.6±1.4
+ ADP (state 3)	274.6±8.3***b	25.8±1.6***b
+ HCys (state 4)	133.8±5.8	12.3±1.3
+ HCys+ADP (state 3)	256.0±12.6***b	26.8±1.9***b

Values are means±SEM from 4 to 8 different animals. State 4, oxygen consumption in the absence of ADP. State 3, oxygen consumption in the presence of ADP. Asterisks represent significant differences (*$P<0.05$; **$P<0.01$; $P<0.001$): (a) comparing assays in the presence and absence of metabolite (Met, SAM, SAH or HCys) in the same state. (b) comparing states 4 and 3 (ADP effect) under otherwise similar conditions. The concentrations of metabolites added were: Met (100 μM), SAM (500 μM), SAH (500 μM), HCys (100 μM with pyruvate/malate and 25 μM with succinate+rotenone)

The effects of the four studied metabolites on the mitochondrial rate of ROS generation was measured using complex I- (pyruvate/malate or glutamate/malate) and complex II- (succinate + rotenone) linked substrates (Figs. 1, 2, 3, and 4). In liver mitochondria with glutamate/malate as substrate methionine (100 μM) significantly increased and HCys (25 and 100 μM) significantly decreased the basal rate of ROS generation, whereas SAM and SAH had no effect (Fig. 1a). In the presence of glutamate/malate plus rotenone 25 μM HCys also decreased ROS generation whereas no significant differences were observed with 100 μM methionine (Fig. 1b). With complex II-linked substrate (succinate+rotenone) 100 μM methionine increased and 25 μM HCys decreased ROS generation (Fig. 1b).

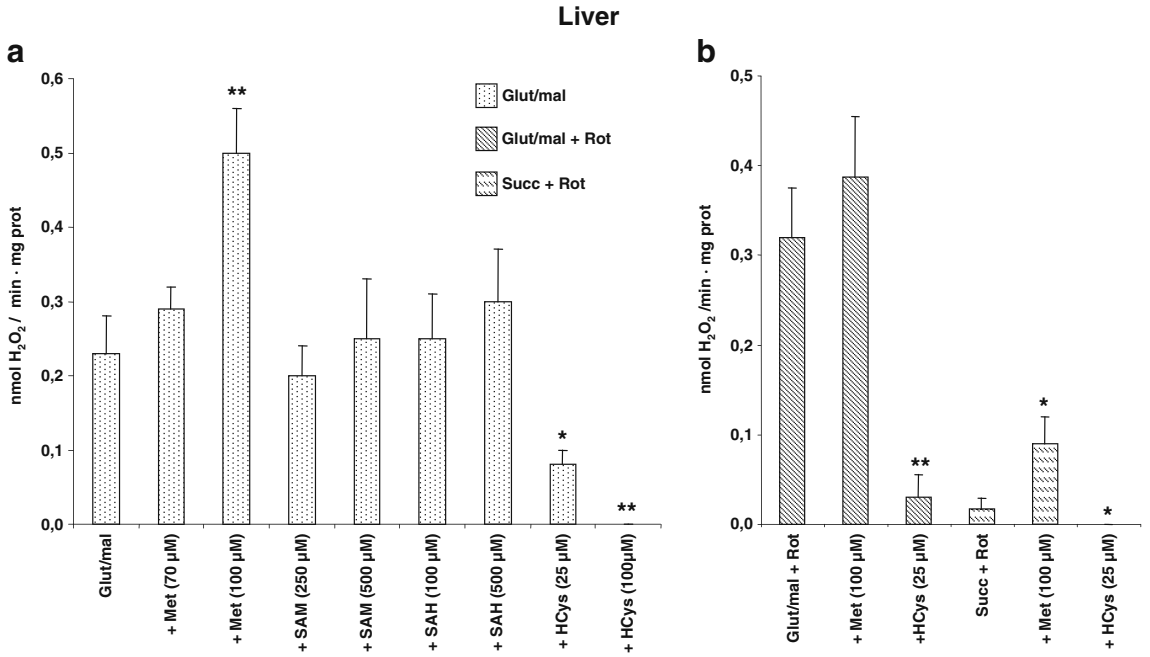

Fig. 1 Rates of H_2O_2 generation (nmoles H_2O_2/min.mg protein) in rat liver mitochondria. The substrates used were: A) glutamate/malate (Glut/mal); B) glutamate/malate plus rotenone (Glu/mal+Rot) or succinate plus rotenone (Succ+Rot). In addition, the assays were performed in the presence of several concentrations of methionine (Met), S-adenosyl methionine (SAM), S-adenosyl homocysteine (SAH) or homocysteine (HCys) as marked in the figure. Asterisks represent significant differences compared to the assay performed without the added methionine cycle metabolite: * ($p<0.05$), ** ($p<0.01$). Values are means ± SEM from 4 to 8 different animals

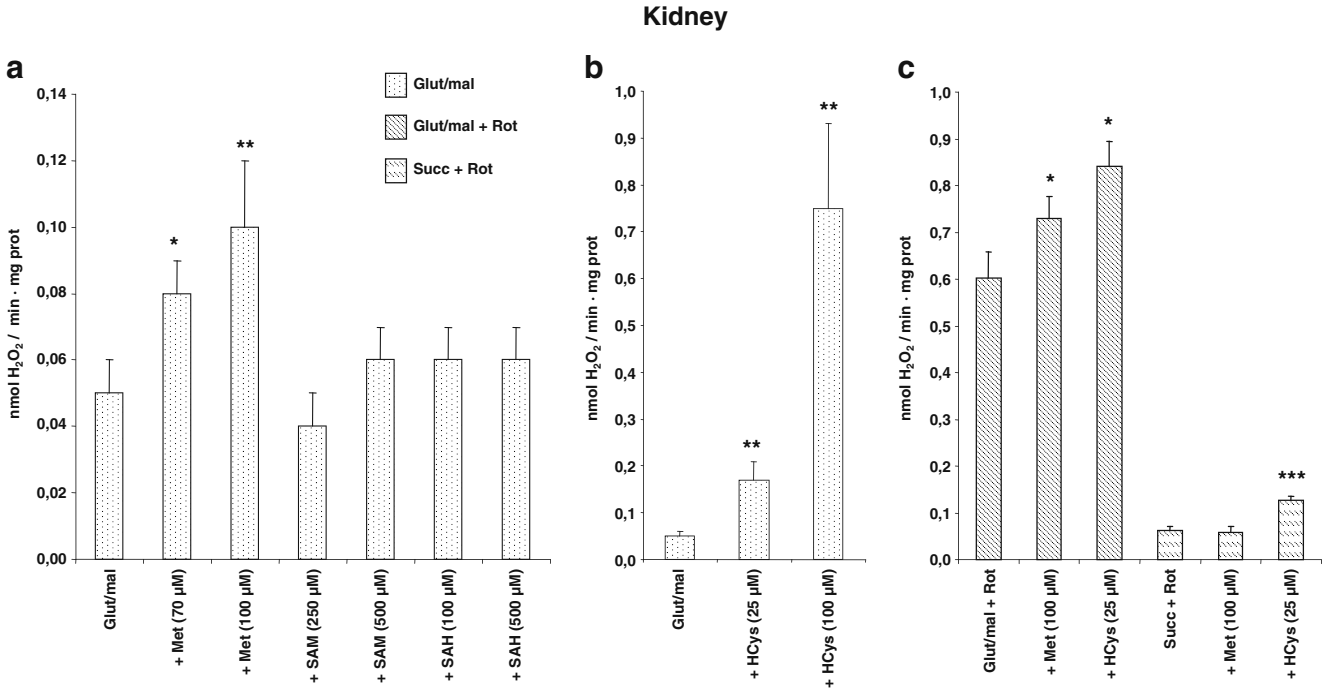

Fig. 2 Rates of H_2O_2 generation (nmoles H_2O_2/min.mg protein) in rat kidney mitochondria. The substrates used were: **a** and **b** glutamate/malate (Glut/mal); **c** Glu/mal+Rot or Succ+Rot. In addition, the assays were performed in presence of several concentrations of methionine (Met), S-adenosyl methionine (SAM), S-adenosyl homocysteine (SAH) or homocysteine (HCys) as marked in the figure. *Asterisks* represent significant differences compared to the assay performed without the added methionine cycle metabolite: * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$). Values are means ± SEM from 4 to 8 different animals

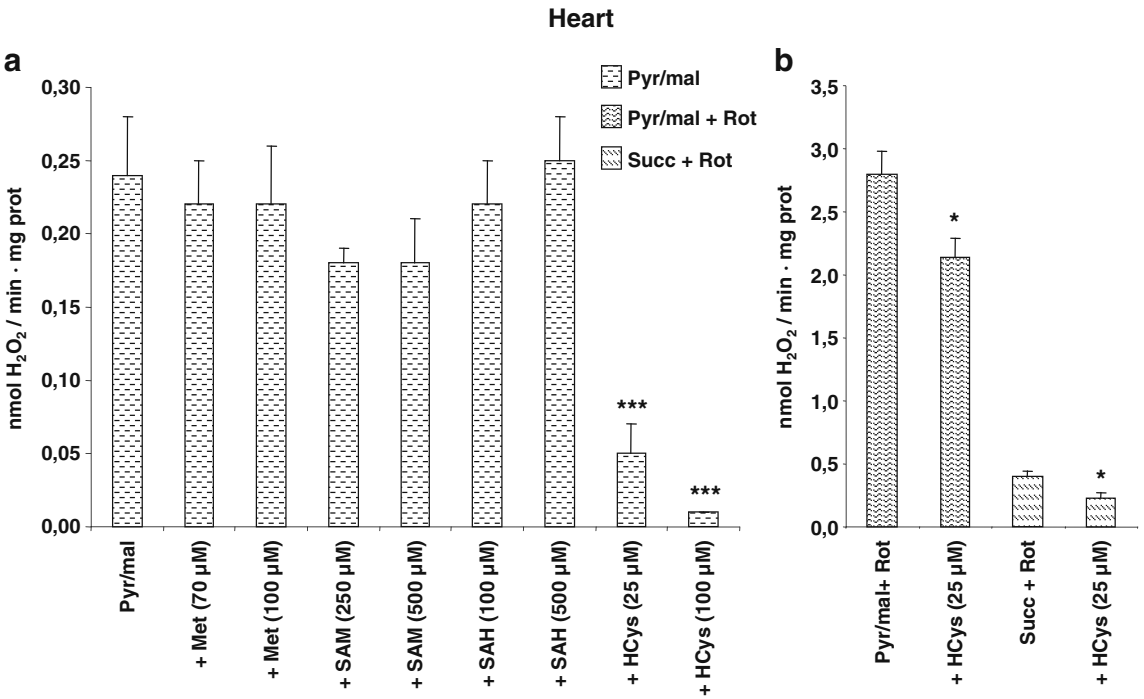

Fig. 3 Rates of H_2O_2 generation (nmoles H_2O_2/min.mg protein) in rat heart mitochondria. The substrates used were: A) Pyruvate/malate (Pyr/mal); B) Pyruvate/malate+rotenone (Pyr/mal+Rot) or succinate plus rotenone (Succ+Rot). In addition, the assays were performed in the presence of several concentrations of methionine (Met), S-adenosyl methionine (SAM), S-adenosyl homocysteine (SAH) or homocysteine (HCys) as marked in the figure. *Asterisks* represent significant differences compared to the assay performed without the added methionine cycle metabolite: * ($p<0.05$), *** ($p<0.001$). Values are means ± SEM from 4 to 8 different animals

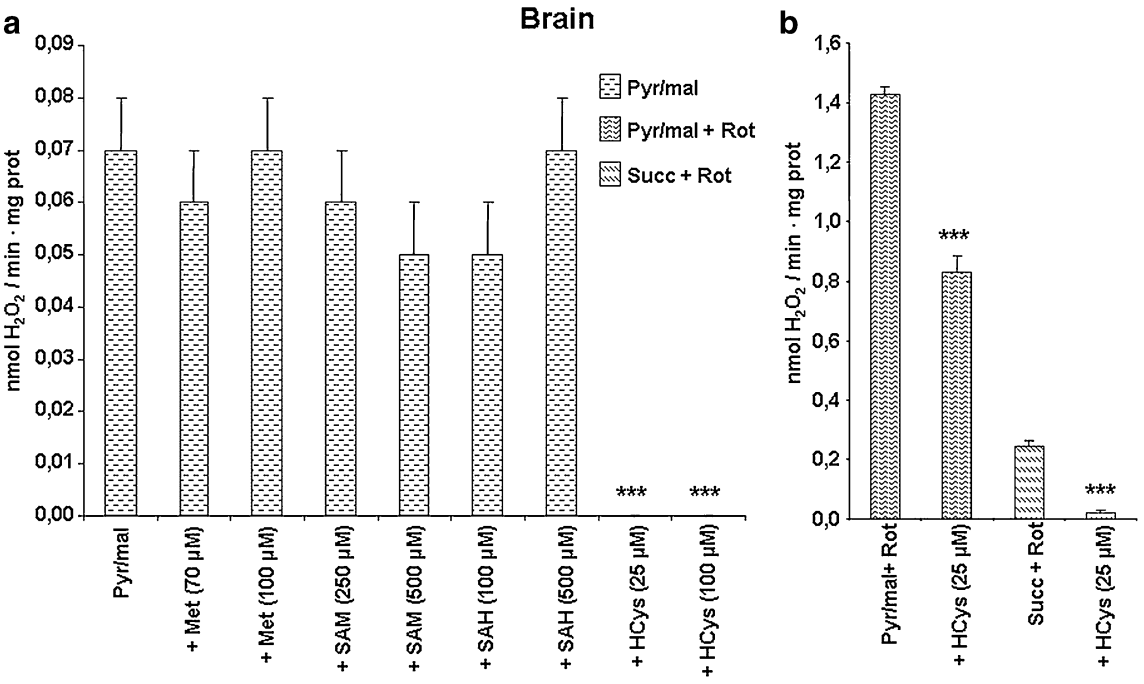

Fig. 4 Rates of H_2O_2 generation (nmoles H_2O_2/min.mg protein) in rat brain mitochondria. The substrates used were: A) Pyruvate/malate (Pyr/mal); B) Pyruvate/malate plus rotenone (Pyr/mal+Rot) or succinate plus rotenone (Succ+Rot). In addition, the assays were performed in the presence of several concentrations of methionine (Met), S-adenosyl methionine (SAM), S-adenosyl homocysteine (SAH) or homocysteine (HCys) as marked in the figure. Asterisks represent significant differences compared to the assay performed without the added methionine cycle metabolite: *** ($p<0.001$). Values are means ± SEM from 4 to 8 different animals

In kidney mitochondria methionine (70 and 100 μM) and HCys (25 and 100 μM) increased ROS production with glutamate/malate whereas SAM and SAH were without effect (Fig. 2a and b). 100 μM methionine and 25 μM HCys also increased ROS production of kidney mitochondria with glutamate/malate+rotenone (Fig. 2c). Twenty-five μM HCys, but not 100 μM methionine, also increased ROS production with succinate+rotenone (Fig. 2c).

In heart mitochondria HCys (25 and 100 μM) decreased ROS production with complex I-linked substrates (pyruvate/malate) and the other three metabolites had no effect (Fig. 3a). 25 μM HCys also decreased ROS production of heart mitochondria with pyruvate/malate+rotenone and with succinate+rotenone (Fig. 3b).

Similarly to heart mitochondria, in brain mitochondria HCys decreased the rate of ROS production with pyruvate/malate, with pyruvate/malate+rotenone and with succinate+rotenone, whereas the other three metabolites studied were without effect (Fig. 4a,b).

Discussion

In this investigation it is shown for the first time that methionine increases the rate of ROS generation of liver and kidney mitochondria when it is directly added to the organelles in vitro. It has been previously observed that increasing dietary methionine increases methionine concentration in rat liver (Gomez et al. 2009). Therefore a decrease in tissue methionine concentration is expected in organs from MetR animals. This decrease would further reduce the rate of ROS production over the decrease in this parameter already observed when the mitochondria isolated from Control and MetR animals are assayed under the same incubation conditions in vitro (Sanz et al. 2006a; Caro et al. 2008, 2009a). This means that the depression in ROS generation in MetR in vivo would be more profound than previously suspected with expected beneficial consequences for oxidative stress in this animal model of longevity extension. Our results also indicate that among methionine cycle metabolites, HCys has also capacity for directly modulating the rate of mitochondrial ROS production.

The increase in ROS production directly induced by methionine in liver and kidney mitochondria is consistent with previous studies showing liver and kidney damage in rodent models of dietary methionine supplementation. Dietary methionine excess produces more damage than any other dietary aminoacid (Harper et al. 1970). Dietary methionine supplementation increases plasma hydroperoxides and LDL-cholesterol (Hidiroglou et al. 2004), raises iron and lipid peroxidation, conjugated dienes and cholesterol in rat liver (Mori and Hirayama 2000; Dever and Elfarra 2008), increases lipid peroxidation in rabbits (Toborek et al. 1996), and increases oxidative stress and nuclear factor kappa B activation in the liver of C57BL/6J mice (Park et al. 2008). Methionine supplementation also leads to tubulointerstitial injury in rat kidney (Kumagai et al. 2002). These effects are of special significance taking into account the high methionine content of western population human diets that far exceed the recommended dietary allowance (RDA) for methionine, as well as the known beneficial capacity of methionine dietary restriction to extend mean and maximum longevity in rats and mice (Lopez-Torres and Barja 2008b). The toxic effects of methionine supplementation could be due to the generation of potentially damaging methionine metabolites like HCys (Hidiroglou et al. 2004) or, alternatively, to methionine itself (Harper et al. 1970; Troen et al. 2007). The results obtained in this investigation would be mainly consistent with this second possibility since direct addition of methionine to liver and kidney mitochondria at physiologically relevant levels significantly raises their rates of ROS production. Thus, the increase in tissue damage during methionine dietary supplementation could be due at least in part to the increase in mitochondrial ROS production induced by this aminoacid which would lead to an increase in tissue oxidative stress. This also agrees with previous results from our laboratory which showed that dietary methionine supplementation in rats increases oxidative damage in mitochondrial DNA in liver but not in heart of Wistar rats (Gomez et al. 2009) and we show here that methionine directly increases liver but not heart mitochondrial ROS generation in Wistar rats. On the other hand, the fact that methionine increases mitochondrial ROS generation in liver and kidney but not in heart and brain indicates that the effect is tissue-specific. This is consistent with the notion that mitochondria are specialized for tissue function and with the observation that the mitochondrial proteome is different even between brown fat and white fat tissues (Forner et al. 2009). Differences in the mitochondrial proteome between liver and kidney on the one hand, and heart and brain on the other, could be responsible for their different response to methionine observed in the present investigation.

Since methionine-dependent ROS production can modulate oxidative stress, organ damage and even longevity, it is important to localize the sites in the respiratory chain where this aminoacid modifies ROS generation. It is well known that the mitochondrial respiratory chain produces ROS at complex I (Barja and Herrero 1998) and at complex III (Boveris et al. 1976). Our experiments allowed us to localize which of these two sites are involved in each organ. In the case of liver mitochondria methionine increased ROS production with glutamate/malate but not with glutamate/malate plus rotenone which rules out complex I as the source of the increase in ROS generation. This agrees with the experiments with complex II-linked substrate. Methionine increased ROS generation with succinate plus rotenone. Under these conditions electrons cannot flow back to complex I but flow

down the respiratory chain through complex III. Therefore, the increase in ROS generation brought about by methionine on liver mitochondria comes from complex III. The situation is different in the case of kidney. In this organ methionine increased ROS generation with glutamate/malate and with glutamate/malate plus rotenone but not with succinate plus rotenone. Therefore, the increase in ROS production induced by methionine in kidney mitochondria comes from complex I.

Concerning the possible mechanisms responsible for the methionine-induced variations in ROS production, an option is that the rate of electron flow in the respiratory chain is modified. In the case of kidney, methionine only decreased the rate of oxygen consumption in state 3 but not in state 4 in which ROS production is measured, and in the case of liver methionine did not affect oxygen consumption in any situation. Therefore, the changes in ROS generation induced by methionine in liver and kidney are not secondary to modifications in the rate of electron flow. Concerning the kind of molecular interaction involved direct modification of proteins in the respiratory chain is possible in principle. It is known that mitochondrial peptides of the respiratory chain can be modified by thiolization (Taylor et al. 2003) or acetylation (Huang et al. 2010) leading to regulatory changes in function. Among these, thiolization of complex I by oxidized glutathione increases ROS production. However, in the case of methionine the sulphur atom is not available for direct reaction. Methylation could be an alternative possibility since methionine has a free methyl group at one end of the molecule available for methylation reactions, and addition of methyl groups at arginine or lysine residues is a known type of protein post-traslational modification (Carr et al. 2011). However, protein methylation is usually catalyzed by methyltransferase enzymes which are not present in the incubation medium in which the increase in ROS production was detected. Therefore, it is difficult that direct methylation was responsible for the increases in ROS generation observed. Further studies are clearly needed to clarify the precise mechanism by which methionine increases mitochondrial ROS generation.

Among the other methionine cycle metabolites assayed in this investigation only HCys caused changes in mitochondrial ROS production, SAM and SAH being without effect. It has been proposed that increases in plasma HCys after protein or methionine dietary supplementation in rodents and humans (Verhoef et al. 2005; Velez-Carrasco et al. 2008) increase ROS production leading to LDL oxidation and atherosclerosis (Hidiroglou et al. 2004) and increases in plasma HCys have been proposed as risk factors for atheroschlerosis and other age-related diseases (Durand et al. 2001; Ninomiya et al. 2004). However, 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). On the other hand, direct incubation in the presence of HCys increases mean ROS levels in endothelial (Zhu et al. 2009) or arterial smooth muscle cells (Ke et al. 2010). However, other studies suggest that HCys-induced increases in ROS levels in endothelial cells are due to induction of NADPH oxidase or decreased thioredoxin expression (Tyagi et al. 2005) and therefore are not necessarily due to modifications in mitochondrial ROS production. In the present investigation HCys changed the rate of ROS production in mitochondria from all the organs studied. However, HCys only increased ROS generation in kidney mitochondria while strongly decreasing it in liver, heart and brain mitochondria showing again that the changes are tissue–specific in agreement with the presence of different mitochondrial proteomes in different organs (Forner et al. 2009). Thus, only in the case of kidney the pathological changes induced by methionine supplementation (Ninomiya et al. 2004) could be directly due to HCys-induced increases in mitochondrial ROS generation. The decrease in ROS generation in heart mitochondria contrasts with a previous report showing HCys-induced increases in ROS production of isolated heart mitochondria (Chang et al. 2004). Although methodological differences can be also involved, a possible explanation for the discrepancy with our results is that those increases were observed at millimolar HCys concentrations (Chang et al. 2004), much higher than the physiological ones used in our investigation.

Concerning the sites of action in the respiratory chain, HCys modified ROS generation at both complex I and complex III in the four tissues studied, because the changes were observed with complex I- and complex II-linked substrates in the presence and absence of rotenone in all cases. The changes in ROS production in liver and brain were independent of mitochondrial oxygen consumption since it was not changed by HCys. The same is true for complex III ROS generation since oxygen consumption was not changed by HCys either. Only the variations in complex I ROS generation in kidney and heart can be related to changes in state 4 oxygen consumption since these were decreased by HCys with complex I-linked substrate.

In relation to the kind of possible chemical interaction between HCys and mitochondrial proteins, a likely candidate is thiolization. It is known that proteins of the electron transport chain are rich in protein thiols (Chen et al. 2007). Some of them have structural roles, while others are reactive/regulatory thiols which are thought to have biological functions including modification of the redox status of the mitochondria (Hurd et al. 2005). An example is the increase in ROS production induced by addition of oxidized glutathione to complex I (Taylor et al. 2003). HCys has a free thiol group that can also lead to the generation of disulfide bridges with complex I or complex III thiols

leading to changes in their rates of ROS generation. The final result would be an increase or a decrease in ROS generation in different tissues depending on the existence of free thiols at different places or subunits or a different response to thiolization of complex I or III subunits in different tissues.

In summary, the results of this investigation show that among methionine metabolites HCys and methionine itself have the capacity to directly modify the rate of mitochondrial ROS production at complex I and complex III. While methionine always increases ROS generation, in the case of HCys the final effect can be an increase or a decrease depending on the tissue. The increases in mitochondrial ROS generation directly induced by methionine and HCys can be involved in the toxic effects of excessive dietary methionine supplementation and the beneficial effects of dietary methionine restriction.

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