Experimental Gerontology 48 (2013) 1030–1042

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Regulation of longevity and oxidative stress by nutritional interventions: Role of methionine restriction

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ARTICLE INFO

Article history:
Received 19 October 2012
Received in revised form 17 January 2013
Accepted 21 February 2013
Available online 27 February 2013

Section Editor: Christiaan Leeuwenburgh

Keywords:
Aging
Reactive oxygen species
Dietary restriction
Mitochondria
Complex I
DNA damage

ABSTRACT

Comparative studies indicate that long-lived mammals have low rates of mitochondrial reactive oxygen species production (mtROSp) and oxidative damage in their mitochondrial DNA (mtDNA). Dietary restriction (DR), around 40%, extends the mean and maximum life span of a wide range of species and lowers mtROSp and oxidative damage to mtDNA, which supports the mitochondrial free radical theory of aging (MFRTA). Regarding the dietary factor responsible for the life extension effect of DR, neither carbohydrate nor lipid restriction seems to modify maximum longevity. However protein restriction (PR) and methionine restriction (at least 80% MetR) increase maximum lifespan in rats and mice. Interestingly, only 7 weeks of 40% PR (at least in liver) or 40% MetR (in all the studied organs, heart, brain, liver or kidney) is enough to decrease mtROSp and oxidative damage to mtDNA in rats, whereas neither carbohydrate nor lipid restriction changes these parameters. In addition, old rats also conserve the capacity to respond to 7 weeks of 40% MetR with these beneficial changes. Most importantly, 40% MetR, differing from what happens during both 40% DR and 80% MetR, does not decrease growth rate and body size of rats. All the available studies suggest that the decrease in methionine ingestion that occurs during DR is responsible for part of the aging-delaying effect of this intervention likely through the decrease of mtROSp and ensuing DNA damage that it exerts. We conclude that lowering mtROS generation is a conserved mechanism, shared by long-lived species and dietary, protein, and methionine restricted animals, that decreases damage to macromolecules situated near the complex I mtROS generator, especially mtDNA. This would decrease the accumulation rate of somatic mutations in mtDNA and maybe finally also in nuclear DNA.

1. Introduction

What are the mechanisms regulating the rate of aging? Although perhaps multi-causal, the main causal factors determining the rate of aging are expected to be relatively few (Barja, 2008) and highly conserved across closely related species like different mammals. Numerous theories of aging have been proposed (Medvedev, 1990). However, any appropriate theory should be able to explain the four main characteristics of aging (Strehler, 1962): it is progressive, endogenous, irreversible, and deleterious (for the individual). Denham Harman first proposed in 1956 that free radicals, and especially those of mitochondrial origin (Harman, 1956; Harman, 1972; Miquel et al., 1980), are among the main causes of aging. The Mitochondrial Free Radical Theory of Aging (MFRTA) is supported by different kinds of experimental and comparative studies (Barja, 2004a,b; Barja et al., 1994a; Pamplona and Barja, 2011; Pérez-Campo et al., 1998; Sohal and Weindruch, 1996). This review summarizes the available evidence concerning the MFRTA focusing in dietary models that increase maximum longevity (dietary, protein and methionine restriction), comparative studies and the underlying mechanisms involved.

2. Mitochondrial free radical theory of aging

In the absence of pathology, mitochondria are an important cellular source of reactive oxygen species (ROS) that can oxidatively damage many different kinds of cellular macromolecules including lipids, proteins and, especially in the case of aging, mitochondrial DNA (mtDNA) (Barja et al., 1994a). MFRTA fits well with the four Strehler's rules of aging: mitochondrial ROS production (mtROSp) comes from endogenous sources (the mitochondrial respiratory chain), progressively and continuously occurs throughout life, and it is finally detrimental (for the individual) in an irreversible way due to the capacity of ROS to give rise to established somatic mutations in mtDNA (Barja, 2004a,b; Barja and Herrero, 2000; Barja et al., 1994a,b; Ku et al., 1993; Sohal

Abbreviations: 8-oxodG, 8-oxo-7,8-dihydro-2′deoxyguanosine; BER, base excision repair; DR, dietary restriction; %FRL, percentage free radical leak; IGF, insulin-like growth factor; IIS, insulin/insulin-like growth factor signaling; MetR, methionine restriction; mtDNA, mitochondrial DNA; MFRTA, mitochondrial free radical theory of aging; $mtVO_2$, mitochondrial oxygen consumption; mtROSp, mitochondrial ROS production; nDNA, nuclear DNA; PR, protein restriction; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethyonine.

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0531-5565/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.exger.2013.02.021

et al., 1990), and maybe finally also in nuclear DNA (nDNA) (Caro et al., 2010). Therefore, mtROSp seems to be one of the main factors that genetically determine the aging rate and the species-specific maximum life span potential (from here on called "longevity").

It is now well known that mtROS generation occurs not only at complex III (Boveris and Cadenas, 1975; Boveris et al., 1976) but also at complex I (Barja and Herrero, 1998; Genova et al., 2001; Herrero and Barja, 1997; Kudin et al., 2004; Kushnareva et al., 2002; Lambert and Brand, 2004). The main electron transport components that can be responsible for complex I ROS generation are the following: those located in the hydrophilic complex I domain facing the mitochondrial matrix, the flavin, and some of the FeS clusters (Genova et al., 2001; Herrero and Barja, 2000), or that situated in the inner membrane arm of complex I, the ubiquinone (Herrero and Barja, 2000; Lambert and Brand, 2004; Murphy, 2009; Treberg et al., 2011). In contrast, complex III would produce ROS directed only to the cytosolic side of the inner membrane, although recent studies suggest that part of the production could also occur towards the matrix (Brand, 2010). Oxygen derived radicals can damage all kinds of macromolecules, but mtDNA is especially important in the case of aging because the final result can be the irreversible loss or alteration of all the copies of relevant DNA-coded information of a cell, which are needed for its survival or proper functioning (Marnett and Plastaras, 2001). Determinant mechanisms of the steady-state level of oxidative mtDNA damage include its location, very close to or even in contact with the site/s of mtROS production at the inner mitochondrial membrane (Barja, 2004b; Barja et al., 1994a).

Comparative studies have shown that long-lived species have low rates of mtROSp and oxidative damage (Barja, 2004a,b; Barja and Herrero, 1998; Barja et al., 1994a,b; Herrero and Barja, 1997, 1998; Lambert et al., 2007; Sohal et al., 1994). Long-lived species produce lower amounts of ROS at their tissue mitochondria than short-lived ones, and this difference seems to occur at complex I, not at complex III. This is due in various cases to the possession of a lower percent leakage of total electron flow in the respiratory chain (%free radical leak: %FRL; Barja, 2004a). The frequently low %FRL of the mitochondria from animals with longevities higher than expected for their body size and metabolic rate means that these animals usually have mitochondria more efficient in avoiding ROS generation. In addition, they can also have (as in pigeons) a lower amount of complex I protein (Lambert et al., 2010; Pamplona et al., 2005; St-Pierre et al., 2002), and then less mtROSp. In summary, available scientific data supports a negative correlation between mtROS production and longevity in vertebrates.

In agreement with their low rates of mtROS generation, long-lived mammals have lower steady-state levels of oxidative damage (estimated by measuring 8-oxo-7,8-dihydro-2′deoxyguanosine: 8-oxodG by HPLC-EC) in their mtDNA (Barja, 2004a,b; Barja and Herrero, 2000). It is interesting to note that long-lived animals do not possess higher levels of molecules protecting from free radicals. Contrarily to this, long-lived animals have lower tissue levels of endogenous antioxidants (reviewed in Pérez-Campo et al., 1998; see also Table 1 in Pamplona and Constantini, 2011) as well as lower repair activities of endogenous DNA damage (base excision repair — BER pathway; Page and Stuart, 2011) and of protein repair through the 20S/26S proteasome (Portero-Otín et al., 2004; Salway et al., 2011) than short-lived ones. The low or very low antioxidant levels of long-lived animals, up to 15 lower in humans than in hamsters in the case of liver GSH-peroxidase (see Fig. 3 in Pérez-Campo et al., 1998), was a seminal observation that led us to propose that long-lived animals should have low rates of mtROSp and that this was the relevant trait for aging, not the antioxidant levels (Barja et al., 1994a; Ku et al., 1993; Lambert et al., 2007; López-Torres et al., 1993a; Pérez-Campo et al., 1994, 1998). Besides, many experimental studies have shown that increasing antioxidant enzymes like SOD, catalase or GSH-peroxidases in transgenic mice or in the diet does not increase animal longevity (Barja, 2004a; Muller et al., 2007; Sanz et al., 2006a) except in *Caenorhabditis elegans* (Melov et al., 2000) but only under specific conditions (Keaney and Gems, 2003). If there is a low rate of mtROS induced damage in long-lived species, there is also a smaller need for endogenous antioxidants, or for protein and DNA repair systems, which could be transitorily induced when needed to come back again to low levels when the episodic increase in oxidative stress has been overcome (Lee et al., 1996; López-Torres et al., 1993b). In this way cells save much energy which otherwise would be invested in the protein synthesis needed to continuously maintain high levels of antioxidants and DNA repair enzymes when they are not needed. Instead, long-lived species decrease mtROSp which is simpler, more efficient, and much less energetically expensive than continuously maintaining high levels of endogenous antioxidants and repair systems. On the other hand, long-lived mammals also have a low degree of fatty acid unsaturation in their cellular membranes which protects them against the deleterious process of lipid peroxidation (Naudi et al., 2011; Pamplona et al., 1996, 2002a; Pamplona and Barja, 2007).

Table 1
Summary of methionine restriction (MetR) longevity experiments in mice and rats.

Manipulation	Species	Number of animals[a]	Change in (maximum) longevity	Reference
80% MetR[b]	Fisher 344 rat	30	↑ 12%	Orentreich et al. (1993)
80% MetR[b]	Fisher 344 rat	16	↑ 44%	Richie et al. (1994)
65% MetR[c]	Mice (CB6F1)	40	↑ 10%	Miller et al. (2005)
65% MetR[c] at middle age (12 months)	Mice (CB6F1)	51	↑ 5.5%	Sun et al. (2009)

↑: increase; CB6F1 = (BALB/cJ × C57BL/6)F1.
[a] Number of different animals per dietary group in each life-long experiment.
[b] Control diet: 0.86% methionine; MetR diet: 0.17% methionine.
[c] Control diet: 0.43% methionine; MetR diet: 0.15% methionine.

The studies summarized below (Sections 3 and 4) show that dietary restriction (DR), protein restriction (PR) and methionine restriction (MetR) increase longevity and decrease mtROS generation and oxidative damage to mtDNA in rodents. Those studies connect longevity and experimental studies with the MFRTA. They offer a plausible mechanism by which both long-lived animal species and DR, PR or MetR animals can slow down the rate of aging: by decreasing mtROSp. This in turn lowers mtDNA oxidative damage and then the long-term accumulation of (irreversible) somatic mutations in mtDNA (Barja, 2004a) including point mutations, as well as deletions, and therefore generating also less mtDNA fragments. These mtDNA fragments show higher levels of 8-oxodG adducts than wild type mtDNA (Suter and Richter, 1999), and are released from mouse liver mitochondria upon opening of the mitochondrial permeability transition pore (Patrushev et al., 2004). Interestingly, these fragments are present also in nDNA, and a recent study has found that the mtDNA fragments present in nDNA increase with age in both rat liver and brain (Caro et al., 2010). Such insertions have the potential to alter the nDNA sequences. According to the "mtDNA fragments insertion inside nDNA" mechanism (Barja, 2010; Caro et al., 2010) the mitochondria would continue to be the source of the aging problem in the MFRTA but the main target would be the nucleus.

In addition to the dietary studies, it has been shown that genetic manipulations also might modulate ageing. Among others, specific mutations in the insulin/insulin-like growth factor (IGF) signalling (IIS) pathway and the target of rapamycin (TOR) pathway extend longevity in a wide range of organisms (Harrison et al., 2009; Mair and Dillin, 2008; Selman et al., 2008; Taguchi and White, 2008). These upstream nutrient signalling pathways might converge and modulate transcription factors. These changes could affect nuclear responses related to mitochondrial functions, as mitochondrial biogenesis or

regulation of ROS production (Csiszar et al., 2008; Page et al., 2010; Sanz et al., 2002). However, little is known about these processes and it is necessarily more work to elucidate the underlying mechanisms.

3. The dietary restriction life-extension effect

3.1. Longevity studies

Dietary restriction (DR; McCay et al., 1935) continues to be the most robust metabolic intervention capable of extending longevity and improving healthspan in diverse organisms including yeast, rotifers, spiders, nematodes, fish, laboratory rodents, and perhaps too in rhesus monkeys and humans (Mair and Dillin, 2008). The life-extension effect of DR in rodents can reach up to 50% (Yu et al., 1982) and it is observed not only when initiated at a young age, but also when started at middle age (Dhahbi et al., 2004; Yu et al., 1985). Besides, DR also mitigates the incidence, time of onset and progression of many age-related pathologies including cardiomyopathy, nephropathy, type-II diabetes, muscle atrophy, hypertension-related diseases, autoimmune diseases and several neurodegenerative disorders like Parkinson's or Alzheimer's disease in rodents (Jang et al., 2012; Martin et al., 2006; Mattson et al., 2002; Weindruch, 2003). Recently, it has been reported that DR also ameliorates detrimental age-related changes in specific metabolic products of lipid and fatty acid metabolism and bile acid biosynthesis in mice (De Guzman et al., 2012).

In rhesus monkeys it was observed that 30% DR strongly decreases age-related mortality (from 37% to 13%), neoplasias (by 50%), cardiovascular diseases (by 50%), diabetes (no incidence at all in the DR group), and age-associated brain atrophy (Wisconsin study, Colman et al., 2009). In aged monkeys, DR also improves glucose regulation, ameliorates task learning and performance (Willette et al., 2012) and preserves total muscle mass (McKiernan et al., 2012). However, a study describing lack of DR effects on the longevity of rhesus monkeys has been recently published (NIA study; Mattison et al., 2012). The reason for the discrepancy between the two available studies with primates is not known although the diets used in the Wisconsin study were semipurified whereas those of the NIA study were not. Using semipurified diets avoids the unexpected presence of undesired substances at levels which could affect the final results obtained. Maybe this methodological difference could justify at least part of the discrepant results concerning the survival outcomes. There were also other differences in dietary composition between both studies including much lower sucrose, the presence of antioxidant flavonoids, or higher vitamin and mineral supplementation in the NIA compared to the Wisconsin study, as well as differences in the source of protein and fat between both investigations. Although the NIA study did not demonstrate lifespan extension, there were benefits concerning age-related diseases (like in the Wisconsin study) in restricted animals and it was concluded that diet composition rather than the calories themselves strongly affect the life-prolonging effect of DR (Mattison et al., 2012). Therefore, taking into account all these considerations, with the information available DR seems to be beneficial regarding mortality and degenerative diseases also in upper primates, although more studies are clearly needed to resolve which are the lifespan effects of DR in primates and human beings.

DR also has benefits for human health (Fontana et al., 2010) similar to those observed in rodents. This nutritional intervention in humans during a mean of six years (3–15 years of 32% DR) protects against obesity, insulin resistance, detrimental effects on heart function and changes in blood pressure, ameliorates hypertension, inflammation and atherosclerosis, and is associated with many of the hormonal changes involved in the signaling of the anti-aging effects of DR in rodents (Fontana et al., 2010; Meyer et al., 2006). Other short-term studies indicated that DR in humans lowers fasting insulin, core body temperature and DNA damage (Heilbronn et al., 2006), lowers risk factors for atherosclerosis and diabetes (Holoszy and Fontana, 2007), and decreases cardiovascular diseases and possibly cancers (Fontana and Klein, 2007; Omodel and Fontana, 2011).

Other effects of DR in animals are: delays in sexual maturation, decreases in fertility and a lowered final body size together with a depressed growth rate of young animals. These negative effects are in part due to physiological changes induced by DR; among others: decreased fat mass and visceral adiposity, lowered body temperature and decreased plasma glucose, growth hormone (GH), insulin, IGF-1, thyroid stimulating hormone, and gonadotropins (Gems and Partridge, 2001; Mobbs et al., 2001).

Despite a large number of investigations and many decades of scientific work, the precise mechanism/s of longevity extension induced by DR have not been clarified. Many hypotheses have been proposed including those involving changes in the insulin/IGF-1 axis and the mammalian target of rapamycin (mTOR) signalling pathway, which affect longevity in a wide range of animals from nematodes to mammals (Harrison et al., 2009; Mair and Dillin, 2008; Selman et al., 2008). These upstream nutrient signaling pathways might converge on common sets of longevity related genes subjected to transcriptional regulation (Liu and Qian, 2011). However, contrasting results have been obtained in the different model organisms. Ames dwarf mice are deficient in pituitary hormones including GH and exhibiting low levels of IGF-1. They live 50% longer than their control siblings (Brown-Borg et al., 1996). DR increases longevity of Ames dwarf mice, supporting the idea that DR and the *df/df* genotype could extend life span at least in part through different mechanisms. However, the observation that DR does not extend the life span of the long-lived growth hormone receptor knockout (GHRKO) mutant mice further complicates the interpretation of these results (Bartke et al., 2001; Bonkowski et al., 2006).

Sirtuins, especially SIRT1 and SIRT3, have been also implicated in the control of life span during DR, although this is currently under debate (Baur, 2010; Burnett et al., 2011). SIRT1 could mediate epigenetic effects during DR, like changes in DNA methylation which could contribute to the longevity response by modifying the expression of nuclear genes (Madrigano et al., 2012; Wakeling et al., 2009). On the other hand, SIRT3, sited at mitochondria, could influence on the regulation of ATP levels, the activity of complex I (Ahn et al., 2008), the modulation of mitochondrial fatty acid oxidation (Hirschey et al., 2010) and mitochondrial ROS production (Koyama et al., 2011). Moreover, it has been found that DR decreases 8-oxodG levels in DNA and prevents age-related hearing loss in wild-type mice but not in SIRT3 −/− deficient mice. Therefore, SIRT3 would be necessary to obtain such beneficial DR-induced effects, which have been attributed to the capacity of this sirtuin to activate mitochondrial isocitric dehydrogenase 2, leading to increased NADPH levels and to a higher GSH/GSSG ratio (Someya et al., 2010).

Other mechanisms possibly involved in the beneficial effect of DR on lifespan are the lowering of mtROSp (reviewed in Gredilla and Barja, 2005) and the induction of mitochondrial biogenesis (Anderson and Prolla, 2009; Lopez-Lluch et al., 2006; Nisoli et al., 2005). On the other hand, in *Drosophila melanogaster* it has been reported that DR influences physiology and aging through the trans-sulfuration pathway (Kabil et al., 2011), a highly conserved mechanism directing methionine and its nearest product metabolites to sulfur-containing compounds like cystathionine, GSH, and perhaps important for the MetR beneficial effects, cysteine (Elshorbagy et al., 2011; reviewed in Perrone et al., 2012a). That pathway, under the control of the enzyme cystathionine β-synthase, which is up-regulated in DR in *Drosophila*, is required for the increase in longevity induced by DR in these flies (Kabil et al., 2011).

In any case, among others, proteins like: GH, insulin, IGF-1, as well as sirtuins and mTOR could participate in *pre-nuclear* signaling mechanisms, which could modulate transcription factors like FOXO (Fontana et al., 2010), while the effectors that finally might affect longevity could be in part those modified by the corresponding nuclear

responses. Mitochondrial biogenesis and mtROSp could fall within these possible final effectors.

3.2. Role of mtROS generation and mtDNA oxidative damage

DR experiments reinforce the conclusions obtained in comparative studies since both kinds of investigations strongly suggest that a causal relationship between mtROS production and aging rate exists. The same oxidative stress-related parameter that is lower in long-lived species than in short-lived ones (mtROSp) also decreases during DR in rodents (see Gredilla and Barja, 2005 for review). Long-term 40% DR (life-long DR started at 1 year of age) significantly decreases the rate of mtROSp in rat tissues including liver, heart and brain (Gredilla and Barja, 2005; Gredilla et al., 2001a,b; Hagopian et al., 2004; Lopez-Torres et al., 2002; Sanz et al., 2005a) as well as in mice (Hagopian et al., 2011; Sohal et al., 1994) and in genetically manipulated mice with total absence of CuZnSOD (Jang et al., 2012). The DR-induced decrease in mtROSp also occurs in all the three different mitochondrial fractions isolated by differential centrifugation in mouse liver (Hagopian et al., 2011). Besides, studies in yeast also demonstrate that DR improves efficiency and capacity of the mitochondrial electron transport chain, lowering mtROSp (Choi et al., 2011). However, after short term DR (6–7 weeks or 4 months), the decrease in mtROSp is observed or not depending on the tissue studied (Gredilla and Barja, 2005; Gredilla et al., 2002). It has been found that 6–7 weeks of DR is enough to decrease mtROSp and oxidative damage to mtDNA and nDNA in the case of rat liver (Gredilla et al., 2001b), while other organs require longer times of DR to show the effect on mtROSp and mtDNA (Gredilla et al., 2002). In contrast, neither the expression (Weindruch et al., 2001) nor the activity (Jang et al., 2012; Sohal et al., 1994) of the antioxidant enzymes SOD, catalase or GSH-peroxidase change in a consistent way in DR, or in long-lived genetic manipulated organisms (Page et al., 2010). It has been also found that the repair of 8-oxodG in mtDNA through the mitochondrial base excision repair (BER) pathway decreases in kidney and brain (not detected in the case of liver) in DR rats (Gredilla et al., 2010; Stuart et al., 2004).

Both complex I and complex III can generate ROS, but DR significantly decreases mtROSp only at complex I (see Gredilla and Barja, 2005). Strikingly, the ROS generator responsible for the low rate of mtROSp is situated at the same respiratory complex in the case of DR and in long-lived animal species: at complex I (Barja, 2004b; Gredilla et al., 2001a,b; Lopez-Torres et al., 2002; Sanz et al., 2005a). In contrast with mtROSp, the rate of mitochondrial oxygen consumption does not change during DR (Gredilla et al., 2001a,b; Lopez-Torres et al., 2002) in agreement with the lack of variation of the basal metabolic rate of animals subjected to DR (McCarter et al., 1985; Yen et al., 2004). The mechanisms allowing the decrease in mtROSp during DR include: 1) quantitative changes: a lower amount of the complex I protein that would produce less ROS; 2) qualitative changes: a decrease in the electronic reduction degree of the complex I generator, because the decrease in mtROSp occurs when complex I is only partially reduced but disappears after fully reducing complex I (with pyruvate/malate plus rotenone) (Gredilla et al., 2001a,b; Lopez-Torres et al., 2002; Sanz et al., 2005a), and a decrease in the percentage of free radical leak in the respiratory chain directed to ROS production (%FRL). These changes occur in rat heart, liver and brain mitochondria from dietary restricted animals (Gredilla et al., 2001a,b; Lopez-Torres et al., 2002; Sanz et al., 2005a). Therefore, mitochondria from animals subjected to DR, similarly to those from long-lived species, are more efficient in avoiding ROS generation at the mitochondrial respiratory chain (Gredilla and Barja, 2005).

The decrease in mtROSp during DR takes place together with significant decreases in oxidative damage to mtDNA alone, or in mtDNA and nDNA (estimated by measuring the level of 8-oxodG) depending on the organ studied (Gredilla et al., 2001a,b; Lopez-Torres et al., 2002; Sanz et al., 2005a), as well as with lowered oxidative, glycoxidative and lipoxidative damage to mitochondrial proteins (Lambert et al., 2004; Pamplona et al., 2002b). The available studies suggest that lowering mtROSp at complex I is a highly conserved evolutionary mechanism shared by both long-lived and dietary restricted animals that lowers steady-state oxidative damage to macromolecules, especially to mtDNA (Barja, 2004a; Barja and Herrero, 2000; Barja et al., 1994a,b; Gredilla and Barja, 2005), and then the accumulation rate of somatic mutations in mtDNA, and possibly the age-related increase in mtDNA fragment-related mutations in nDNA (Caro et al., 2010), and finally the aging rate (Fig. 1).

Much of the available evidence points to decreases in mitochondrial oxidative stress and insulin signaling as possible contributors to the increase in longevity during DR. Is mitochondrial oxidative stress under the control of insulin signaling? In a study performed in our laboratory with control and DR animals treated with 80%slow–20%fast insulin or GH it was found that the effects of these hormones are complex and can include both increases and decreases in mitochondrial oxidative stress (Sanz et al., 2005b). Animal models capable of dissociating the decrease in mitochondrial oxidative stress from the decreases in insulin-like signaling during DR have been obtained using the every other day feeding model in C57BL/6 mice. When these mice were maintained on this regimen, they showed increases in longevity (Goodrick et al., 1990) and improvements in neuronal resistance to injury, just like in DR, but their plasma IGF-1 levels did not decrease and were even somewhat increased (Anson et al., 2003). This dissociates the increase in longevity from the decrease in IGF-1 observed in DR. In another study from our laboratory it was found that restricting the diet using the every other day feeding method in C57BL/6 mice decreases, like classic DR, mtROSp and oxidative damage in mtDNA (Caro et al., 2008a; Gredilla and Barja, 2005) which dissociates the decrease in mtROS generation from insulin/IGF-1 signaling. These studies suggest that the decrease in mtROSp in DR would not be under the control of insulin-like signaling, although more studies are needed before firmly establishing this conclusion.

4. Protein and methionine restriction: the effect of a single amino acid

4.1. Effect on longevity extension

After many decades of highly relevant, long and expensive research on the effects of life-long DR in rodents, a rather general consensus was reached among the specialists: calorie intake per se would be exclusively responsible for the increase in lifespan induced by DR in rodents. However, now many studies question this classical consensus (Archer, 2003; Iwasaki et al., 1988; Lopez-Torres and Barja, 2008; Mair et al., 2005; Miller et al., 2005; Orentreich et al., 1993; Piper et al., 2011; Richie et al., 1994; Sun et al., 2009). The results of many investigations are consistent with the possibility that part of the life-extending effects of DR are due to the decreased intake of particular components of the diet, such as proteins, and more specifically the amino acid methionine (Caro et al., 2008b, 2009a,b; Grandison et al., 2009; Lee et al., 2008; Lopez-Torres and Barja, 2008; Mair et al., 2005; Miller et al., 2005; Orentreich et al., 1993; Pamplona and Barja, 2006; Richie et al., 1994; Sanchez-Roman et al., 2011, 2012; Sanz et al., 2004, 2006b; Simpson and Raubenheimer, 2009; Sun et al., 2009). Neither life-long isocaloric carbohydrate nor lipid restriction seems to increase rodent life span. Two available investigations of carbohydrate restriction or supplementation reported contradictory and minor changes in rat longevity (Khorakova et al., 1990; Ross, 1976), whereas it was found that the longevity of Fisher 344 rats does not change after life-long lipid restriction (Shimokawa et al., 1996).

In contrast, the large majority of the investigations on the effects of isocaloric protein restriction in rats and mice found increases in longevity. Ten out of eleven PR studies in rats or mice (16 out of 18

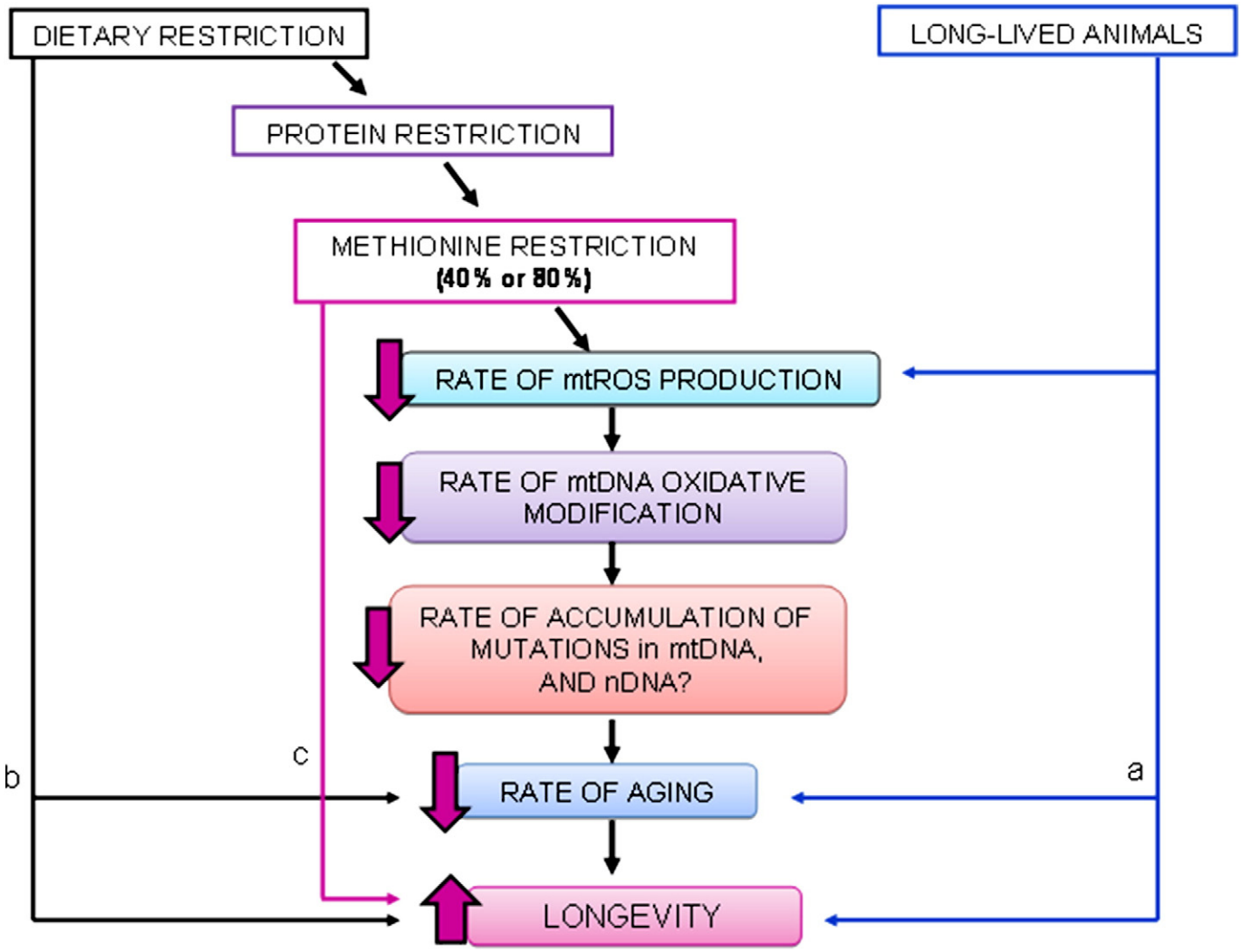

Fig. 1. Mitochondrial oxidative stress and longevity. The rate of mitochondrial ROS production (mtROSp) and the ensuing oxidative damage to mtDNA are low in long-lived mammals and birds, as well as in dietary (DR), protein (PR) and methionine (MetR) restricted rodents. The lower the endogenous rate of mtROSp and oxidative damage to mtDNA, the lower the progressive accumulation rate of (irreversible) somatic mutations in mtDNA would be, and maybe also in nDNA through the "insertion of mtDNA fragments inside nDNA" mechanism (Caro et al., 2010). This would contribute to slow down the aging rate which, together with many other necessary differences/changes[a–c] (Barja, 2008), would increase (maximum) longevity. At least three different longevity gene clusters should exist. The cluster involved in modifying the longevity of different animal species during evolution must be the largest one, followed by that operating in DR, and the likely somewhat smaller one would work in MetR. The low mtROSp and 8-oxodG in mtDNA from DR and PR animals seem to be induced by their lower level of methionine ingestion. Other unknown mechanisms (b) different from the MetR-mtROSp-DNA/s mutation pathway should contribute to decrease aging rate in DR.

different life-long survival experiments) reported increases in longevity (reviewed in Pamplona and Barja, 2006, and Lopez-Torres and Barja, 2008), although the mean magnitude of this increase (19.6 %) was lower than that usually found in 40% DR (around 40% increase). Thus PR would be responsible for around half of the life-extension effect of DR.

Which is the amino acid responsible for the increase in longevity exerted by PR? It has been demonstrated that isocaloric 80% methionine restriction (MetR) increases longevity in F344 rats (Orentreich et al., 1993; Richie et al., 1994) and mice (Miller et al., 2005; Sun et al., 2009) to a similar extent than PR (around 18% mean increase) (Table 1). This occurs even when MetR is started as late as at 12 months of age in C6BF1 mice (Sun et al., 2009). Studies performed in *D. melanogaster* have also shown that casein restriction (Min and Tatar, 2006) and methionine restriction (Troen et al., 2007) extend longevity independently of the caloric intake. Moreover, other recent studies link essential amino acids, and again especially methionine, with the positive effect of DR on longevity in yeast (Petti et al., 2011) and *D. melanogaster* (Grandison et al., 2009; Kabil et al., 2011). Interestingly, PR performed in rats, results in profound changes in methionine and serine metabolism (including lowering cystathionine β-synthase and cystathionine γ-lyase activities) and increases in fatty acid oxidation (Kalhan et al., 2011).

In addition to extending lifespan, 80% MetR also decreases disease-associated markers and the incidence of age-related degenerative diseases. The beneficial effects of this intervention in rodents include decreases in serum glucose, insulin, IGF1, cholesterol, triglycerides and leptin (Table 2). Besides, MetR protects against age-related changes in immunity, slows cataract development (Miller et al., 2005), improves colon tight junction barrier function (Ramaligan et al., 2010) and improves metabolic flexibility and increases respiratory uncoupling (Hasek et al., 2010). MetR may be also an important strategy to inhibit tumor growth particularly in many cancers that exhibit the known phenomenon of "methionine dependence". These include bladder, breast, colon, glioma, kidney, melanoma, prostate and other cancers in which tumor cells have a much greater reliance on methionine than normal cells do (Hoffman, 1985; Komninou et al., 2006). They need this amino acid for survival and proliferation and their growth seems seriously limited or inhibited in the absence of methionine (reviewed in Cavuoto and Fenech, 2012). Adding homocysteine instead of

methionine in the culture medium can halt the growth of many cancer cells while allowing the continued growth of non-transformed cells (Cao et al., 2002).

Regarding the beneficial effects of MetR (80%) on adiposity, this intervention reduces total adipose tissue mass and lowers visceral fat by 70% (by more than 40% after correcting for the decrease in body mass; Malloy et al., 2006) in association with an improvement in insulin sensitivity (Malloy et al., 2006) (Table 2). In addition, MetR decreases leptin and increases adiponectin in rodents in agreement with the decrease in visceral adiposity and the size of white adipose tissue depots. These beneficial effects seem to be mediated by tissue-specific responses that favor increased mitochondrial function and biogenesis, fatty acid oxidation and total energy expenditure possibly mediated by β-adrenergic receptor signaling and changes in lipid homeostasis (Perrone et al., 2012a). In this line, a recent metabolomic and genomic MetR study found changes in the expression of a large number of genes and proteins that led the authors to conclude that MetR increases lipid metabolism in adipose tissue and muscle whereas it decreases lipid synthesis in the liver (Perrone et al., 2012b). Therefore, these changes in lipid metabolism seem to be involved in the strong decrease in adiposity and increased insulin sensitivity observed in isocaloric MetR.

MetR also leads to altered levels of sulfur-containing amino acids: serum levels of methionine, cysteine, cystathionine, and taurine decrease in MetR rats, whereas homocysteine levels (Elshorbagy et al., 2011) and GSH (Richie et al., 1994) increase. Interestingly, adding cysteine to the MetR diet reverses most of the studied beneficial changes on adiposity and insulin resistance (Elshorbagy et al., 2011) and increases the transcription of various genes associated with inflammation and carcinogenesis (Perrone et al., 2012b). Therefore, the beneficial changes of MetR diet have been attributed to the decrease of cysteine in serum (Elshorbagy et al., 2011) or liver (Perrone et al., 2012b) observed in animals subjected to MetR diet.

On the other hand, excessive intake of dietary methionine is toxic. This toxicity far exceeds that produced by any other amino acid (Harper et al., 1970), leading to damage in some vital organs and increases in tissue oxidative stress (Gomez et al., 2009; Park et al., 2008) with similar negative effects to those observed in rats fed diets with a high protein content. Chronic and excessive methionine supplementation increases plasma hydroperoxides and LDL-cholesterol (Hidiroglou et al., 2004), induces vascular (Troen et al., 2003) and kidney damage with tubular hypertrophy (Kumagai et al., 2002), raises iron accumulation and lipid peroxidation, and leads to liver dysfunction (Mori and Hirayama, 2000), besides other alterations in other organs. In addition, methionine supplementation strongly increases methionine and its two more nearly derived methionine cycle metabolites, S-adenosylmethyonine (SAM) and S-adenosylhomocysteine (SAH), in rat liver and kidney (Gomez et al., 2009). Some of the harmful effects have been attributed to these methionine-related metabolites like SAM, SAH, or homocysteine, rather than to methionine itself, although in other cases a direct methionine toxic effect has been suggested (Harper et al., 1970; Troen et al., 2007). This last case fits well with our observation that direct addition of methionine to isolated mitochondria in vitro increases their rate of mtROSp in liver and kidney although not in heart or brain rat mitochondria (Gomez et al., 2011).

Oxidation of methionine residues in proteins generates methionine sulfoxide depriving them of their function as methyl donors and may lead to loss of their biological activity (Ciorba et al., 1997). This modification can be repaired by methionine sulfoxide reductase in a thioredoxin-dependent reaction. In this context it is interesting that overexpression of methionine sulfoxide reductase increases longevity in *D. melanogaster* (Chung et al., 2010) and the opposite manipulation, knocking out the same enzyme, increases protein carbonyls and decreases longevity (Moskovitz et al., 2001). There is evidence that this enzyme plays an important role in protection against oxidative, cold, and heat stress and in the regulation of aging in *D. melanogaster* (Lim et al., 2012). Also in agreement with a methionine role in aging, it has been reported that long-lived Ames dwarf mice have an altered methionine metabolism showing a marked increase in the transulfuration pathway compared to their wild-type siblings (Uthus and Brown-Borg, 2006). All the above results point to methionine as the single dietary factor responsible for part of the longevity extension effect of DR.

Table 2
Beneficial effects of methionine restriction (MetR) in rats and mice.

Species	Parameter	Reference
Fisher 344 rat	↓ Fat pad	Orentreich et al. (1993)
	↓ Visceral adiposity	Malloy et al. (2006)
	↓ Adipocyte size in all WAT depots[a]	Perrone et al. (2008)
	↓ Serum insulin and glucose	Perrone et al. (2010)
Mice (CB6BF1)	↓ Serum insulin and glucose	Miller et al. (2005)
Fisher 344 rat	↓ Serum IGF-1	Malloy et al. (2006), Perrone et al. (2010)
Mice (CB6BF1)	↓ Serum IGF-1	Miller et al., 2005
Fisher 344 rat	↓ Serum cholesterol	Malloy et al. (2006), Perrone et al. (2010)
Mice (CB6BF1)	↓ Serum cholesterol	Miller et al. (2005)
Fisher 344 rat	↓ Serum triglycerides	Perrone et al. (2010)
Fisher 344 rat	↓ Serum leptin	Perrone et al. (2010)
Fisher 344 rat	↓ Serum thyroxine levels	Malloy et al. (2006), Perrone et al. (2010)
Mice (CB6BF1)	↓ Total T4 levels	Miller et al. (2005)
Mice (CB6BF1)	↓ Cataract development	Miller et al. (2005)
Mice (CB6BF1)	↓ Of the decrease in T cells with age	Miller et al. (2005)

CB6F1 = (BALB/cJ × C57BL/6) F1; ↓ = decrease.
[a] Inguinal, epididymal, mesenteric and retroperitoneal. WAT = White adipose tissue.

4.2. Role of mtROS generation and oxidative damage

What is the specific dietary component responsible for the decreases in mtROS production and oxidative damage to mtDNA during DR? In agreement with their lack of effect on longevity (Khorakova et al., 1990; Ross, 1976; Shimokawa et al., 1996), neither isocaloric 40% lipid restriction (Sanz et al., 2006c) nor isocaloric 40% carbohydrate restriction (Sanz et al., 2006d) changes mtROSp or 8-oxodG in mtDNA. However, isocaloric 40% PR does decrease mtROSp and oxidative damage to mtDNA in rat liver (Sanz et al., 2004) in a strikingly similar way, quantitatively and qualitatively (Sanz et al., 2004), to 40% DR. The effect of PR was studied in rat liver without changing the amount eaten per day of the other dietary components and it was found, like in 40% DR, that 40% PR decreases liver mtROSp specifically at complex I, lowers %FRL and 8-oxodG in mtDNA (Sanz et al., 2004), and decreases the five specific markers of protein purely oxidative, glycoxidative and lipoxidative modification studied, as well as the complex I protein content in rat liver mitochondria and tissue (Ayala et al., 2007). Strikingly, the direction of change, the magnitude, mechanisms and site of action exerted by PR on mtROSp and 8-oxodG in mtDNA are very similar to those found in 40% DR (Lopez-Torres and Barja, 2008). Taken together, those studies suggest that proteins are the dietary components responsible for the decreases in mtROSp and oxidative damage to mitochondrial macromolecules that takes place in DR, and for part of the increase in longevity during DR.

It was logical to suspect that dietary methionine could be involved in those PR and DR effects since it was already known that MetR, independent of energy restriction, increases rat (maximum) longevity

Table 3
Changes in mitochondrial oxidative stress-related parameters in methionine restricted (MetR), amino acid restricted (except for methionine; RESTAAS) and methionine supplemented (MetS) rats.

Dietary manipulation	Organ	mtROSp	Content of respiratory complexes	FRL (%)	8-oxodG in mtDNA	Oxidat. protein modific. [a]	Reference
80% MetR[b]	Liver	↓at Cx I ↓at CxIII	↓CxI/IV	↓at Cx I	↓	↓	Sanz et al. (2006b)
	Heart	↓at CxI	↓CxI/IV	↓at CxI	↓	↓	
80% MetR[b]	Liver	↓at Cx I ↓at CxIII	↓CxI/II/III/IV	↓at CxI	↓	↓	Caro et al. (2008b)
40% MetR[c]	Liver	↓at CxI ↓at CxIII	↓CxI/II/III/IV	↓at CxI	↓	↓	Caro et al. (2008b)
40% MetR[c]	Brain	↓at Cx I	↓CxI/II/III/IV	↓at Cx I	=	↓	Caro et al. (2009a)
	Kidney	↓at Cx I	↓Cx IV	↓Cx I	↓	↓	
40% MetR[c]	Heart	↓at Cx I	=	n.d.	↓	↓	Sanchez-Roman et al. (2011)
40% MetR[c]at old age	Liver	↓at Cx I	↑CxIV	↓	↓	↓	Sanchez-Roman et al. (2012)
40% RESTAAS[d]	Liver	=	=	=	=	↓	Caro et al. (2009b)
2.5% MetS[e]	Liver	↑at CxI	↓Cx IV	↑at CxI	↑	=	Gomez et al. (2009)
	Heart	=	=	=	=	=	

Cx = respiratory complex; mtROSp = rate of mitochondrial ROS production; %FRL = percent free radical leak at the respiratory chain; 8-oxodG (8-oxo-7,8-dihydro-2o-deoxyguanosine) by HPLC-EC.

[a] Purely oxidative (the protein carbonyls glutamic and aminoadipic semialdehydes), glycoxidative (Carboxyethyl-lysine; Carboxymethyl-lysine-CML), and lipoxidative (CML and MDA-lysine) protein modifications (GC-MS) in mitochondria (except for Gomez et al., 2009, in total tissue). = No change; ↑ increase; ↓ decrease; n.d. not determined. Semipurified diets (Biolink USA) based on AIN-76G or AIN-93G formulations were given during 6–7 weeks starting at 6–7 weeks of age except in Sanchez-Roman et al., 2012 (started at 24 months of age).

[b] Control diet: 0.86% methionine; MetR diet: 0.17% methionine.

[c] Control diet: 0.86% methionine; MetR diet: 0.52% methionine.

[d] The RESTAAS diet contained 40% less of all the dietary amino acids, except for methionine that was present at same concentration in the two diets.

[e] Control diet: 0.86% methionine; methionine supplemented diet: 2.5% methionine.

(Orentreich et al., 1993; Richie et al., 1994) while such effect had not been described for any of the other dietary amino acids. This is why in our laboratory we decided to study the effects of MetR on mtROSp and oxidative stress (Table 3). Isocaloric MetR (80% and 40%), applied to young rats during 7 weeks, lowers mtROSp (mainly at complex I), the % FRL, the complex I content, 8-oxodG in mtDNA, and specific markers of protein oxidative, glycoxidative and lipoxidative modification in rat heart (80% MetR; Sanz et al., 2006b; 40% Sanchez-Roman et al., 2011) or liver (40% and 80% MetR; Caro et al., 2008b) mitochondria, similarly to what occurs after 7 weeks of 40% MetR in rat kidney and brain mitochondria (Caro et al., 2009a; Naudi et al., 2007). In order to obtain these decreases it was enough to restrict methionine by 40%. 80% MetR leaded to a similar decreases in 8-oxodG than 40% MetR, while the decrease in mtROSp in 80% MetR was only somewhat more intense than in 40% MetR, being the decrease in mtROSp from control to 40% MetR more pronounced than that occurring between 40% MetR and 80% MetR.

Interestingly, in another experiment, when all the dietary amino acids except methionine were restricted (by 40%) during 7 weeks, neither the rate of mtROSp nor the level of 8-oxodG in mtDNA was modified. In addition, we have recently found that 40% MetR also decreases mtROSp, %FRL and 8-oxodG in mtDNA and reverses aging-related increases in protein modification when implemented at old age (during 7 weeks in 24 month old rats; Sanchez-Roman et al., 2012). All those results, taken together, indicate that the lowered ingestion of methionine during MetR (and PR and DR) is responsible for the decreases in mitochondrial ROSp and oxidative stress observed in MetR (and PR and DR), and possibly for all (during PR and MetR) or part (during DR) of the life-extension effect observed during these dietary manipulations. Moreover, the extraordinary capacity of a *single dietary molecule* to induce the decrease in mtROSp is still present in old rats.

Concerning the mechanisms responsible for the decrease in mtROS production during MetR (Fig. 2), there are various possibilities described previously in the DR section. A simple mechanism is based on a decrease in the content of the complex I protein in MetR that would directly lead to a decreased rate of mtROSp. This has been reported under 40% MetR in the majority of tissues studied (Table 3), also during DR and PR, as well as in long-lived birds (pigeons, canaries and parakeets) compared to the much short-lived mammals (rats and mice) of similar body size (Lopez-Torres and Barja, 2008; Pamplona et al., 2005; St-Pierre et al., 2002). But this cannot be the whole explanation. MetR also induces qualitative changes in mitochondria since not only generate less ROS but they have a lower %FRL and a lower electronic reduction state of the complex I ROS generator (the decrease in mtROSp is observed, like in DR, with partial complex I reduction but not with full reduction). Thus, MetR mitochondria (from both young and old animals) are more efficient in avoiding mtROS generation. They leak less radicals per unit of electron flow in the respiratory chain, similarly to what has been found in long-lived compared to short-lived animals as well as in DR and PR rats compared to their controls (Barja, 2004a,b).

These quantitative and qualitative changes can be due to: a) direct interaction of methionine, or more likely, of a more chemically reactive methionine metabolite with the mitochondria or some critical complex I polypeptide/s; b) changes in cellular signaling molecules and the ensuing modification of specific gene expression; and c) decreases in the matrix NADH (which feeds electrons to complex I) thus lowering the state of electronic reduction of the complex I generator, due to decreases in the amounts of mitochondrial substrates.

Regarding mechanism "a": recent studies have shown that the direct addition of methionine to isolated functional mitochondria freshly obtained from rats increases their rates of mtROSp (Gomez et al., 2011). Therefore a rather direct and rapid effect of methionine (or a closely derived reactive metabolite) on complex I in vivo seems to occur. However, this action could be due to a chemically reactive methionine metabolite. This possibility is most relevant because in the methionine molecule, differing from homocysteine or cysteine, the potentially reactive sulfur is located inside the molecule and it is not available for direct covalent chemical reaction with protein thiols. Interestingly, it has been recently observed that the reaction of methionine with hydroxyl radicals generates methionine radical carbon-, nitrogen- and sulfur-centered radicals as intermediates in the formation of the methanethiol product, as detected by EPR spin trap techniques and GC–FID and GC–MS techniques (Spasojević et al., 2012). These radicals or methanethiol (CH_3SH) itself could react with complex I or some of its subunits leading to increases in mtROS generation. Since it is known that GSSG thiolization of isolated complex I increases its rate of ROS production (Taylor et al., 2003) a similar reaction of methanethiol, or cysteine (which also has a free thiol group available for direct reaction) with

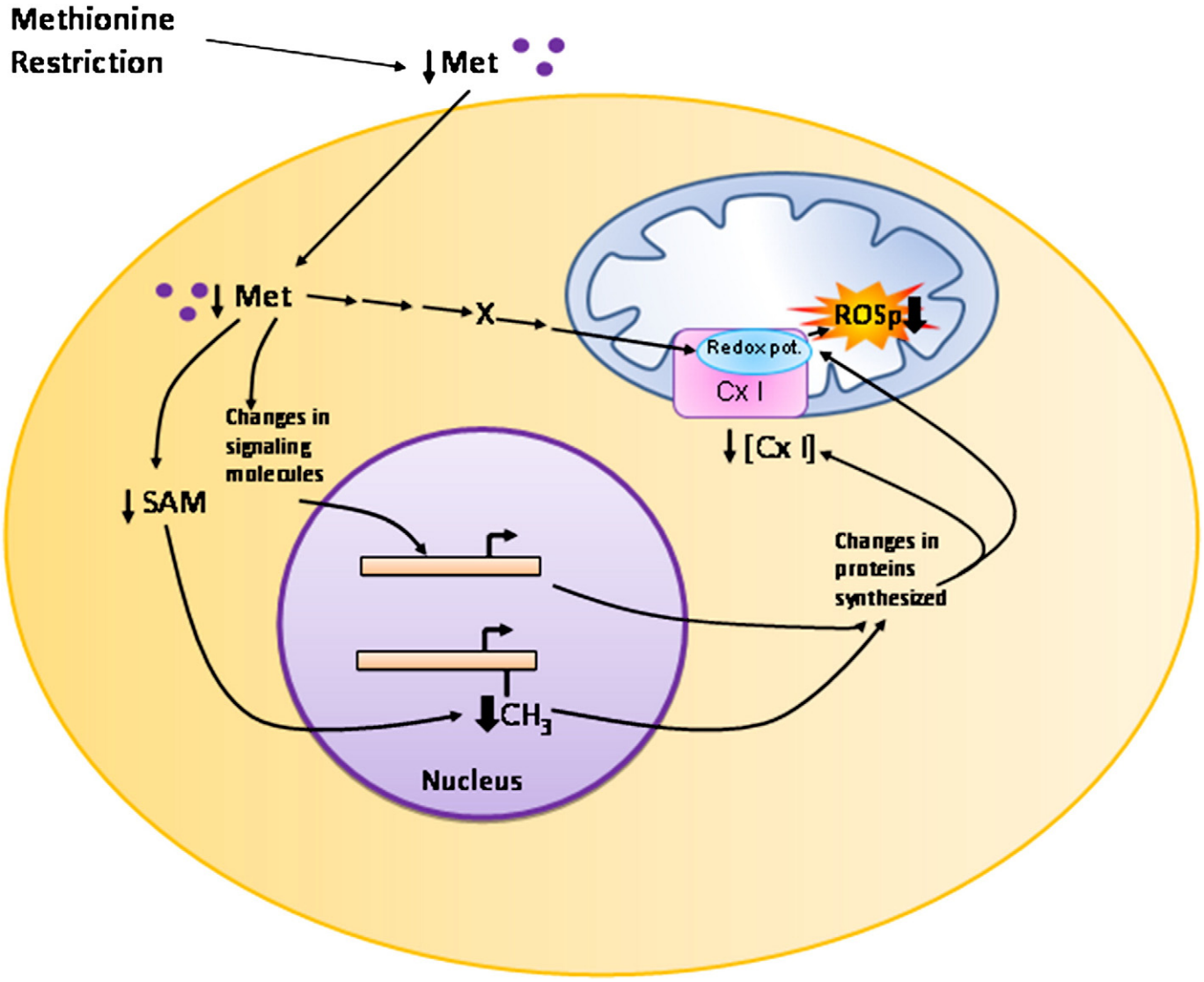

Fig. 2. How MetR can decrease mitochondrial ROS production. The scheme summarizes the possible pre- and post-nuclear acting mechanisms potentially responsible for the decrease in mtROS production induced by MetR. Dietary methionine restriction decreases tissue cytosolic methionine levels. This can: a) increase the midpoint redox potential of the complex I ROS generator (thus lowering mtROSp and %FRL), possibly due to decreased thiolization or chemical interaction with some complex I subunit/s. These effects have been attributed to methionine metabolites rather than to methionine itself; b) modify cellular cytosolic signaling molecules which modulate specific gene expression; and c) decrease the S-adenosylmethionine (SAM) cytosolic concentration which would decrease nuclear DNA methylation and it could modify gene expression. The resulting post-nuclear mechanisms can: 1) decrease the amount of complex I per mitochondrial mass, which would tend to decrease mtROSp; and 2) increase the midpoint redox potential of the complex I ROS generator (thus lowering mtROSp and %FRL). There are multiple pre-nuclear signaling mechanisms involved in DR (and likely in MetR), but few post-nuclear final effector mechanisms have been described: the decrease in mtROSp in MetR, PR and DR as well as the increase in mitochondrial biogenesis (better documented in DR). The simultaneous operation of these two post-nuclear changes will produce more mitochondria with a lower generation rate of mtROS and DNA damage. This could contribute to increase longevity in coordination with the many other necessary changes modulated by the DR and MetR longevity gene clusters. SAM = S-adenosyl-methionine; Redox pot. = midpoint redox potential of the complex I ROS generator.

complex I thiol groups could be involved in the decrease in mtROSp in MetR. This dietary manipulation decreases hepatic methionine and cysteine (Perrone et al., 2012b) and likely methanethiol levels, which can decrease thiolization of complex I subunits and then their rates of mtROSp. Alternatively, cysteine could also interact with the protein cysteines of some of the FeS clusters of the hydrophilic arm of complex I, leading to iron release or availability for reaction and then ROS generation. Lower cysteine levels in MetR could also decrease mtROSp through this mechanism.

Concerning the changes in gene expression possibly involved in the MetR effects (mechanism "b"), a recent genomic MetR study found changes in the expression of a large number of genes and proteins involved in lipid metabolism (Perrone et al., 2012b). In addition, modifications of DNA methylation could be also involved (Passarino et al., 2010; Robert et al., 2010). Methionine is an essential amino acid with many key roles in mammalian metabolism including protein synthesis and function, as well as protein and DNA methylation (Brosnan and Brosnan, 2006). Since ageing seems to be associated with site-specific changes in DNA methylation (Cedar and Bergman, 2012; Christensen et al., 2009; Heyn et al., 2012; Maegawa et al., 2010; Wakeling et al., 2009), MetR diets could extend longevity in rodents through various changes including modulation of DNA methylation patterns, specific changes in gene expression, and changes in translation rates, whose effects could include decreases in mtROS generation and oxidative damage (Fig. 2). In agreement with that, we have recently detected that MetR induces a small but statistically significant decrease in global genomic DNA methylation in rat liver of young immature rats (Sanchez-Roman et al., 2011), whereas when

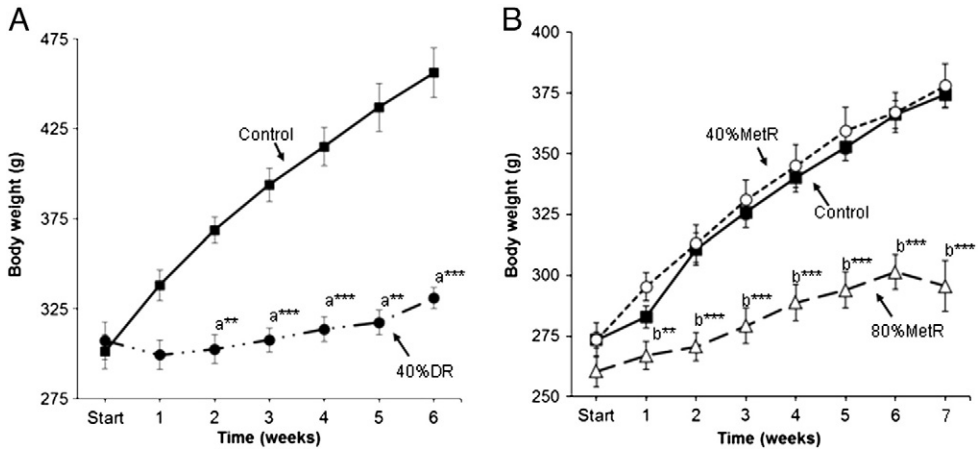

Fig. 3. Body weight and growth rate of male Wistar rats in 40% DR (A), 40% and 80% MetR (B), and their respective controls (A and B). Animals were fed semipurified diets (ICN Biolink, USA) based on the AIN-93G diet. Each point represents the mean ± SEM from 10 different individuals. The experiments started with animals of 6–7 weeks of age. Significant differences: [a]between 40% DR and their Controls; [b]between 80% MetR and their controls or between 80% MetR and 40% MetR; $^{**}p < 0.01$, $^{***}p < 0.001$. No significant differences in body weights were found between control and 40% MetR at any time of experimentation. Data come from experiments in Gredilla et al. (2001a,b) (A) or Caro et al. (2008b) (B).

this manipulation was performed in old rats the decrease in this parameter did not reach statistical significance (Sanchez-Roman et al., 2012).

Concerning mechanism "c": decreased NADH, it is more likely in DR than in MetR, due to the large number of metabolites than can potentially be decreased because of the lower caloric ingestion. In fact there is a published study in which it was shown that pyruvate, malate, and succinate, as well as NADH and the NADH/NAD+ ratio are decreased in the tissues of DR animals (Burch et al., 1970).

In summary, DR, PR and MetR are nutritional interventions that increase longevity in rodents, although the magnitude of the longevity extension of MetR and PR in rodents is around 50% that of DR. This lower but significant life extension effect in MetR than in DR would agree with the widely held notion that aging and longevity have more than one cause. Restriction of methionine intake can be responsible for part of the aging-delaying effects of DR by decreasing mtROSp and oxidative damage to mtDNA and macromolecules, acting at least in this sense as a "DR-mimic". All that suggests that methionine is the single dietary substance responsible for the beneficial changes of DR on mitochondrial oxidative stress. The remaining effects of DR on aging rate could be due to decreases in other dietary components or in the calories themselves through different additional mechanisms (Fig. 1, arrow marked as "?"). In any case, it is interesting that 40% MetR can decrease mitochondrial oxidative stress, because this dietary manipulation, (or PR), does not imply the strong behavioral and nutritional stress of caloric restriction and thus seems a much more feasible option for wide application to human populations. Most importantly, negative effects such as delays in puberty, and decreases in growth rate and final body size are shared by DR and 80% MetR but do not occur in 40% MetR (Fig. 3). Methionine restriction at the 40% level is potentially advantageous because it lowers mtROSp and 8-oxodG in mtDNA to a similar extent than 80% MetR, while totally avoiding the decreases in body and organ weight, growth rate, maturation and likely final body size that takes place in 80% MetR and 40%DR (Fig. 3).

It is becoming clear that health benefits can be obtained using "prudent" diets largely based on the consumption of complex carbohydrates, emphasizing vegetables with proteins rich in essential amino acids but low in the sulphur-containing amino acids methionine and cysteine (like pulses), or almost totally lacking methionine and cysteine (like fruits and vegetables), and avoiding the presently excessive intake of animal proteins (as well as fats). The results already available about PR in humans seem to be positive for human health and of similar character than those found in DR after up to 6 years of intervention in human beings (Fontana et al., 2008). These studies suggest that DR and PR can protect from obesity, mortality, and degenerative diseases including at least cardiovascular ones, diabetes and cancer, and can increase the human healthspan. MetR, implemented at 40%, should be investigated in more depth concerning mechanisms, detailed effects, and times of application, as well as its possible effects on longevity.

5. Conclusions

1. Long-lived mammals have low rates of mitochondrial ROS production and oxidative damage in mtDNA.
2. Dietary restriction (DR) decreases the rate of mitochondrial ROS production at complex I and oxidative damage to mtDNA and proteins, and extends the maximum life span of most or all the animal species studied to date.
3. The respiratory complex related to aging, longevity, and mtROS generation, both concerning comparisons between mammalian and bird species with different longevities, as well as dietary restriction, is complex I, not complex III.
4. Both protein restriction (PR) and methionine restriction (MetR) similarly increase maximum longevity in rodents, although the magnitude of these increases seems to be around half that usually found in DR. Both interventions also decrease mitochondrial ROS production at complex I, the %FRL, and 8-oxodG in mtDNA to a similar extent, quantitatively and qualitatively, as DR.
5. Restriction of all the dietary amino acids except methionine does not modify mitochondrial ROS production, the %FRL, or oxidative damage to mtDNA.
6. Methionine is the single dietary nutrient responsible for the decrease in mitochondrial ROS production and oxidative stress, and possibly for part of the increase in longevity that takes place during DR (and PR).
7. MetR animals resemble DR animals in many traits including low levels of insulin and IGF-1, low adiposity, low rates of mitochondrial ROS generation and oxidative stress, and longer mean and maximum lifespan. However, DR seems to exert a stronger life extension effect than MetR.

Conflict of interests statement

The authors declare no conflict of interest.

Acknowledgments

The results obtained at our laboratory described in this review have been supported by grant nos. BFU2008-0335 and BFU2011-23888 from the Ministry of Science and Innovation, and no. UCM/BIO-2648 GR74/07-CCG07 Madrid Community-UCM to G. Barja. I. Sanchez-Roman received a pre-doctoral FPU fellowship from the Spanish Ministry of Education.

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