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Review

Mitochondrial metabolism in aging: Effect of dietary interventions

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ABSTRACT

Mitochondrial energy metabolism and mitochondrially-derived oxidants have, for many years, been recognized as central toward the effects of aging. A body of recent work has focused on the relationship between mitochondrial redox state, aging and dietary interventions that affect lifespan. These studies have uncovered mechanisms through which diet alters mitochondrial metabolism, in addition to determining how these changes affect oxidant generation, which in itself has an impact on mitochondrial function in aged animals. Many of the studies conducted to date, however, are correlative, and it remains to be determined which of the energy metabolism and redox modifications induced by diet are central toward lifespan extent. Furthermore, dietary interventions used for laboratory animals are often unequal, and of difficult comparison with humans (for whom, by nature, no long-term sound scientific information on the effects of diet on mitochondrial redox state and aging is available). We hope future studies will be able to mechanistically characterize which energy metabolism and redox changes promoted by dietary interventions have positive lifespan effects, and translate these findings into human prevention and treatment of age-related disease.

1. Introduction

The importance of energy metabolism in aging has been recognized since 1928, when Pearl proposed that rates of aerobic metabolism correlated with aging. Rate of living is well demonstrated today *not* to be directly correlated with lifespan, in particular when oxidative metabolism is corrected for animal mass (Barja, 2002). However, the idea that aerobic metabolism (and in particular mitochondrial metabolism) is central toward the development of the aging phenotype has not only stood the test of time, but is gaining further strength in recent years. Indeed, it is to be expected that mitochondria, as the site of oxidative phosphorylation and providing the vast majority of high energy phosphates the cell requires to function, are strongly involved in aging effects. This concept is further supported by the very early finding that the restriction of dietary calories (calorie restriction, CR), an intervention which clearly alters mitochondrial metabolism, extends rodent lifespan (McCay et al., 1989 (reprinted from 1935)).

In addition to their central role in energy metabolism, mitochondria were found to be a source of free radicals and other reactive oxygen species (ROS) in the 1960s (Hinkle et al., 1967). Based on his earlier proposal that oxidative damage was a limiting factor in lifespan (Harman, 1956) and the experimental evidence that mitochondria were a significant source of intracellular oxidants, Harman revisited his free radical theory of aging to suggest mitochondria were centrally involved in aging (Harman, 1972; reviewed in Sohal and Weindruch, 1996; Wallace, 2005).

Indeed, CR decreases mitochondrial ROS formation and results in a decrease in levels of markers of oxidized biomolecules (revised in Sohal and Weindruch, 1996; Merry, 2004; Gredilla and Barja, 2005; Kowaltowski, 2011), evidence that supports the mitochondrial/free radical theory of aging (Barja, 2002; Yu et al., 2008). Despite this correlative experimental support, the concept that global oxidative damage is limiting in lifespan is but in check by results showing that the manipulation of antioxidant enzyme expression and use of chemical antioxidants failed to extend lifespans in most models (Seto et al., 1990; Huang et al., 2000; Van Remmen and Jones, 2009; Alexeyev, 2009; Jang and Van Remmen, 2009; Perez et al., 2009, 2012). However, one must remember that antioxidants cannot be expected to remove all types of ROS from the large variety of biological microenvironments. Mitochondrially-targeted antioxidant interventions seem to be more successful in terms of impact on lifespan compared to less specific antioxidants (Schriner et al., 2005; Wanagat et al., 2010; Anisimov et al., 2011). In addition, mildly uncoupling mitochondria, which among other effects decreases ROS generation by this organelle, extends lifespans of yeast and mice (Barros et al., 2004; Caldeira da Silva et al., 2008; Mookerjee et al., 2010; Cunha et al., 2011). Together, these findings suggest that mitochondrial oxidant levels are a more important limiting factor in lifespan than overall oxidative damage.

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Oxidants are not all equal, and recently nitric oxide radical ($NO^{\bullet}$) levels have been shown to be increased by CR (Nisoli et al., 2005; Cerqueira and Kowaltowski, 2010; Cerqueira et al., 2011a,b). $NO^{\bullet}$ is a poorly reactive and highly diffusible species (Moncada and Higgs, 1993) associated with multiple signaling pathways (Patel et al., 1999), but which can also react with superoxide radicals ($O_2^{\bullet-}$) generating the powerful nitrating agent peroxynitrite ($ONOO^-$) (Beckman et al., 1990; Radi et al., 1991; Jessup et al., 1992; Radi, 2004).

Interestingly, $NO^{\bullet}$ generated by the endothelial nitric oxide synthase (eNOS) has recently been shown to activate PGC1-α (Nisoli et al., 2003), resulting in an increase in mitochondrial mass (Nisoli et al., 2005; Cerqueira et al., 2011b; Civitarese et al., 2007). Since mitochondrial mass decreases during aging (Oberley et al., 2008; Picard et al., 2010), the stimulation of mitochondrial biogenesis and maintenance of mitochondrial energetic metabolism over time may be a key mechanism through which CR acts.

It is thus clear that the interplay between diet, mitochondrial energy metabolism, levels of intracellular oxidants and healthy aging is complex. This review will cover aspects of these factors and their interrelationships.

2. Mitochondrial changes during aging

It is well established that animals accumulate oxidatively modified DNA as they age, and that this accumulation is more substantial in mitochondria (reviewed in Shigenaga et al., 1994; Balaban et al., 2005; Kim et al., 2007). This has lead to the idea that a vicious cycle may occur, in which damaged mtDNA leads to the production of defective respiratory chains which generate more ROS, resulting in further damage to these mitochondria, although it should be noted that the vicious cycle hypothesis is not a necessary condition for the mitochondria/free radical theory of aging to be true.

In support of the vicious cycle hypothesis, mice deficient in mtDNA proofreading exhibit shortened lifespans and a phenotype of premature aging (Trifunvic et al., 2004). On the other hand, evidence that electron transport activity is affected by aging is not clear cut (reviewed by Maklashina and Ackrell, 2004). This may be an artifact of isolating mitochondria for these studies, a process that removes damaged organelles (Picard et al., 2010). Furthermore, damaged mitochondria are eliminated *in vivo* through mitochondrial autophagy (mitophagy, reviewed by Cuervo et al., 2005), which may also explain the lack of a consistent decline in mitochondrial function over time. Indeed, aging is associated with enhanced mitophagy (Oberley et al., 2008) and total mitochondrial mass decreases with age (Byrne and Dennett, 1992; Müller-Höcker et al., 1992). Interestingly, CR (see Table 1) increases both mitophagy (Cuervo et al., 2005) and mitochondrial biogenesis (Cerqueira et al., 2011b; Civitarese et al., 2007; Nisoli et al., 2005), possibly resulting in higher organelle turnover and a quantitatively and qualitatively healthier mitochondrial pool. This idea is well in line with the emerging concept that spare respiratory capacity of mitochondria, above the normal energetic demands of the cell, is a key feature ensuring cellular survival under stressful conditions (Nicholls, 2009; Dranka et al., 2010).

Another dietary intervention that enhances lifespans in laboratory animals is the restriction of methionine (Richie et al., 1994; Miller et al., 2005; Pamplona and Barja, 2006). Interestingly, methionine restriction (see Table 1) also enhances mitochondrial biogenesis (Naudí et al., 2007; Perrone et al., 2010), may enhance mitophagy (Hipkiss, 2008) and decreases mitochondrial ROS generation and oxidative damage (Sanz et al., 2006).

3. Mitochondrial ROS and reactive nitrogen species (RNS) metabolism

While larger respiratory capacities seem to be beneficial during aging, and are a possible mechanism through which CR delays the aging phenotype, it is certainly undesirable for this larger mitochondrial mass to be associated with larger levels of oxidant production, leading to enhanced oxidative damage. That does not appear to be the case in CR, since it not only regulates mitochondrial mass, but also oxidant levels.

Mitochondria continuously reduce a small quantity of oxygen by one electron, generating $O_2^{\bullet-}$ (reviewed by Kowaltowski et al., 2009). $O_2^{\bullet-}$, a reasonably reactive ROS, is dismutated to more stable H_2O_2 through the activity of matrix Mn-superoxide dismutase (Mn-SOD) as well as Cu,Zn-superoxide dismutase (Cu,Zn-SOD) in the intermembrane space. H_2O_2 is removed by antioxidant enzymes present redundantly in mitochondria and the cytosol, including glutathione peroxidase, catalase and thioredoxin peroxidase (reviewed by Kowaltowski et al., 2009).

H_2O_2 can produce highly potent hydroxyl radicals ($HO^{\bullet}$; Sies, 1993), in the presence of transition metals. Some criticism exists regarding the availability of free copper and iron to generate $HO^{\bullet}$ *in vivo*, but evidence for its formation exists based on the presence of oxidatively damaged biomolecules (Burkitt and Mason, 1991; Halpern et al., 1995). In this regard, iron and copper-rich mitochondria, which generate substantial levels of H_2O_2, are the most feasible intracellular site for site $HO^{\bullet}$ generation *in vivo*.

$O_2^{\bullet-}$ also reacts with $NO^{\bullet}$ producing peroxynitrite ($ONOO^-$) a strong and biologically relevant oxidant, known to react with DNA bases, tyrosine residues and unsaturated fatty acids (reviewed by Radi, 2004). Since both SOD and $NO^{\bullet}$ react with $O_2^{\bullet-}$ at diffusion controlled rates (Iwabu et al., 2010), they essentially complete as $O_2^{\bullet-}$ reactants. $ONOO^-$ and carbon dioxide (CO_2), which is in equilibrium with intracellular HCO_3^-, can react, resulting in the production of carbonate ($CO_3^{\bullet-}$) and nitrogen dioxide ($^{\bullet}NO_2$) radicals (Augusto et al., 2002; Szabó et al., 2007). $^{\bullet}NO_2$ nitrates cystein and tyrosin residues in proteins, lipids and 2′-deoxyguanine. $CO_3^{\bullet-}$ oxidizes a wide range of aminoacids as well as 2′-deoxyguanine (Augusto et al., 2002; Kalyanaraman et al., 2012). Lipid peroxides produced from $ONOO^-$, $^{\bullet}NO_2$; $O_2^{\bullet-}$ or $HO^{\bullet}$ are an abundant product of biomolecule oxidation. Lipid peroxides generate highly oxidative intermediates such as aldehydes, which can amplify oxidative reactions.

Evidence demonstrating a mitochondrial source of $NO^{\bullet}$ is controversial (see Giulivi, 2003; Brookes, 2004; Ghafourifar and Cadenas, 2005), but this radical is certainly diffusible enough to affect mitochondria even if generated at significant distances (Cardoso et al., in press). Three NOS isoforms have been identified: endothelial (eNOS), neural (nNOS) and inducible (iNOS). All are expressed in most mammalian cells, and are located in the cytoplasm, plasma membrane and cytoplasmatic vesicles (Pollock et al., 1993). In mitochondria, $NO^{\bullet}$ at nanomolar concentrations inhibits cytochrome c oxidase (Brown and Borutaite, 2001) by binding to its metal centers forming metal nitrosyls (Brown and Borutaite, 2001; Brown, 1995). Thiol nitrosylation promoted by $NO^{\bullet}$ regulates enzymatic activities (Cooper, 1999; Foster et al., 2009) and is reversible (Sengupta and Holmgren, in press). In excess, S-nitrosylation can lead to protein missfolding and aggregation found in age-related diseases (reviewed by Nakamura and Lipton, 2011).

4. Damaging effects of oxidants during aging

A rough inverse correlation has been observed between the rate of $O_2^{\bullet-}$ or H_2O_2 production and maximum life span potential in different species (Sohal et al., 1989, 1990; Barja, 1998; Lambert

Table 1
Impact of different dietary interventions on ROS, NOS activity and expression, mitochondrial mass and function.

Dietary intervention	NOS activity and expression	Tissue ROS release	Mitochondrial mass/function	References
Calorie restriction	Increased expression/phosphorylation of eNOS; increased nNOS and NO• products	Reduced	Increased mitochondrial biogenesis and mass	Merry (2004), Balaban et al. (2005), Gredilla and Barja (2005), Pamplona and Barja (2006), Civitarese et al. (2007), Cerqueira and Kowaltowski (2010), Cerqueira et al. (2011a)
Dietary restriction[a]	Unaltered phospho-eNOS/NOS	Tissue-dependent increase	No increased in skeletal muscle respiratory rates	Cerqueira and Kowaltowski (2010)
Intermittent feeding	Increased eNOS content and tissue-dependent increased eNOS phosphorylation	Increased/decreased, depending on animal model		
	Increased mitochondrial biogenesis and mass	Nisoli et al., 2005; Caro et al., 2008; Cerqueira et al., 2011a		
Methionine restriction	Undetermined	Reduced	Increased	Sanz et al., 2006, Naudí et al. (2007), Perrone et al., 2010

[a] Limitation of total food intake (without vitamin and mineral supplementation).

et al., 2007), albeit with notable exceptions (Rodriguez et al., 2011). Furthermore, aged animals accumulate oxidatively damaged biomolecules in their mitochondria, in a manner prevented by life-span enhancing interventions such as CR and methionine restriction (reviewed by Shigenaga et al., 1994; Balaban et al., 2005; Pamplona and Barja, 2006; Kowaltowski et al., 2009). Some groups have found that mitochondria from aged animals generate higher levels of ROS (Bowling et al., 1993; Genova et al., 2004), while others have not found this to be the case (reviewed by Maklashina and Ackrell, 2004), possibly due to mitochondria isolation (Perez et al., 2009) and mitophagy (reviewed by Cuervo et al., 2005; Kim et al., 2007), as discussed above.

mtDNA is particularly prone to oxidative damage not only due to its proximity to a large intracellular source of ROS, but also due to its structural and repair characteristics (Sohal and Weindruch, 1996; Larsen et al., 2005). It encodes respiratory complex proteins, many of which are subunits of complex I, which could lead to defective assembly and activity of this complex, increasing $O_2^{\bullet-}$ generation from NADH-linked substrates (Genova et al., 2006). However, it should be noted that reverse electron flux from $FADH_2$-linked substrates, a significant source of mitochondrial ROS, substantially decreases upon Complex I inhibition (reviewed by Turrens, 2003; Kowaltowski et al., 2009). Complex I function may be further impaired in aged animals due to oxidative modification of cardiolipin, which is closely associated to this complex in the membrane (Petrosillo et al., 2009). Changes in mitochondrial electron transport and ROS release may also be related to oxidative damage to nuclear DNA during aging, which can also affect the expression of antioxidant enzymes (Semsei et al., 1991; Brown-Borg and Rakoczy, 2000).

In animals, CR involves a shift from carbohydrate to fatty acid oxidation (Hagström-Toft et al., 2001; Bruss et al., 2010). Based on this fact and the importance of Complex I as a source of electron leakage leading to ROS release, Guarente (2008) proposed that CR prevents ROS release in mitochondria by increasing $FADH_2$ relative to NADH, thus bypassing the generation of ROS at Complex I. This proposal, however, does not account for reverse electron transfer from coenzyme Q to complex I, a major source of ROS, at least in isolated mitochondria (Kowaltowski et al., 2009; Tahara et al., 2009, but see Schönfeld et al., 2010). Furthermore, acyl-CoA and glycerol-phosphate dehydrogenases can be significant sources of superoxide radicals themselves (St-Pierre et al., 2002; Tahara et al., 2009; Tretter et al., 2007), which may explain the higher generation of ROS observed in some tissues upon use of lipid substrates (Lambertucci et al., 2008; Tahara et al., 2009).

CR may also prevent hyperglycemia, which occurs in aged animals fed *ad libitum*. Interestingly, Yu et al. (2008) demonstrated that high glucose levels induce mitochondrial fission resulting in the enhanced oxidation of ROS-sensitive fluorescent probes in cardiovascular cells. Since the inhibition of mitochondrial fission prevented the oxidation of these probes and associated mitochondrial oxidative damage, this work suggests that the regulation of mitochondrial morphology can be an important determinant of oxidant generation under different dietary conditions. Nevertheless, it should be stressed that other authors saw no effect of regulators of mitochondrial fission and fusion on ROS-sensitive fluorescent probe signals in fibroblasts (Wu et al., 2011), while studies in fungi have found increases in probe oxidation associated with increased mitochondrial fission (Scheckhuber et al., 2007). Furthermore, all these studies used fluorescent probes to evaluate mitochondria ROS generation *in situ*, which may lead to a myriad of artifactual measurements, in particular when considering the metabolic alterations that occur with aging and changes in substrates metabolized (reviewed by Kowaltowski, 2011; Kalyanaraman et al., 2012).

$NO^{\bullet}$ levels in the mitochondrial microenvironment are mostly expected to decrease with aging, since eNOS and nNOS decrease in tissues from aged animals (Cernadas et al., 1998; Colas et al., 2006). On the other hand, iNOS expression increases during aging (Chou et al., 1998), together with the general upregulation of pro-inflammatory mechanisms (Chung et al., 2002). While eNOS and nNOS generate nanomolar amounts of $NO^{\bullet}$ for short periods of time, iNOS stimulation can result in the release of micromolar levels of $NO^{\bullet}$ (Nathan, 1992; Moncada and Higgs, 1993). These high levels of $NO^{\bullet}$ greatly favor $ONOO^{-}$ formation, further stimulated by potentially high $O_2^{\bullet-}$ generation in aged mitochondria.

5. Beneficial effects of oxidants during aging

While micromolar levels of $NO^{\bullet}$ are associated with oxidative damage, nanomolar levels of $NO^{\bullet}$ produced by eNOS induce mitochondrial biogenesis in a cGMP-dependent manner (Nisoli et al., 2003, 2004, 2005; Nisoli and Carruba, 2006). cGMP activates PGC-1, leading to increased expression of mitochondrial proteins encoded in the nucleus and mtDNA (Nisoli and Carruba, 2006; Wadley and McConell, 2007; Ventura-Clapier et al., 2008). Interestingly, eNOS is regulated by the insulin-Akt pathway, as indicated by the fact that insulin sensitizers rosiglitazone (Strum et al., 2007) and adiponectin (Hattori et al., 2003; Iwabu et al., 2010; Li et al., 2011) induce mitochondrial biogenesis. Nonetheless, the Akt pathway is also activated by CR (Cerqueira et al., 2011a,b), despite the fact that insulin levels are decreased, due to enhanced adiponectin levels promoted by this diet (Chen et al., 2005; Zhu et al., 2007; Cerqueira et al., 2011a,b, 2012).

PGC-1 also regulates the expression of mitofusin-2, involved in the control of mitochondrial fusion (Zorzano et al., 2010), a finding consistent with the observation that CR enhances mitofusin expression (Nisoli et al., 2005; van Diepeningen et al., 2010; Cerqueira et al., 2011b). At least in yeast, inhibition of mitochondrial fission is sufficient to extend lifespan (Scheckhuber et al., 2007; Braun and Westermann, 2011). Mitochondrial fusion also allows the complementation process, a quality control mechanism in which a damaged mitochondrion recovers by exchanging genetic material with healthy mitochondria (Ono et al., 2001; Chen et al., 2005). The association of higher mitochondrial biogenesis and complementation induced by $NO^{\bullet}$ may be a central and not yet fully explored effect mediating the beneficial effects of CR, by generating a larger and healthier pool of mitochondria and enhancing reserve respiratory capacity (Nicholls, 2009; Dranka et al., 2010).

6. Dietary effects on oxidant generation

As stated previously, CR prevents the oxidation of biomolecules observed in aging animals, an effect that has mostly been associated with a decrease in ROS production by mitochondria (revised by Sohal and Weindruch, 1996; Merry, 2004; Gredilla and Barja, 2005; Kowaltowski, 2011), since modifications in the expression of antioxidant enzymes in CR have not been consistently demonstrated (Lee et al., 1999; Weindruch et al., 2001; Kowaltowski, 2011). The question that emerges is how oxidant release is decreased in mitochondria by CR, while the total mitochondrial mass increases with these dietary interventions.

The increase in mitochondrial mass and respiratory rates may in itself contribute toward the prevention of ROS formation in CR, by decreasing oxygen tensions in the mitochondrial microenvironment and thus the probability of monoelectronic oxygen reduction to $O_2^{\bullet-}$ (Turrens, 2003). Higher respiratory rates also result in lower reduction of electron transport chain intermediates capable of generating $O_2^{\bullet-}$ (Turrens, 2003; Kowaltowski et al., 2009) and lower NADH/NAD^+ levels, decreasing $O_2^{\bullet-}$ generation by matrix flavoenzymes (Starkov et al., 2004; Tretter and Adam-Vizi, 2004; Tahara et al., 2007). This may be particularly true in CR, in which the availability of reducing equivalents is expected to be lower (Lambert and Merry, 2004) and the production of ROS per oxygen consumed in mitochondria is lower (Sanz et al., 2005; Ash and Merry, 2011).

Higher respiratory rates and lower levels of ROS release from mitochondria are often observed when these organelles are mildly uncoupled (reviewed by Skulachev, 1998), also due to decreases in oxygen tensions in the mitochondrial microenvironment and lower reduction states of intermediates (Turrens, 2003; Kowaltowski et al., 2009). Interestingly, some authors have found that CR and methionine restriction may lead to mitochondrial uncoupling (Lambert and Merry, 2004; Sanz et al., 2006). Furthermore, animals that are spontaneously or chemically uncoupled (Speakman et al., 2004; Caldeira da Silva et al., 2008) present enhanced lifespans and lower biomolecule oxidative damage, similarly to CR animals.

While CR consistently prevents the formation of oxidized biomolecules and the release of ROS by mitochondria (reviewed in Kowaltowski, 2011), a work of caution is necessary regarding the dietary intervention adopted (Cerqueira and Kowaltowski, 2010). In the recent literature, many groups have adopted full food restriction (or dietary restriction, see Table 1), lacking the supplementation of micronutrients, as equivalent to CR. Other groups have used intermittent or every other day feedings. Although these interventions present similarities to CR short-term, we found that when adopted long-term they can be strikingly different, in particular in their effect on redox state: both food restriction and intermittent feeding increase ROS release from insulin-sensitive tissues, while CR lowers this release (Cerqueira et al., 2011a). Since these diets also increase the activity of NOSs, $ONOO^-$ formation under these conditions is expected to increase. Indeed, enhanced ROS release observed in intermittent feeding associated with higher levels of $NO^{\bullet}$ promoted by this diet (Nisoli et al., 2005) lead to significant nitration (a modification mediated by $ONOO^-$) and protein functional loss (Cerqueira et al., 2011a). Overall, these results indicate that, while increased $NO^{\bullet}$ signaling promoted by CR, which is associated with low levels of $O_2^{\bullet-}$ formation, is beneficial, increased $NO^{\bullet}$ in the presence of enhanced ROS release such as intermittent feeding is not.

Further caution must be applied when comparing results using CR in laboratory animals to possible results human settings (Maalouf et al., 2009). Indeed, due to the very nature of human activity, very little information is known regarding the effects of highly controlled diets on redox state and longevity. Most healthy humans are not under the equivalent of rodent *ad libitum* diets, which result in significant obesity and characteristics of the metabolic syndrome. Furthermore, the human diet is much more varied and inconsistent than standard laboratory chow, and reductive diets are accompanied by changes in micronutrient, and not only calorie, content. Finally, epidemiological evidence indicates that both obese and underweight humans present lower life expectancy than normal-weight individuals, but that moderately overweight humans did not present excess mortality (Flegal et al., 2005), suggesting overt limitations of caloric ingestion in humans do not result in lifespan benefits. However, these studies are based almost exclusively on the use of body mass indexes, the reliability of which has been recently questioned (Donini et al., 2012; Livingston, 2012), as measures of weight adequacy.

7. Conclusions

Overall, a complex and fascinating interrelationship between mitochondrial energy metabolism, redox state, diet and aging has been uncovered in recent years. Further studies should help refine the signaling mechanisms involved in the modulation of these effects, as well as uncover which alterations promoted by diet have positive and significant impacts on lifespan.

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