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Review

Mitochondrial redox metabolism: Aging, longevity and dietary effects

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ABSTRACT

Mitochondrial redox metabolism has long been considered to play important roles in mammalian aging and the development of age-related pathologies in the major oxidative organs. Both genetic and dietary manipulations of mitochondrial redox metabolism have been associated with the extension of lifespan. Here we provide a broad overview of the circumstantial evidence showing associations between mitochondrial reactive oxygen species (ROS) metabolism, aging and longevity. We address most aspects of mitochondrial ROS metabolism, from superoxide production, to ROS detoxification and the repair/removal of ROS-mediated macromolecular damage. Finally, we discuss the effects of dietary manipulations (e.g. caloric restriction, methionine restriction), dietary deficiencies (e.g. folate) and dietary supplementation (e.g. resveratrol) on mitochondrial ROS metabolism and lifespan.

1. Introduction

For several decades, mitochondrial redox metabolism has attracted great interest for its potential role(s) in aging and longevity. Three lines of evidence implicate mitochondrial redox metabolism in aging. Firstly, mitochondria are the primary source of reactive oxygen species (ROS) in most cell types, and cumulative oxidative damage to cellular macromolecules may underlie the cellular dysfunction observed in aging animals. Secondly, signalling proteins that mediate apoptotic cell death are in the mitochondrial compartment and are redox sensitive. Tissue degeneration due to cumulative cell loss is a common observation in aging animals. Alterations of mitochondrial redox metabolism that influence the propensity to release apoptotic factors may therefore modulate the rate of tissue degeneration and thus aging. Finally, mitogenic signals originating from mitochondria modulate rates of cell division, which may be significant in cancer and the gradual decline of tissue progenitor cell populations in adults.

Dietary factors, including restriction of caloric intake, restriction of protein or methionine intake, or the ingestion of specific nutrients, have been shown to alter mitochondrial redox metabolism, cellular oxidative stress and animal lifespans. In this review, we consider the evidence that specific aspects of mitochondrial redox metabolism affect aging and longevity of animals. We then discuss dietary manipulations known to modulate these mitochondrial properties, and examine evidence that they are linked to extension of lifespan. Due to the vast quantity of relevant data, we have confined our focus primarily to experimental results from mammals, and discuss results from other animals when mammalian data are limiting. In addition, while we are able to cover most aspects of mitochondrial redox metabolism that are relevant to aging and nutrition, we can provide only a brief overview of particular subfields. In these instances, the reader is referred to recent in-depth reviews.

2. Mitochondrial respiration and ROS production

In the mitochondrial respiratory chain, electrons entering at complexes I and II are transferred to complex III, then IV where they are combined with molecular oxygen and hydrogen to form H_2O. Redox reactions at respiratory complexes I, III and IV are coupled to the extrusion of protons from the mitochondrial matrix into the intermembrane space. The re-entry of protons into the matrix is coupled to the synthesis of ATP from ADP and P_i. This oxidative phosphorylation is responsible for the vast majority of ATP production and oxygen consumption in most types of animal cells (Rolfe and Brown, 1997). In many types of cells, including the highly oxidative cell types that are vulnerable to the degenerative effects of aging, mitochondrial respiration is also the source of most intracellular ROS production (reviewed in Lambert and Brand, 2009). Mitochondrial ROS have been considered primarily for their pathological consequences, as they are capable of oxidizing and destabilizing most mitochondrial macromolecules. However, ROS also play key roles in intracellular signalling events

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that are implicated in cell death, growth and transformation. Both aspects of mitochondrial ROS may be relevant to aging.

3. Mitochondrial ROS production and lifespan

Electron transfer between respiratory complexes is favoured both energetically and perhaps also due to the proximity of respiratory complexes to each other within respiratory supercomplexes (Acín-Pérez et al., 2008). Nonetheless, under conditions in which complexes I and III are highly reduced, a significant proportion of electrons are diverted from them directly to molecular oxygen, thus forming the radical superoxide ($O_2^{\bullet -}$) (reviewed in Lambert and Brand, 2009). In addition to these reactions, it recently has been shown that $p66^{shc}$, a protein present in the intermembrane space (IMS), can also catalyse superoxide production via electron transfer from reduced cytochrome *c* to molecular oxygen (reviewed in Pinton and Rizzuto, 2008). Superoxide production occurs on both the matrix and IMS faces of the mitochondrial inner membrane (IMM), and is thus active both within mitochondria and within the cytosol.

Superoxide participates directly in a variety of reactions, including: (1) spontaneous or enzyme-catalysed dismutation to hydrogen peroxide. In the presence of ferrous iron, hydrogen peroxide can give rise to highly oxidative hydroxyl radicals; (2) reaction with nitric oxide (NO) to produce peroxynitrite; (3) direct oxidation of proteins, such as the TCA cycle enzyme aconitase. These reactions can be problematic if they produce sufficiently high quantities of their products. Hydroxyl radicals indiscriminately damage proteins, lipids and DNA. Similarly, peroxynitrite is highly oxidative, with a wide range of potential molecular targets that includes both cytochrome *c* (Jang and Han, 2006) and cytochrome *c* oxidase (Cooper and Davies, 2000).

A number of studies have found an inverse correlation between the rate of respiratory ROS production and lifespan. The data in these studies were collected using assays that measure hydrogen peroxide diffusion out of respiring isolated mitochondria. Long-lived bats have lower rates of hydrogen peroxide production by heart, brain and kidney mitochondria compared to a short-lived shrew species (Brunet-Rossinni, 2004). Similarly, long-lived birds produce less heart mitochondrial hydrogen peroxide than do rats (Ku and Sohal, 1993; Herrero and Barja, 1998). Also, mitochondrial hydrogen peroxide production is lower in cells of the exceptionally long-lived naked mole rat compared to Fischer 344 rats (Csiszar et al., 2007). Barja's group reported a negative correlation between ROS production of isolated heart mitochondria and MLSP in mammals and birds (reviewed in Barja, 2002), a result that was later confirmed and extended in 14 mammal and bird species by Lambert et al. (2007).

Taken together, these studies provide substantial evidence for a negative correlation between mitochondrial ROS production and species longevity. However, this conclusion is tempered by examples in which the opposite trend has been found. Vascular endothelial cells of long-lived Ames dwarf mice produce more hydrogen peroxide than those of normal littermates (Csiszar et al., 2008). Similarly, no relationship between mitochondrial ROS production and longevity is evident in *Drosophila melanogaster* (Miwa et al., 2004). Finally, a weakness of virtually all of these studies is the non-physiological conditions under which the measurements were performed (see Robb et al., 2009 for review). Nonetheless, the weight of the evidence at this time suggests that mitochondrial ROS production is generally lower in longer lived species.

4. Dietary effects on mitochondrial ROS production

Mitochondrial ROS production is particularly amenable to dietary interventions that restrict intake of total calories, protein, or the amino acid methionine. Caloric restriction of rodents, which confers increased longevity, is also associated with reduced mitochondrial hydrogen peroxide production. Hydrogen peroxide production by mitochondria isolated from liver (Hagopian et al., 2005), skeletal muscle (Bevilacqua et al., 2004) and brain (Sanz et al., 2005) is reduced in calorie-restricted rats. Mitochondrial hydrogen peroxide production is similarly reduced in mice fed ad libitum every other day (Caro et al., 2008a), or consuming diets with reduced protein or methionine content (Sanz et al., 2006a; Caro et al., 2008b). All of these diets also confer increased longevity in mice (reviewed in López-Torres and Barja, 2008; Sun et al., 2009). In contrast, restricting the dietary intake of lipids (Sanz et al., 2006b) or carbohydrates (Sanz et al., 2006c) in mice neither alters mitochondrial ROS production nor extends longevity. These results suggest an inverse correlation between mitochondrial ROS production and longevity in rodents. However, they do not indicate mitochondrial ROS production as a determinant of longevity. Some evidence suggesting that this may be the case is discussed below.

The mechanism(s) by which the rate of mitochondrial ROS production is reduced in long-lived species and by caloric restriction has not been fully elucidated. However three studies have shown a relationship between the rate of ROS production and respiratory complex I content. As respiratory complex I is a major source of superoxide, the relative abundance of complex I in mitochondria could directly affect the rate of its production. Pigeon heart mitochondria have lower levels of complex I than rat heart mitochondria, based on relative levels of flavine nucleotides (St. Pierre et al., 2002; Lambert et al., 2010). Similarly, Ayala et al. (2007) found by western blot that complex I levels were reduced in liver mitochondria of calorie-restricted rats. In contrast, Valle et al. (2007) found that the activity of complex I in liver mitochondria isolated from the same rat strain was unaffected by a similar duration of caloric restriction. Thus, while modulation of complex I levels may represent one mechanism by which mitochondrial ROS production is reduced in parallel with extended longevity, more work will be required to confirm this.

5. Mitochondrial cardiolipin peroxidation and lifespan

As the redox centres of the respiratory complexes are within the inner membrane bilayer, most superoxide produced at complexes I and III likely originates within the lipid interior of the bilayer. Oxygen solubility is up to 8-fold greater in this milieu than in the aqueous matrix or cytosol (Smotkin et al., 1991), and this relatively high concentration of oxygen favours superoxide production. Products of superoxide readily undergo peroxidative reactions with membrane phospholipids. Membrane lipid peroxidation in turn alters the biophysical properties of the bilayer (e.g. Megli and Sabatini, 2003, 2004), affecting the lateral mobility of proteins. The products of lipid peroxidation also form adducts on inner membrane proteins, impacting specific enzyme catalysed activities (Chen et al., 1998a,b), and may thus directly affect aspects of oxidative phosphorylation.

In addition to these non-specific effects, peroxidation of specific phospholipids may be critical. Recently, evidence has accumulated that peroxidation of cardiolipin is a key step in mitochondria permeability transition and apoptosis. Cardiolipin is a unique phospholipid that is found almost exclusively in the mitochondrial inner membrane. Most respiratory complexes as well as associated enzymes such as the adenine nucleotide translocator require cardiolipin for maximal activity (Fry and Green, 1981; reviewed in Schlame et al., 2000 and Bogdanov et al., 2008), and this phospholipid may also be essential for the formation of respiratory 'supercomplexes' (Pfeiffer et al., 2003; Zhang et al., 2005). Peroxidation of cardiolipin fatty acyl chains inhibits the activities

of respiratory complexes (reviewed in Pope et al., 2008), and thus impacts mitochondrial redox metabolism directly.

In addition to being a target of non-specific peroxidation due to its proximity to sites of ROS formation, cardiolipin appears to be directly peroxidized by cytochrome *c* (see Kagan et al., 2009 for review). Although cardiolipin is localized primarily to the matrix face of the IMM, a portion of it is found on the IMS face of the IMM, associated with contact sites. This IMS-facing cardiolipin is involved in electrostatic interactions with cytochrome *c*, which may be important in mediating the association of the latter with respiratory complexes. However, an early step in mitochondrial-mediated apoptosis is the redistribution of cardiolipin from the matrix face of the IMM to the IMS face, facilitating interactions between cardiolipin and cytochrome *c*. Under these conditions, conformational changes in cytochrome *c* induced by cardiolipin binding stimulate a cytochrome *c* peroxidize activity that specifically targets cardiolipin. This peroxidation of cardiolipin is in turn associated with a number of key steps in the apoptotic death cascade: (1) loss of the cytochrome *c* association; (2) permeabilization of the outer membrane, possibly via interactions with other apoptotic proteins; (3) stimulation of mitochondrial permeability transition pore opening, thus depolarizing the inner membrane and facilitating mitochondrial destruction and promoting cell death.

Mammalian mitochondria contain an isoform of glutathione peroxidase, GPx4 (or mitochondrial phospholipid hydroperoxide glutathione peroxidase) that has the unique ability to catalyse the reduction of peroxidized acyl groups in phospholipids (Ursini et al., 1985). GPx4 has been hypothesized to prevent stress-induced apoptosis by protecting against cardiolipin oxidation (reviewed in Imai and Nakagawa, 2003). Deletion of the GPx4 gene in mice is embryonic lethal (Yant et al., 2003). In contrast, overexpression of GPx4 protects mouse embryonic fibroblasts from cell death induced by potent oxidants, such as t-butylhydroperoxide and, in vivo, protects against liver damage caused by diquat (Ran et al., 2003, 2004). GPx4 prevents a decrease in electron transport chain complex activity, and protects cardiac function following ischemia/reperfusion in mice (Dabkowski et al., 2008), perhaps due to its ability to prevent cardiolipin peroxidation. Recently, Liang et al. (2009) reported that transgenic overexpression of GPx4 attenuates cardiolipin peroxidation and the release of mitochondrial apoptotic factors following oxidative stress. Surprisingly, despite these observations of GPx4s protective functions, $GPx4^{+/-}$ mice which have only about 50% of normal GPx4 protein levels in brain, liver, kidney and spleen have a reduced incidence of cancer and longer lifespan than wild type mice (Ran et al., 2007). A possible explanation for this result is that reduced GPx4 activity predisposes cancerous cells to apoptotic death. However, it should be noted that in these experiments levels of GPx4 in subcellular compartments other than mitochondria were also affected, making it impossible to attribute all observed effects to mitochondrial functions. Also, the implications of GPx4 underexpression in other species with a lower propensity of developing cancer are unknown.

6. Dietary effects on cardiolipin fatty acyl composition

Given the central role that cardiolipin appears to play in the mitochondrial apoptotic pathway, it is important to ask whether this phospholipid might be a useful target for modulating the aging process (see Pepe, 2005 for review). Cardiolipin is particularly susceptible to peroxidation because of its high content of unsaturated fatty acyl chains. In heart mitochondria, 80–90% of cardiolipin fatty acyl chains are linoleic acid (18:2$n-6$). Lee et al. (2006) reported that the fatty acyl composition of heart mitochondrial cardiolipin is altered with age: 24-month-old rats have almost 35% less linoleic acid, and higher levels of the highly unsaturated arachadonic acid (20:4$n-6$) and docosahexaenoic acid (22:6$n-3$). These more highly unsaturated fatty acids are more susceptible to peroxidation. Cardiolipin linoleic acid content is amenable to dietary manipulation (for example, see Dannenberger et al., 2007). Dietary supplementation with linoleic acid can attenuate the age-related depletion of linoleic acid from rat heart mitochondrial cardiolipin, while improving overall heart function and resistance to oxidative challenges (Chicco et al., 2008). These results indicate that dietary supplementation is capable of promoting the incorporation of linoleic acid into cardiolipin, which may in turn be significant in maintaining mitochondrial function with age and thus perhaps promote longevity. More research into this possibility is therefore warranted.

7. Uncoupling of oxidative phosphorylation, mitochondrial ROS production and longevity

Most vertebrate tissue mitochondria contain uncoupling proteins (UCPs; summarized in Stuart et al., 2001), though levels of expression of this family of proteins is generally low with the exception of UCP1 in brown adipose tissue. UCPs are IM-spanning proteins that catalyse proton conductance from the IMS to matrix. UCP2 is widely expressed, at low levels, in many mammalian cell types; UCP3 expression is highest in skeletal muscle. One function of UCPs may be suppressing superoxide formation via a reduction of membrane potential that increases the rate of electron transfer and oxygen consumption while maintaining respiratory complexes in a more oxidized state (Echtay et al., 2002). As a result, the increased rate of oxygen consumption reduces local PO_2, while the decreased proportion of respiratory complexes I and III in a reduced state decreases the likelihood of electron donation to oxygen. The proton conductance activity of UCPs is stimulated by lipid peroxidation products such as 4-hydroxynonenal (Echtay et al., 2005). Thus, phenomena that promote membrane phospholipid peroxidation stimulate UCP activity, possibly as part of a negative feedback circuit that reduces superoxide formation to defend the structure and function of the IM. Evidence for this includes observations that mice deficient in UCPs have higher levels of membrane lipid peroxidative damage (Brand et al., 2002). Also consistent with this role, transgenic UCP2 overexpression can partially rescue the early neonatal lethality of mitochondrial superoxide dismutase (MnSOD) deficiency (Andrews and Horvath, 2009), and confers neuroprotection in adult mice under conditions related to oxidative stress (Mattiasson et al., 2003; Deierborg et al., 2008). Andrews and Horvath (2009) report that UCP2 gene deletion dramatically reduces maximum lifespan in mice; however, simultaneous overexpression of UCP2 and UCP3 has minimal effect on longevity (McDonald et al., 2008). Evidence from invertebrate experiments shows no clear outcome with respect to lifespan (Fridell et al., 2005; Iser et al., 2005; Sánchez-Blanco et al., 2006; Humphrey et al., 2009).

Although UCP overexpression is not consistently associated with increased longevity, this could be related to the requirement of these proteins for effector molecules. Maximal activity of UCPs requires stimulation with fatty acids, including endproducts of fatty acid peroxidation. UCP activities are also inhibited by adenine nucleotides. Thus, increasing UCP expression without modifying levels of stimulators and inhibitors of their activities may not affect membrane potential under normal conditions.

8. Dietary interventions affecting mitochondrial coupling

UCP levels in various tissues are affected by dietary manipulations including caloric restriction (Xiao et al., 2004), short-term starvation (Jikumaru et al., 2007) and dietary fat intake (Samec et al., 1999). However, these changes have not always been

associated with altered proton leak, and their significance is thus not well understood. Therefore there is little compelling evidence to suggest that dietary modulation of UCP activities can be usefully applied to modulate lifespan.

Caloric restriction has been reported to induce a marginal increase in liver mitochondrial proton leak that is accompanied by reduced substrate oxidation activity, thus lowering the proton motive force (Lambert and Merry, 2004). Liver mitochondria lack UCPs, indicating that this difference cannot be due to differential expression of UCPs. However, other IMM proteins contribute to proton leak and these may be altered by caloric restriction.

The uncoupling of oxidative phosphorylation can also be realized by dietary supplementation with chemical uncouplers, such as dinitrophenol (DNP). Low doses of DNP (or other chemical uncouplers) that provide a marginal reduction in the mitochondrial inner membrane potential reduce ROS production while maintaining ATP synthesis (Korshunov et al., 1997). DNP confers neuroprotection against oxidative stress (reviewed de Felice and Ferreira, 2006). A subtle uncoupling of oxidative phosphorylation, via chronic dietary intake of DNP, reduced rates of ROS production and levels of oxidative damage in brain, heart and liver tissues of rats (Caldeira da Silva et al., 2008). Concomitantly, this treatment provided a marginal increase in median and mean lifespan. While these results are consistent with a direct role for mitochondrial uncoupling in reducing oxidative and extending longevity, it is equally possible that chronic DNP treatment of rodents elicits tissue-specific responses that could invoke a caloric restriction type of response.

9. Mitochondrial superoxide dismutase in aging and longevity

Attenuating respiratory superoxide production at its source is an effective strategy for reducing mitochondrial ROS production and oxidative stress. However, many of the same outcomes can be achieved by upregulating mitochondrial antioxidant enzymes. In addition, antioxidant enzyme activities can alter the relative levels of different ROS, such as superoxide and hydrogen peroxide, which is of regulatory significance. Matrix superoxide is dismutated to hydrogen peroxide by the mitochondrial superoxide dismutase (MnSOD). MnSOD is localized exclusively within the matrix, and a portion of the mitochondrial MnSOD pool is associated with the matrix face of the IM (Okado-Matsumoto and Fridovich, 2001). MnSOD may thus be physically close to sites of respiratory superoxide production. Manipulation of MnSOD expression has been attempted in a variety of species and experimental contexts, and the majority of reported results suggest beneficial effects on age-related disease, if not on aging *per se* (Chen et al., 1998a,b; Jones et al., 2003; Dumont et al., 2009).

In vitro, MnSOD overexpression provides protection against stress-induced cell death in a wide range of cell types (for examples see Keller et al., 1998; Epperly et al., 2003; Lee et al., 2004; Cruthirds et al., 2005; Silva et al., 2005; Hu et al., 2007). MnSOD levels in dermal fibroblasts correlate positively with cellular stress resistance and mammalian species lifespan (see Kapahi et al., 1999; Brown and Stuart, 2007). In vivo evidence is clearly supportive of a protective role for MnSOD against apoptotic cell death. In mice, deletion of the MnSOD gene causes severe defects from birth including cardiomyopathy and neurodegeneration, limiting lifespan to several weeks (Li et al., 1995; Lebovitz et al., 1996). Mitochondria from MnSOD$^{+/-}$ mice have reduced mitochondrial respiratory control ratios (RCR) and increased propensity of isolated mitochondria to undergo permeability transition (Williams et al., 1998; Van Remmen et al., 2003). MnSOD$^{+/-}$ mice show increased incidence of apoptotic cell death induced by oxidative stress (Van Remmen et al., 2003; Loch et al., 2009). Overexpression of MnSOD in mice provides protection from stress-induced cell death. Transgenic mice that overexpress human MnSOD are protected from oxidative damage and neuron loss induced by ischemia (Keller et al., 1998). MnSOD overexpression confers resistance to a wide variety of degenerative disorders that are common in advanced age (Dumont et al., 2009; Chen et al., 1998a,b; Jones et al., 2003). MnSOD activity in brain, standardized to mitochondrial content using citrate synthase activity, is positively correlated with maximum species lifespan in 14 species of mammals and birds (Page et al., in press).

Given the results outlined above, it is somewhat surprising that MnSOD overexpression in mice fails to extend lifespan (Jang et al., 2009; Pérez et al., 2009), though a contradictory report suggests it may (Hu et al., 2007; see Jang et al., 2009 for discussion of this discrepancy). Equally surprising is the report that MnSOD$^{+/-}$ mice do not have significantly shorter lifespans than their wild type counterparts (Van Remmen et al., 2003). These results are obviously significant to our understanding of relationships between mitochondrial redox metabolism, stress resistance and longevity, and challenge the basic hypothesis that stress resistant mitochondria that produce less ROS promote extended longevity. It nonetheless appears to be the case that higher levels of MnSOD expression are associated with increased resistance to neurodegenerative disorders and reduced rates of cancer cell growth, suggesting health benefits to the elderly if similar results could be achieved in humans.

MnSOD overexpression is significant also for its role in altering the relative levels of superoxide and hydrogen peroxide (Beuttner et al., 2006). ROS are hypothesized to act as intracellular signalling molecules in cell replication (for review see Sauer et al., 2001). The conversion of superoxide anion, thought to act as a mitogen, to hydrogen peroxide by SOD may provide a signal to slow replicative growth. The change in redox state that accompanies differences in mitochondrial MnSOD activity may therefore be important in the regulation of cell proliferation. Also, because of its high reactivity with NO, by reducing steady state superoxide levels, MnSOD probably mediates reduced rates of reaction with NO to produce peroxynitrite. Some of the effects of manipulating the MnSOD expression level are thus undoubtedly due to effects on signalling pathways modulated by ROS and NO/RNS (reactive nitrogen species).

Cullen et al. (2003) correlated MnSOD activity to the growth of human pancreas and human pancreatic cancer cell lines and found significantly lower MnSOD activity in rapidly replicating pancreatic cancer cell lines compared to normal pancreatic cell lines. Transgenic MnSOD overexpression has also been shown to slow the replicative growth rates of human pancreatic cancer cells in vitro, and result in slower tumour growth in vivo (Ough et al., 2004; Weydert et al., 2003). Decreased cellular replication rate is also observed following MnSOD overexpression in the transformed mouse fibroblast cell line NIH/3T3 (Li et al., 1998). Although the majority of experiments concerning cellular replication rate and SOD have involved MnSOD, Weydert et al. (2006) found that adenoviral MnSOD or adenoviral CuZnSOD overexpression were similarly able to inhibit the growth rate of three human breast cancer cell lines in vitro. The absence of a MnSOD specific effect may suggest that the observed change in replicative growth rate is not dependent on a mitochondrial, but rather an overall change in intracellular redox state.

The ability of MnSOD to slow replicative growth appears to be related to a role in regulating progression through the cell cycle. Sarsour et al. (2008) reported that manipulation of MnSOD activity in mouse embryonic fibroblasts influenced the ability of these fibroblasts to move through the cell cycle. An increase in MnSOD activity favoured a transition into quiescence. In contrast, fibroblasts deficient in MnSOD were unable to successfully exit the cell cycle (Sarsour et al., 2008). In vitro, transgenic MnSOD

overexpression in mice protects the replicative capacity of mouse myoblasts (Lee et al., 2009), suggesting that progenitor cell turnover is slowed by MnSOD in vivo. MnSOD's ability to protect replicative capacity in vivo may be especially important in the context of stem cell depletion and aging, as a slowed rate of division may extend the replicative capacity of these cells. This would also suggest a role for MnSOD in aging and longevity. Taken together, the data regarding MnSOD's role in cell proliferation are sufficiently strong to support the targeting of this enzyme to modulate cell growth rates in vivo.

10. Dietary interventions and nutrient signalling pathways affecting mitochondrial superoxide dismutase

Mitochondrial antioxidant enzymes are targets of nutrient sensing pathways involved in the regulation of longevity. This has been studied thoroughly for MnSOD, though there is evidence that mitochondrial glutathione peroxidase activity (Valle et al., 2007) and mitochondrial peroxiredoxin III (Chiribau et al., 2008; see below for discussion of peroxiredoxin III) are similarly affected. In mice, disruption of insulin/insulin-like growth factor-1 signalling (IIS) extends maximum lifespan in a variety of genetic backgrounds, though the magnitude of the effect is almost always greater in females than males (Baba et al., 2005; Holzenberger et al., 2003; Selman et al., 2008). The FOXO family of transcription factors mediate some of the effects of IIS signalling (reviewed by Gross et al., 2009). One downstream target of FOXO3a is MnSOD, whose expression is upregulated by activated FOXO3a (Kops et al., 2002). This MnSOD upregulation is evident in heterozygous insulin receptor knockout ($IR^{+/-}$) mice (Baba et al., 2005). FOXO3a activity is stimulated by deacetylation during caloric restriction (Wang et al., 2007), thus providing a link between this dietary intervention and mitochondrial MnSOD. The *klotho* gene, whose protein products include a hormone that inhibits IIS similarly extends lifespan in mice (Kurosu et al., 2005) and is also associated with increased MnSOD levels (Yamamoto et al., 2005). $p66^{shc}$ gene disruption, which increases longevity in mice, also increases MnSOD expression in mouse fibroblasts (Haga et al., 2008). Taken together, these results suggest that MnSOD is a target of genetic manipulations that extend lifespan in mice (Fig. 1).

There are two caveats to this interpretation of the above results. Firstly, because MnSOD is a mitochondrial protein, changes in the cellular abundance of mitochondria will directly affect cellular MnSOD levels, even though they may not affect the amount of this enzyme per unit of mitochondria. For this reason it is necessary to standardize MnSOD to another mitochondrial marker, so that the relative amount of MnSOD per mitochondrion under different conditions can be inferred. This standardization is rarely performed, even in instances in which alterations of mitochondrial abundance are likely to occur. A second caveat is that other long-lived mutants with disrupted IIS signalling (e.g. Snell dwarf mice) do not have elevated MnSOD levels in fibroblasts (Page et al., 2009), or heart or brain tissue (Page et al., in press) compared to normal littermates, despite their enhanced cellular stress resistance (Maynard and Miller, 2006; Murakami et al., 2003; Salmon et al., 2008). Thus, elevated MnSOD levels are characteristic of many, but not all, long-lived mouse strains.

In addition to caloric restriction, mitochondrial redox metabolism can also be modified by the intake of a variety of bioactive dietary molecules, some of which are considered caloric restriction mimetics (Baur and Sinclair, 2006a). Perhaps most notable amongst these are the phytoestrogens found in soy products (e.g. genestein) and red wine (e.g. resveratrol). Resveratrol has anti-aging effects in some animal species (for review see Baur and Sinclair, 2006a), and a broad range of mitochondrial effects (Lagouge et al., 2006). Dietary delivery of resveratrol increases mitochondrial abundance and aerobic capacity in cultured endothelial cells and mice (Csiszar et al., 2009; Lagouge et al.,

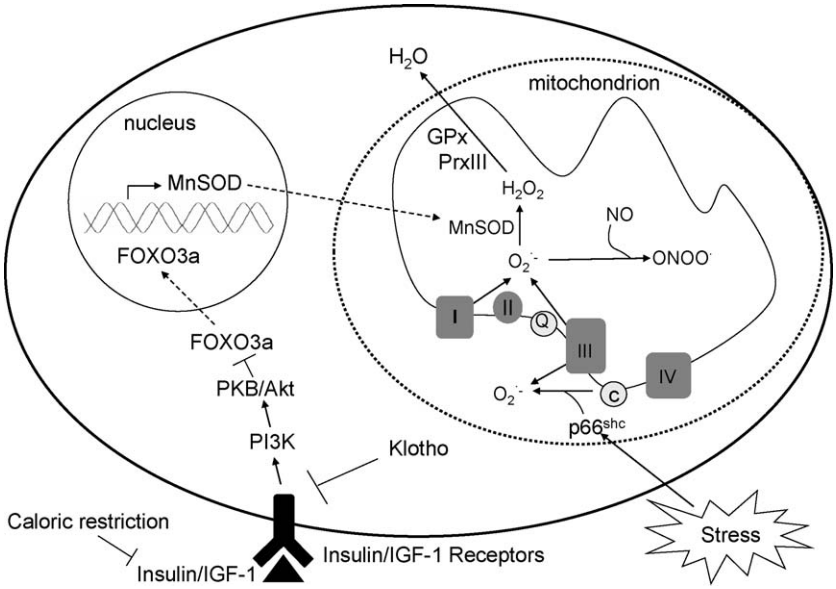

Fig. 1. A model of insulin/IGF-1 signalling effects on mitochondrial ROS metabolism. Insulin/IGF-1 receptor binding triggers a signalling cascade that inhibits FOXO3a translocation from cytosol to nucleus. Reduced IGF-1 signalling, or disruption of the pathway (including insulin receptor deficiency (Baba et al., 2005) and inhibition by Klotho (Kurosu et al., 2005)) promotes the translocation of FOXO3a to the nucleus where it induces expression of a number of mitochondrial genes, including MnSOD (Yamamoto et al., 2005), peroxiredoxin III (Chiribau et al., 2008) and probably glutathione peroxidizes (Valle et al., 2007). Enhanced MnSOD activity lowers steady-state superoxide levels, attenuates peroxynitrite formation and may increase local hydrogen peroxide levels. However, increased glutathione peroxidize and peroredoxin III activities promote the removal of hydrogen peroxide. MnSOD activity has been associated with altered progression through the cell cycle (Sarsour et al., 2008; Lee et al., 2009) and reduced propensity to undergo permeability transition (Williams et al., 1998; Van Remmen et al., 2003), release cytochrome *c*, and initiate apoptotic cell death (Van Remmen et al., 2003; Loch et al., 2009). The translocation of $p66^{shc}$ to the mitochondrial intermembrane space is promoted by cellular stress (Nemoto and Finkel, 2002). In the intermembrane space, $p66^{shc}$ interacts with cytochrome *c* to catalyse superoxide formation, promoting permeability transition and apoptosis (reviewed in Pinton and Rizzuto, 2008). Deletion of the $p66^{shc}$ gene results in increased MnSOD activity, and also reduces the production of superoxide from reduced cytochrome *c*. IGF-1, insulin-like growth factor-1; PI3K, phosphoinositide 3-kinase; PKB/Akt, protein kinase b; GPx, glutathione peroxidize; PrxIII, peroxiredoxin III.

2006; Baur et al., 2006b). Direct interactions between resveratrol and various components of the mitochondrial electron transport chain, including the ATP synthase are also observed in vitro. At micromolar concentrations, resveratrol is able to bind and inhibit the activity of complex III in isolated mitochondria (Zini et al., 1999). Similarly, a resveratrol binding site on the F1-ATPase has been identified (Gledhill and Walker, 2005). The importance of these observations in terms of mitochondrial membrane potential and rates of ROS production is currently unknown.

Both genestein (Borrás et al., 2006) and resveratrol (Robb et al., 2008b) stimulate MnSOD expression. Chronic exposure of human cells in culture to resveratrol stimulates an approximately 6-fold increase in MnSOD protein levels and activity (Robb et al., 2008b). When fed to mice as part of a high fat diet, resveratrol supplementation for six weeks stimulates an approximately 1.5-fold increase in brain MnSOD (Robb et al., 2008a). Resveratrol-induced upregulation of MnSOD is concomitant with increased cellular stress resistance and reduced rates of cell proliferation in replicating cells in vitro (Robb and Stuart, 2010). In cardiomyocytes, resveratrol pretreatment has been shown to protect against ROS production by the chemotherapeutic drug doxorubicin and to maintain mitochondria membrane potential, observations the authors attribute to an increase in MnSOD (Danz et al., 2009). Similarly, resveratrol pretreatment provides protection against neurotoxic compounds associated with oxidative damage both in vitro and in vivo (Alvira et al., 2007; Okawara et al., 2007; Blanchet et al., 2008), a result that can be achieved through genetic manipulation of MnSOD. Recently, a vegetarian diet has been shown to induce MnSOD expression in human buccal mucosa cells by decreasing DNA methylation of the MnSOD promoter region (Thaler et al., 2008). These exciting results highlight the importance of further research into how diet and specific dietary molecules, including resveratrol, can be used to manipulate mitochondrial redox metabolism in humans as a means of protection against the degenerative diseases of aging.

11. Mitochondrial protein oxidative damage and longevity

Mitochondrial ROS interact directly with a wide variety of mitochondrial proteins, and the resultant modifications are both regulatory and pathological (Bulteau et al., 2006). For example, oxidation of methionine and cysteine residues constitutes a homeostatic regulatory mechanism for some mitochondrial proteins, but excessive redox imbalance may compromise the functions of other proteins. Moosmann and Behl (2008) suggest that detrimental aspects of cysteine oxidation in mitochondria are sufficient to have driven the replacement of this amino acid during the evolution of longevity in mammalian species. Cysteine content of mitochondrial DNA (mtDNA) encoded proteins is inversely correlated with species longevity, or with adoption of an aerobic metabolism (Moosmann and Behl, 2008). Mitochondria also contain isoforms of several protein redoxins that function to repair oxidized methionine and cysteine residues. These activities may also be subject to evolutionary pressures associated with increased lifespan, though they have not been studied in this context. Examples of mitochondrial protein redoxins include thioredoxin 2, peroxiredoxin 3, and thioredoxin reductase 2 (Table 1). All of these are localized to the mitochondrial matrix, where they function by reducing disulphide bridges that form following oxidation by hydrogen peroxide. A fourth mitochondrial enzyme, glutaredoxin 2, catalyses *S*-glutathionylation and deglutathionylation of proteins to protect thiol groups from oxidation and restore the reactivity of active thiols. Finally, an isoform of methionine sulphoxide reductase (MsrB2), which catalyses the reduction of oxidized methionine sulphoxides to methionine, is also localized to mitochondria. Most of these 'protein repair' enzymes have been studied by gene knockout and overexpression. Similarly to other antioxidant enzymes, these enzymes are found to play a key role in cellular stress resistance (Table 1).

Only a limited subset of amino acid residues is amenable to reductive repair following oxidation, while the majority of proteins whose function has been impaired by oxidation must be removed via proteolytic degradation. Within the matrix, this degradation is catalysed by the Lon protease, an enzyme that appears to fulfil some similar functions to the 20S/26S proteasome, which is not present in mitochondria. The Lon protease catalyses the ATP-dependent degradation of damaged or oxidized proteins, preventing their accumulation and aggregation (Ngo and Davies, 2007). The recognition system utilized by the mammalian Lon protease is poorly understood. However, it has been shown in *Escherichia coli*

Table 1
Enzymes involved in maintaining protein methionine and cysteine residues within the mitochondrial compartment: effects on mitochondrial ROS metabolism and cellular stress resistance.

Enzyme	Activity	Experimental manipulation	Effects
Glutaredoxin 2	Thioltransferase, catalyses reduction and formation of protein-glutathione mixed disulphides	Overexpression	Attenuates doxorubicin- or 2-deoxy-D-glucose-induced respiratory dysfunction and cytochrome *c* release (Enoksson et al., 2004) Prevents cardiomyocyte death during ischemia (Nagy et al., 2007)
Thioredoxin 2	Maintains protein thiols in reduced state	Overexpression	Attenuates effects of tert-butylhydroperoxide and inhibits apoptosis (Chen et al., 2002)
		Heterozygous $Trx2^{+/-}$	Increases cytochrome *c* release and procaspase 3 cleavage (Nonn et al., 2003) Increases H_2O_2 production and oxidative damage
Peroxiredoxin 3	Thioredoxin peroxidase, uses thioredoxin 2 as electron donor to eliminate H_2O_2	Overexpression	Protects heart function and reduces oxidative damage following myocardial infarction (Matsushima et al., 2006)
		siRNA silencing	In human neuroblastomas, causes increase in oxidative damage and increase in apoptosis with exposure to MPP^+ (de Simoni et al., 2008)
Methionine sulphoxide reductase B2	Catalyses reduction of oxidized methionine sulphoxides to methionine	Overexpression	Reduces ROS formation, maintains inner membrane potential and prevents cell death following H_2O_2 treatment (Cabreiro et al., 2008)
		siRNA silencing	No effect on total cellular methionine sulphoxide reductase activity or H_2O_2 toxicity (Cabreiro et al., 2008)

Lon that specific sequences rich in aromatic residues that are exposed but hidden in the protein's native state are imperative to recognition (Gur and Sauer, 2008).

12. Effects of aging and diet on mitochondrial protein repair and degradation

While non-mitochondrial protein redoxins have been generally well studied in the context of aging and nutritional intervention, much less is known about the mitochondrial redoxins in this context. Thioredoxin reductase 2 levels are reduced with aging, coincident with increased sensitivity to oxidative stress, and caloric restriction prevents this age-related deterioration (Rohrback et al., 2006). Thioredoxin-2 gene deletion in *Drosophila* results in sensitivity to oxidative stressors and reduced lifespan (Svensson and Larsson, 2007). However, the effect of thioredoxin-2 gene deletion on lifespan in mammals is not yet known. Similarly, effects of aging and caloric restriction on activities of other mitochondrial protein redoxins have not yet been studied in detail.

Lon protease expression is stress-responsive. Following exposure of cultured human Rhabdomyosarcoma cells to hydrogen peroxide**,** Lon protease is dramatically upregulated, and siRNA silencing to prevent this upregulation results in the persistence of high levels of protein carbonyls (Ngo and Davies, 2009) and cell senescence in culture (Bota et al., 2005). Data describing the effects of aging on Lon protease expression and/or activity generally indicate an age-associated decline in a variety of mouse and rat tissues (Lee et al., 1999; Bota et al., 2002; Bakala et al., 2003; Delaval et al., 2004). Similarly, RNAi knockdown of Lon protease levels is associated with mitochondrial dysfunction and structural irregularities reminiscent of those seen in aging (Ngo and Davies, 2007). Interestingly, caloric restriction has been shown to prevent the age-related decline in Lon protease expression (Lee et al., 1999), suggesting that manipulation of this activity might favour increased longevity.

13. Age-related mitochondrial DNA mutations and dietary strategies for their prevention

Two laboratories have demonstrated reduced lifespans in mice genetically manipulated to accumulate mtDNA mutations at high rates (Kujoth et al., 2005; Trifunovic et al., 2004, 2005). This suggests that mtDNA mutations may cause some of the phenotypes associated with aging, though the specific mechanisms linking mtDNA oxidative damage, mutation, dysfunction and aging remain to be determined. This topic has been reviewed in depth recently (e.g. Terzioglu and Larsson, 2007; Reeve et al., 2008) and will therefore be only briefly considered here.

Mitochondrial ROS may initiate the damage leading to mtDNA mutation. mtDNA is localized primarily at the matrix face of the inner membrane, putting it in close proximity to the sites of superoxide production and therefore vulnerable to attack from ROS (reviewed in Stuart and Brown, 2006) and phospholipid peroxidation products that form adducts on DNA (Blair, 2008). Some forms of mtDNA damage, such as 8-oxodeoxyguanine (8-oxodG), can be repaired by enzymes of the base excision repair (BER) pathway, which are associated with the inner membrane (Stuart et al., 2005a). Mitochondrial BER activities are stimulated by oxidative stress (Stuart et al., 2004a), and are thus not surprisingly downregulated by caloric restriction (Stuart et al., 2004b), a nutritional intervention that decreases mitochondrial ROS production. There have been many observations of reduced mtDNA damage and mutation in calorically restricted (e.g. Hamilton et al., 2001; Lezz et al., 2008) and every other day fed (e.g. Caro et al., 2008a,b) animals, indicating that these dietary interventions are effective in reducing the age-related accumulation of mtDNA mutations. This may be explained simply by the ability of these interventions to reduce rates of ROS production and therefore the incidence of damage. Indeed, the relevance of mitochondrial BER to aging and longevity remains to be proven, as very high levels of BER substrates such as 8-oxodG can be tolerated without apparent effect on mitochondrial function (see de Souza-Pinto et al., 2001; Stuart et al., 2005b) or lifespan. In any case, further work will be required to characterize the mechanism(s) by which caloric restriction protects mtDNA integrity.

Dietary vitamin deficiencies also have been associated with the accumulation of mtDNA mutations and subsequent mitochondrial dysfunction. Folate, or vitamin B9, deficiency has been linked to the major diseases of aging: cancer, heart disease and neurodegeneration (Li et al., 2003). Folate deficiency causes the accumulation of mtDNA deletions in a variety of tissues, concomitantly with mitochondrial oxidative stress and dysfunction (Crott et al., 2005; Chang et al., 2007; Chou et al., 2007). Folate deficiency increases the susceptibility of brain tissue to stress associated with ischemic events (Endres et al., 2005). Thus, current evidence indicates that maintenance of dietary sufficiency of folate throughout the lifespan is important for preserving mtDNA integrity and thus mitochondrial function.

Recently, the nuclear DNA maintenance enzyme telomerase has been implicated in the protection of mitochondria from oxidative stress (Passos et al., 2007; Ahmed et al., 2008). Oxidative stress causes exclusion of telomerase from the nucleus and colocalization with mitochondria. Whether telomerase interacts directly with mtDNA has not been established. However, telomerase overexpression reduces the incidence of oxidative stress-induced mtDNA damage in MRC-5 human lung fibroblasts (Ahmed et al., 2008; however see also Santos et al., 2004). Ornish et al. (2008) demonstrated the ability of diet and stress reduction to increase telomerase activity in some cell types. It is therefore possible that such lifestyle modifications could have positive effects on mitochondrial function via the uncharacterized interaction of telomerase with mitochondria. Future research should address this possibility.

14. Mitochondrial autophagy in aging and caloric restriction

In addition to the repair and removal of oxidatively damaged individual mitochondrial macromolecules, degradation of mitochondria and other organelles via autophagy has been implicated in aging and longevity. Autophagy results in the degradation of mitochondria (mitophagy), typically in concert with mitochondrial biogenesis, thus promoting the maintenance of a fully functional population of organelles within cells (reviewed in Lemasters, 2005). Aging has been associated with reduced autophagic activity, and the resultant accumulation of damaged mitochondria. Caloric restriction and other dietary manipulations can promote autophagic activity in advanced age and thus maintain mitochondrial function. Several recent reviews have suggested a role for mitochondrial ROS in the stimulation of autophagy (Scherz-Shouval and Elazar, 2007; Yen and Klionsky, 2008). However, given the findings that caloric restriction generally reduces mitochondrial ROS production, it is unclear how this might work. Generally, a wealth of data supports the idea that autophagy becomes limited with advanced age and this phenomenon can be attenuated by caloric restriction. Recently, Harrison et al. (2009) demonstrated that dietary delivery of rapamycin, which stimulates some of the downstream effectors of caloric restriction including autophagy, extends lifespan in mice. This result provides hope that a molecule deliverable orally might be capable of stimulating autophagy in humans, with similar outcome with respect to longevity. However, much work remains to determine whether this is possible.

15. Conclusions

A vast body of data has accumulated linking mitochondrial redox metabolism to the aging process. Similarly, a growing number of dietary interventions have been demonstrated to modulate mitochondrial ROS production, detoxification and oxidative damage repair. Many (but not all) of these dietary interventions are associated with lifespan extension, or protection against age-related disease, in mammals. Emerging nutraceuticals such as resveratrol are showing promise as modulators of mitochondrial redox metabolism capable of eliciting beneficial outcomes that are similar to those of caloric restriction. If the goal of dietary interventions impacting mitochondrial redox metabolism is to promote the health of aging populations, rather than to extend lifespan *per se*, then these approaches hold significant promise. Evidence of their positive effects on cardiovascular and brain function (Sanz et al., 2005; Chicco et al., 2008; Caldeira da Silva et al., 2008), and cancer growth (Ran et al., 2007; Ough et al., 2004; Weydert et al., 2003) is extensive. Generally, significant advances over the past several decades in the identification of key processes and target molecules associated with mitochondrial ROS metabolism have greatly enhanced the prospect of beneficially modulating these phenomena to promote healthy longevity.

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