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Mitochondrial dysfunction as a cause of ageing

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Mitochondrial dysfunction is heavily implicated in the ageing process. Increasing age in mammals correlates with accumulation of somatic mitochondrial DNA (mtDNA) mutations and decline in respiratory chain function. The age-associated respiratory chain deficiency is typically unevenly distributed and affects only a subset of cells in various human tissues, such as heart, skeletal muscle, colonic crypts and neurons. Studies of mtDNA mutator mice has shown that increased levels of somatic mtDNA mutations directly can cause a variety of ageing phenotypes, such as osteoporosis, hair loss, greying of the hair, weight reduction and decreased fertility. Respiratory-chain-deficient cells are apoptosis prone and increased cell loss is therefore likely an important consequence of age-associated mitochondrial dysfunction. There is a tendency to automatically link mitochondrial dysfunction to increased generation of reactive oxygen species (ROS), however, the experimental support for this concept is rather weak. In fact, respiratory-chain-deficient mice with tissue-specific mtDNA depletion or massive increase of point mutations in mtDNA typically have minor or no increase of oxidative stress. Mitochondrial dysfunction is clearly involved in the human ageing process, but its relative importance for mammalian ageing remains to be established.

Keywords: ageing, life span, mitochondria, mitochondrial dysfunction, mtDNA, respiratory chain.

Introduction

Ageing can be defined as 'a progressive, generalized impairment of function, resulting in an increased vulnerability to environmental challenge and a growing risk of disease and death' [1]. It is generally assumed that accumulated damage to a variety of cellular systems is the underlying cause of ageing [1]. Ageing is not frequently observed in nature as most wild animal species succumb due to a variety of environmental causes (e.g. cold, starvation, predation, accidents) before they reach old ages. Ageing is therefore mainly observed in animal populations that live in protected environments, e.g. in an animal house. It has been argued that there has been little evolutionary pressure to maintain efficient repair and maintenance mechanisms at high ages as most animals do not reach high ages in the wild [1]. The disposable soma theory predicts that stochastic accumulated damage and decline of somatic maintenance and repair systems cause ageing in somatic tissues, whereas the germ line is carefully maintained and kept immortal [1]. The robustness of the maintenance and repair mechanisms will of course be under genetic control, but there are strong arguments that the decay associated with ageing is not genetically programmed [1, 2].

Over three decades ago Harman was one of the first to propose that mitochondria play a central role in ageing [3]. The initial theory suggested that ageing, as well as associated degenerative diseases, could be attributed to the deleterious effects of reactive oxygen species (ROS) on various cell components. The mitochondrial respiratory chain is a major site of ROS production in the cell and it has therefore been suggested that mitochondria are prime targets for oxidative damage. Hence, the mitochondrial theory of ageing, a refined version of the free radical theory,

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was proposed [3]. This theory is intellectually very appealing, as mitochondria are the only organelles in animal cells that posses their own DNA, mtDNA, localized in the physical proximity to the RC allowing irreversible damage to be introduced.

Mitochondrial ATP production and ageing

Oxidative phosphorylation of mitochondria

A very important function of the mitochondrial network in the cell is the production of ATP, the energy currency used for a variety of metabolic reactions. Degradation of nutrients absorbed from digested food transfers electrons to carrier molecules such as NAD^+ and FAD^+ thus generating NADH and $FADH_2$, which, in turn, deliver the electrons to the electron transport chain (ETC) located in the inner mitochondrial membrane (Fig. 1) [4]. The ETC, composed of respiratory chain complexes I–IV, transfers electrons in a stepwise fashion until they finally reduce oxygen to form water (Fig. 1). The energy harvested by the stepwise electron transfer is used by the ETC to translocate protons from the matrix to the intermembrane space of the mitochondria (Fig. 1). The ETC thus creates an electrochemical gradient across the inner mitochondrial membrane resulting in a potential of ~150–180 mV [4]. This transmembrane proton gradient is used to drive the ATP synthesis as protons re-enter the mitochondrial matrix through the ATP synthase (complex V) (Fig. 1) [5]. There is usually a tight coupling between electron transport and ATP synthesis and inhibition of ATP synthase will therefore also inhibit electron transport and cellular respiration [6]. Protonophores (e.g. FCCP) and uncoupling proteins (e.g. UCP1) provide alternate routes for proton entry to the mitochondrial matrix and will thereby uncouple electron transport from ATP synthesis (Fig. 1). As a consequence, uncoupling leads to low ATP production concomitant with high levels of electron transfer and high cellular respiration [6].

Studies of the link between mitochondrial respiration/ATP production and longevity have given conflicting results that are not easy to reconcile in a unifying theory. Different genetic and dietary manipulations that are known to prolong lifespan have been shown to both decrease and increase ATP production in the cell.

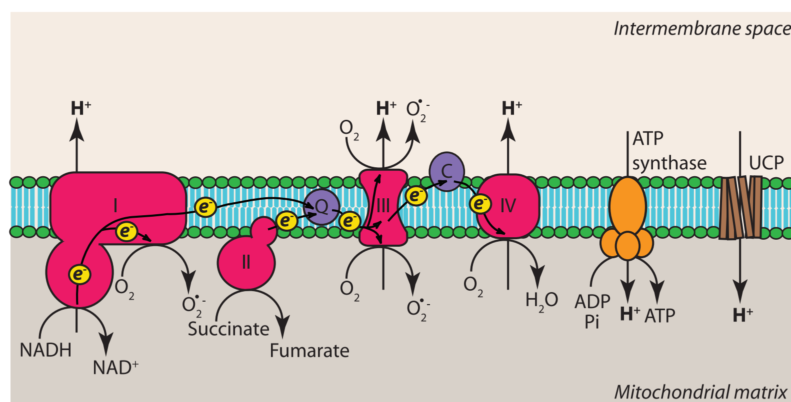

Fig. 1 Mitochondrial ATP production (oxidative phosphorylation) is driven by the transfer of electrons from NADH and $FADH_2$ to O_2 through Complexes I–IV of electron transport chain. The released energy allows components of Complexes I, III and IV to pump protons (H^+) across the inner mitochondrial membrane. This creates an electrochemical H^+ gradient (called proton motive force, $\Delta\mu_H{}^+$) that provides energy for ATP synthesis. The movement of protons back across the inner mitochondrial membrane driven by the proton motive force is *coupled* to the synthesis of ATP by the mitochondrial ATP synthase. The whole process is called the oxidative phosphorylation. Any proton re-entry bypassing ATP synthase leads to *uncoupling* of oxidative phosphorylation. A well-known example of such an uncoupling of respiration from ATP synthesis is represented by uncoupling proteins (UCPs).

Caloric restriction prolongs life by increasing respiration rate

Caloric restriction (CR), also called dietary restriction (DR), is the only known nongenetic manipulation that prolongs life span in a variety of experimental organisms, e.g. budding yeasts, worms, flies and rodents [7, 8]. It is currently not clear if CR also can extend life span in nonhuman primates and humans. However, it has been shown that CR reduces the incidence of age-associated diseases such as cancer, cardiovascular diseases and diabetes in mammals [7, 8]. The CR response thus seems to have been conserved during animal evolution and there is intense ongoing research to define the underlying molecular pathways [9].

The *SIR2* gene encodes a deacetylase that is mediating aspects of the CR response in budding yeast (*Saccharomyces cerevisiae*) under moderate CR conditions (0.5% glucose in contrast to normal 2% glucose) [9]. It has been proposed that CR in the yeast shifts carbon metabolism from glycolysis towards oxidative metabolism involving the tricarboxylic acid cycle and the ETC. Moderate CR in yeast leads to 25% increase in the replicative lifespan and a twofold increase in respiration rate [10]. Overexpression of Hap4, a transcription factor that causes a switch from glycolysis to respiration, significantly prolongs lifespan (35%) of yeast cells grown on glucose medium and also increases the respiration rate [10]. Furthermore, growth at 0.5% glucose fails to extend replicative lifespan in yeast strains lacking an intact electron transport chain (ETC), suggesting that a metabolic shift towards respiration is necessary for CR-induced increase of lifespan [10]. However, other studies argue that respiration and the *Sir2* protein are not required for lifespan extension by CR [11, 12]. CR at low glucose concentrations (0.05%) significantly increases lifespan in, at least, some yeast strains lacking mtDNA (rho^0 cells) [11]. The fact that alternate pathways promoting longevity are induced in strains lacking respiratory capacity does not negate a role of mitochondrial metabolism in CR when the organelles are functional.

Both genetic and environmental DR manipulations have been shown to increase lifespan in *Caenorhabditis elegans*, including mutations that decrease feeding rate (*eat* mutants), growth in axenic culture (chemically defined medium without bacteria), and dilution of the bacterial food source. Initial results indicated that DR does not change metabolic rate in worms grown in reduced food conditions [13]. Similar findings have been reported in *Drosophila melanogaster,* where DR had no effect on resting metabolic rate [14]. Opposing results have recently been offered by Bishop and Guarente who have shown that DR worms do have higher respiration rates [15]. Furthermore, it has been proposed that increase in respiration is necessary for the DR induced longevity, because ETC inhibitors (myxothiazol and antimycin) suppressed its effect, without shortening the lifespan of worms fed *ad libitum* [15]. The effect of ETC inhibition is likely to be specific to DR-induced longevity, as the long life of a *daf-2* mutant (insulin-signalling pathway) was not affected by antimycin treatment [15].

The mammalian homologue of the yeast *Sir2* protein, *SIRT1*, deacetylates and thereby activates the nuclear hormone receptor Pgc1α, which, in turn, induces mitochondrial biogenesis [16]. Recently it has been shown that short term CR of overweight, but not obese individuals, lead to increased mitochondrial biogenesis and increased Pgc1α levels independently of their exercise status [17]. Nitric oxide (NO) has also been shown to have the capacity to induce mitochondrial biogenesis in mice [18]. Interestingly, CR increases NO production by inducing the expression of endothelial NO synthetase (eNOS) and thereby causes increased mitochondrial biogenesis [19].

Single gene mutations affecting mitochondrial function can extend life span

Pioneering genetic experiments in *C. elegans* showed that it is possible to perform genetic screens to obtain long-lived mutants [20]. Subsequent work lead to the identification of the *age-1* gene, the first gene whose altered expression resulted in prolonged lifespan in *C. elegans* [21]. A more recent systemic RNA interference (RNAi) screen for gene inactivation that increase

lifespan in worms, revealed a 10-fold overrepresentation of genes encoding mitochondrial proteins such as mitochondrial carriers, ETC components, and mitochondrial ribosomal subunits [22]. Most of the detected, long-lived mitochondrial mutants were smaller, had altered mitochondrial morphology (disorganized, swollen and/or fused mitochondria) and lower ATP levels and lower oxygen consumption rates [22]. The observed effect on the lifespan extension was most comparable to that induced by inactivation of insulin/IGF-signalling pathway [22]. However, mitochondrial genes influenced lifespan independently of the insulin/IGF-signalling pathway [22].

Several *C. elegans* models have been developed showing that a direct disruption of the ETC can have a significant effect on lifespan. A mutation in the *C. elegans* iron sulphur protein (*isp*-1) of complex III slow down embryonic development and doubles maximal life span [23]. The *isp*-1 mutant has decreased mitochondrial respiration with low oxygen consumption and decreased sensitivity to ROS [23].

Smaller, but long-lived worms have been obtained by RNAi knockdown of various respiratory chain subunits of complexes I, III, IV and V [24]. Intriguingly, antimycin treatment gives a similar lifespan extension in *C. elegans* despite increasing superoxide production [24, 25]. RNAi treatment can decrease mitochondrial function and lower ATP production in both juvenile and adult worms, but, surprisingly, only RNAi treatment of juvenile worms prolongs lifespan [24]. It has therefore been proposed that *C. elegans* possesses a regulatory mechanism during development that sets a respiratory rate that will be maintained throughout the worm's life and will determine lifespan [24].

The *clk*-1 mutant provides another link between defective RC and lifespan. These mutant worms lack an enzyme required for ubiquinone (coenzyme Q) synthesis that is essential for both complex I and II-dependent respiration [26]. The *clk*-1 mutant worms have slower development, slower rhythmic behaviour and an increased lifespan [26]. Although some have reported that *clk*-1 mutant animals have normal respiration levels [27], others have proposed that the main mechanism promoting longevity in these worms is a lower metabolic rate leading to decreased ROS production and damage [28, 29].

How can disruption of the mitochondrial RC cause life extension in worms, when many mutations that compromise mitochondrial function in humans lead to variety of severe, life-shortening diseases? Recent results from Rea *et al.* may provide an explanation for this paradox [30]. They report that life extension is only possible in juvenile worms, under a certain window of partial RC disruption. Milder disruption of the RC has no effect on lifespan, whereas a more severe impairment actually shortens lifespan [30].

Further support for this phenomenon comes from mammalian models. Homozygous inactivation of *Mclk1*, the mouse ortholog of *clk-1*, leads to severe developmental delay and embryonic lethality, whereas partial deficiency of *Mclk-1* increases lifespan by on average by 31% in mice of three different genetic backgrounds [31]. Inactivation of *Surf1,* a gene encoding an assembly factor for complex IV of the RC, increases lifespan in mice [32]. However, *SURF1* mutations in humans lead to a drastic reduction in the amount of fully assembled complex IV and an early onset of a severe, often fatal mitochondrial encephalomyelopathy denoted Leigh syndrome. Inactivation of *Surf1* in mice results in a much milder reduction in the amount of fully assembled complex IV, which may explain the drastic difference in observed phenotypes between *SURF1* deficient mice and humans [32]. There are thus data indicating that the effect of mitochondrial dysfunction on lifespan are evolutionarily conserved in nematodes and mammals.

Some studies of long-lived *C. elegans* mutants are in agreement with the idea that reduced energy metabolism results in decreased ROS production and slower accumulation of damage allowing lifespan to increase. On the other hand, the *mev*-1 mutation in the *Cyt*-1 gene, encoding the *C. elegans* large anchoring subunit (SDHC) of succinate:ubiquinone oxidoreductase (complex II of the ETC), leads to a decreased respiration rate and a shorter lifespan in mutant worms [33]. The *mev*-1 mutant animals are intolerant to hyperoxia

and their mitochondria have severe structural abnormalities [33]. The accelerated ageing of *mev*-1 mutants has primarily been attributed to increased generation of ROS at complex II. This was further supported by the finding that ROS scavengers, such as EUK-8 and EUK-134, could restore normal life span in the *mev*-1 mutant animals [34].

Another model organism, frequently used to study the effects of mitochondrial metabolism on longevity, is the filamentous fungus *Podospora anserina*. It has been shown that inactivation of *COX5* (subunit 5 of the complex IV of the RC) leads to the use of an alternative respiratory pathway (AOX) and to a significant decrease in ROS production. This inactivation results in a striking increase of longevity (more than 5 years versus 16 days) associated with stabilization of mitochondrial genome [35]. The increased mtDNA stability and longevity are associated with a decrease in ATP production, which is in agreement with studies in worms showing that decreased mitochondrial function can prolong life span.

Mitochondrial DNA mutations and ageing

Genetics of mitochondrial DNA (mtDNA)

Mitochondria are the only organelles in animal cells, besides the nucleus, that contain their own DNA. The human mitochondrial DNA (mtDNA) is a 16 569 bp circular, double-stranded molecule that encodes 13 protein subunits, all of which are respiratory chain subunits, and 24 RNA components (22 tRNAs and two rRNAs) necessary for mitochondrial protein synthesis [36]. The majority of the ∼1500 different mitochondrial proteins are encoded by nuclear DNA (nDNA), translated in the cytoplasm and transported into mitochondria [36]. The biogenesis of the respiratory chain is therefore dependent on an intricate and still poorly understood cross talk between mitochondrial and nuclear genomes. Even more, mtDNA is completely dependent on nuclear-encoded proteins for its maintenance and expression.

A mammalian cell have around 1000–10 000 copies of the mitochondrial genome. Although the high copy number of mtDNA ensures that mutations affecting a single copy will not impact overall mitochondrial function, it cannot prevent expansion of *de novo* mutations arising in a single mtDNA molecule. This was confirmed in 1988 when two groups, independently, reported the first pathogenic mtDNA mutations in human patients [37, 38]. The affected patients either had homoplasmy, i.e. only mutated mtDNA [38], or heteroplasmy, i.e. a mixture of wild-type and mutated mtDNA [37]. Heteroplasmic mtDNA mutations segregate during cell division because there is no mechanism to ensure that every mtDNA molecule is replicated once and only once during each cell cycle (Fig. 2) [39]. Random distribution of mtDNA molecules during cell division can lead to increased amounts of mutant mtDNA molecules in one of the daughter cells. Thus, a cell carrying low levels of

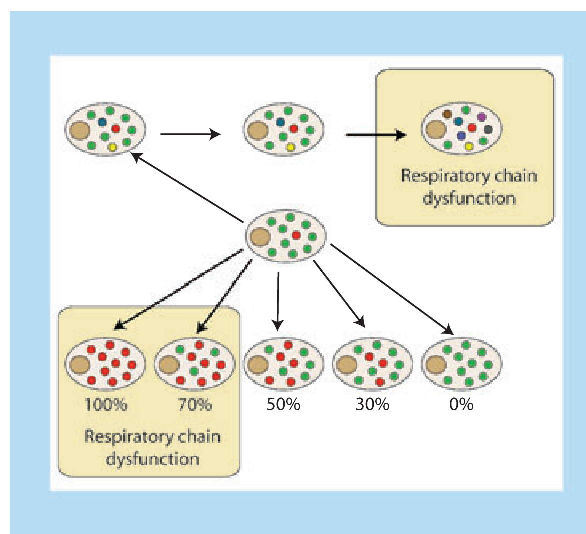

Fig. 2 Clonal expansion of mtDNA mutations. Different *de novo* mutations of mtDNA affecting a single cell may cause respiratory chain dysfunction (upper row). MtDNA replication is not coupled to cell cycle and therefore a somatic mtDNA mutation affecting a single mtDNA molecule may undergo clonal expansion (lower row). A similar mechanism is probably responsible for clonal expansion in postmitotic cells. Clonal expansion of an mtDNA molecule containing a pathogenic mutation above a certain threshold (e.g. >70%) will lead to respiratory deficiency in some cells, whilst others with lower mutational levels will still be respiratory active. This will then create a mosaic pattern of respiratory chain deficiency in a tissue and could lead to organ failure.

mutated molecules can give rise to a daughter cell with high mutation levels (Fig. 2).

A minimal threshold level of a pathogenic mtDNA mutation must be present in a cell to cause respiratory chain deficiency (Fig. 2). It has been shown that different types of heteroplasmic mtDNA mutations have different thresholds for induction of respiratory chain dysfunction, ranging from 90% for some tRNA mutations to 60% for mtDNA deletions [36]. Patients carrying heteroplasmic mtDNA mutations often have widely varying levels of mutated mtDNA in different organs and even in different cells of a single organ [36]. Furthermore, it has been suggested that the intracellular distribution of mitochondria could play a role in the manifestation of the effects of mtDNA mutations. Recent evidence suggests that there is an age-dependent, selective decrease in the rate of oxidative phosphorylation in the intermyofibrillar (located between myofibrils) cardiac mitochondria, but not in the subsarcolemal mitochondria (located below the plasma membrane) [40].

Increased levels of somatic mtDNA mutations in ageing

Probably, the first report on mtDNA damage involvement in ageing comes from a study of the integrity of mtDNA in young adult and senescent rats, performed by Piko in 1988 [41]. Using electron microscopy of reconstituted mtDNA duplexes, Piko reported an increased abundance of structural aberrations consistent with mtDNA deletions in aged animals [41]. Similar reports showing the existence of mtDNA rearrangements in tissues from aged humans quickly followed [42–44]. In addition, age associated decrease in the respiratory chain capacity was reported in various tissues, such as skeletal muscle [45] and liver [46]. Hypotheses were put forward that acquired mutations of mtDNA would increase with time and segregate in mitotic tissues to eventually cause decline of respiratory chain function leading to age-associated degenerative disease and ageing [47].

It is widely recognized that mtDNA deletions accumulate in different tissues during human ageing. However, there are only a few reports on accumulation of mtDNA point mutations. This is surprising as more than 100 different mtDNA mutations have been associated with inherited mitochondrial diseases [48]. One possible explanation for the few reports on acquired mtDNA point mutations in ageing could be lack of reliable methods for accurately quantifying low levels of point mutations in ageing tissues.

A study of fibroblasts from young and aged humans revealed the occurrence of age-associated, site-specific point mutations in the control region, with some of them altering highly conserved sequences necessary for transcription of mtDNA [49]. Age-dependent accumulation of mtDNA mutations in the control region has also been reported in human skeletal muscle [50]. Unfortunately, experimental studies demonstrating any functional importance of these mutations are still lacking.

The data obtained from studies of tissues from humans and other mammals have provided circumstantial evidence for the involvement of mtDNA mutations in ageing [51]. Recently, we have developed a mouse model that provided the first experimental evidence for a causative link between mtDNA mutations and ageing phenotypes in mammals [52]. The mtDNA mutator mice were engineered to have a defect in the proofreading function of mitochondrial DNA polymerase (*Polg*), leading to the progressive, random accumulation of mtDNA mutations during the course of mitochondrial biogenesis. As the proofreading in these mice is efficiently prevented, they develop an mtDNA mutator phenotype with a three- to fivefold increase in the levels of point mutations, as well as increased amounts of deleted mtDNA molecules [52]. In contrast to the mitochondrial theory of ageing, the levels of somatic mtDNA mutations accumulate at a higher rate during early embryonic development of mtDNA mutator mice, whereas mtDNA mutations accumulate in rather linear fashion during the rest of their life [53]. The mtDNA mutator mice display a completely normal phenotype at birth and in early adolescence, but subsequently acquire many features of premature ageing. The increase in somatic mtDNA mutations is associated with reduced lifespan

and premature onset of ageing-related phenotypes such as weight loss, reduced subcutaneous fat, alopecia, kyphosis, osteoporosis, anaemia, reduced fertility, heart disease, sarcopenia, progressive hearing loss and decreased spontaneous activity [52]. Results obtained from this model confirm that mtDNA point mutations can cause ageing phenotypes if present at high enough levels. However, it does not prove that the levels present in normal ageing are sufficient to cause ageing phenotypes.

Focal distribution of mtDNA mutations and respiratory chain deficiency in ageing

The levels of mtDNA deletions and point mutations found in ageing human tissues are in most cases very low and are therefore only present in a small proportion of the mitochondrial genomes. It was therefore considered unlikely that ageing-related mtDNA mutations played a significant role in the loss of mitochondrial function that occurs in cells from old individuals. Some more recent studies have taken into account a possible intercellular or intracellular mosaicism in the distribution of the mtDNA mutations, and performed analysis on single cells rather than total tissue extracts.

In 1989, Müller-Höcker published a carefully conducted extensive enzyme histochemical study of heart from individuals of different ages. Surprisingly, he found that increasing age was associated with focal respiratory chain deficiency in a subset of the cardiomyocytes [54]. Sporadic occurrence of respiratory chain deficient cells is detected from age 20 years and onwards, with being characteristic for all individuals above the age of 60 years [54]. In a follow up study, age-associated focal respiratory dysfunction was confirmed in skeletal muscle specimens [55]. Furthermore, age-associated focal respiratory chain deficiency has also been reported in colonic crypts [56] and a variety of neuronal cell types, such as hippocampal neurons and midbrain dopaminergic neurons [57, 58]. Many recent studies have demonstrated that focal respiratory chain deficiency is often associated with clonal expansion of deleted mtDNA mutations [57, 58].

Analysis of mtDNA from single substantia nigra neurons from individuals with Parkinson disease and age matched controls (mean age 76–77 year), showed that levels of deleted mtDNA molecules accumulated to between 52% and 43% respectively [59]. Individual cells carried unique, clonally expanded deletions, and high levels of these rearrangements are associated with respiratory chain deficiency. The level of deleted mtDNA was greater in cells proven to have compromised RC activity in comparison with neighbouring respiratory chain competent cells (67% vs. 48%) [59]. Clonally expanded single large mtDNA deletions are is a ubiquitous finding in aged skeletal muscle [60]. In addition, a number of individual skeletal muscle cells from old human subjects also contain high amounts (up to >90%) of one or very few mtDNA point mutations [60]. Overall, it seems that intracellular mosaicism caused by uneven distribution of acquired mtDNA mutations cause respiratory chain deficiency, which may lead to tissue dysfunction even if the overall levels of mtDNA mutations are low.

Mitochondrial ROS production and ageing

Mitochondrial ROS production

Mitochondria are assumed to be the main cellular producers of ROS, consistent with the fact that mitochondrial enzymes transport electrons and consume most of the cellular oxygen in the process of oxidative phosphorylation [61]. Electrons escaping from the ETC can reduce oxygen to form the highly reactive free radical superoxide anion ($O_2^{\bullet -}$) (Fig. 1), which, in turn, can be further reduced to hydroxyl radical ($OH^{\bullet -}$) and hydrogen peroxide (H_2O_2) [62]. Furthermore, the superoxide anion can initiate the oxidation of sulphite or nitric oxide, resulting in additionally ROS such as sulphur pentoxy anion or peroxynitrite [63]. Hydrogen peroxide, on the other hand, is more stable than superoxide and can diffuse freely through membranes into the cytosol or the nucleus, causing oxidative damage to many cell compartments. In the presence of metal ions (e.g. iron–sulphur clusters in complex I, complex II, or the mitochondrial aconitase) the highly reactive hydroxyl radical can be generated by the Fenton reaction [63]. The cell is equipped with

a variety of defence mechanisms to remove ROS. Superoxide dismutases (SODs) convert superoxide into hydrogen peroxide, which in turn can be transformed into water by catalase or glutathione peroxidase (GPX). The cell also contains nonenzymatic scavengers such as ascorbate, pyruvate, flaveniods, carotenoids and glutathione which may inactivate potentially damaging ROS [64].

Early studies on isolated mitochondria suggested that 1–4% of the total oxygen consumed was used to generate ROS [65]. However, subsequent studies, under more physiological conditions, have estimated this basal value to ~0.2% of the consumed oxygen [66]. Even with this lower estimated value of ROS generation, the RC still remains the major site (~90%) of ROS production in the cell. This fact has lead to the suggestion that mitochondria also are the prime targets for oxidative damage and that accumulation of defective mitochondria is a major contributor to ageing [3].

Does mitochondrial ROS production determines lifespan?

Age-related increases in oxidative damage to lipids, proteins and DNA are well established in humans and model organisms [67]. The decreased oxidative stress reported in a number of the well-characterized longevity mutants further supports the importance of ROS in the ageing process. Then again, discrete interventions to lower ROS or to mitigate their effects have not produced consistent results in terms of lifespan extension.

A number of different models have been generated to elucidate the role of ROS in the ageing process. For instance, mice expressing peroxisomal catalase targeted to the mitochondria had a 5-month increase in lifespan in comparison to their wild-type littermates [68]. In addition to increased lifespan the authors also reported decreased hydrogen peroxide production, decreased levels of oxidative DNA damage and normal aconitase enzyme activity in these transgenic mice. However, extended lifespan was not observed in mice overexpressing human catalase targeted to the peroxisome or nucleus, suggesting that only mitochondrial ROS could influence lifespan [68].

If ROS production is essentially important for the ageing process any manipulation that decrease ROS production should lead to extension of lifespan. Flies overexpressing the mitochondrial adenine nucleotide translocase (ANT) had significantly decreased ROS production, but failed to get an extended lifespan [69]. Furthermore, overexpression of human cytosolic superoxide dismutase *(SOD1)* in mice resulted in animals with increased resistance to superoxide-mediated brain injury, including oedema after cold injury, traumatic damage and ischaemia, but the lifespan was comparable to wild-type mice [70].

Mice partially lacking mitochondrial superoxide dismutase (*Sod2*$^{+/-}$) have increased oxidative damage to mitochondrial proteins and mtDNA, increased incidence of cancer and reduced aconitase activity [71]. Surprisingly, no detectable changes in oxidative damage to cytoplasmic or nuclear compartments of the cell were observed and these mice had lifespans comparable to wild-type littermates [72].

Disruption of the gene encoding the H_2O_2 -scavenging enzyme GPX1 in mice (*Gpx1*$^{-/-}$) causes only a mild phenotype with 20% reduction in body size. Furthermore, mice deficient in two of the major mitochondrial ROS scavenging enzymes (*Sod2*$^{+/-}$/*Gpx1*$^{-/-}$) were extremely sensitive to oxidative stress, but appeared normal, had normal reproduction and a normal lifespan, thus questioning a role for O_2^- and H_2O_2 overproduction in determining the lifespan [73].

Early hypothesis assumed that cutting down calories increased longevity by simply slowing metabolism and thereby causing less ROS production and less cellular damage. It has indeed been shown that CR decreases the level of oxidative damage to cellular components. However, recent evidence suggest that induction of mitochondrial metabolism during CR might initially increase ROS production and thereby induce an increase in stress defence, which, in turn, results in a reduction of net stress levels [13, 74–76].

Impaired glucose metabolism extends lifespan in worms by increasing the mitochondrial respiration accompanied by increased ROS formation. This in turn increases oxidative stress resistance and survival rates. Accordingly, treatment of worms with different antioxidants and vitamins prevents extension of the life span [74]. There is even evidence to suggest that antioxidant food supplements may decrease lifespan in humans [77].

Mitochondrial energy production and ROS

Mitochondrial ROS production is not simply a byproduct of mitochondrial respiration, tightly linked to the rate of oxygen consumption, as often assumed. Although increased respiration rate, frequently found in different CR models, came as a surprise to some [10], it is in accordance with known principles for mitochondrial respiration. When the rate of electron flow is slow, electrons tend to accumulate in the respiratory chain, thus increasing the degree of reduction of RC complexes, leading to strong increase in their capacity to form ROS [78]. As a consequence, mitochondrial ROS generation will be high during resting (state 4) respiration because in this state the rate of oxygen consumption (VO_2) is low. As a consequence of adenosine diphosphate (ADP) addition to the mitochondria, the rate of electron flow and oxygen consumption strongly increases (the so-called state 4 to state 3 energy transition occurs). When electrons flow quickly through the respiratory chain reducing O_2 to water, rate of ROS production is usually lower [78]. Beside the degree of reduction of the RC complexes, mitochondrial ROS production strongly depends on the local oxygen concentration inside mitochondria. When mitochondrial oxygen consumption acutely increases, as during active exercise, local depletion of O_2 within mitochondria occurs, thus limiting ROS production due to lack of one of its substrates [79].

There is an unfortunate tendency to automatically associate respiratory chain dysfunction with increased ROS production. The experimental support for this link is weak and there are in fact many examples of mouse models with impaired mitochondrial function that only exhibit minor or no oxidative stress [53, 80, 81].

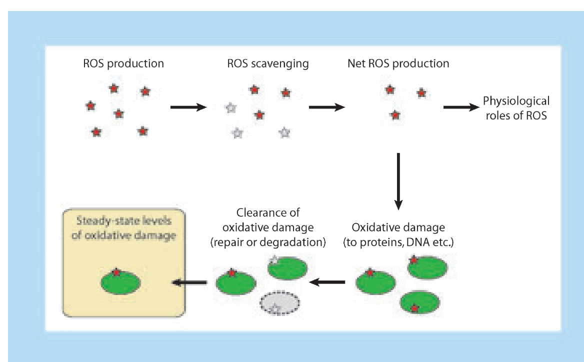

Fig. 3 Schematic representation of reactive oxygen species (ROS) formation and oxidative damage. ROS production is counteracted by a variety of ROS defence mechanisms resulting in net ROS production. ROS causes damage to a variety of macromolecules such as DNA, lipids and proteins, but some ROS are important signalling molecules and have a function in normal cell physiology. The steady-state levels of oxidatively damaged molecules are determined by rates of their formation and clearance.

An important factor determining the amount of oxidative damage to the cell is the antioxidant capacity of the defence mechanisms that inactivate the different ROS species (Fig. 3). It has been shown that there is are tissue-specific differences in the antioxidant capacity as levels of different antioxidant enzymes varies between tissues [82]. In addition, the steady-state levels of oxidatively damaged molecules are depending both on net ROS formation and clearance of damaged molecules. It is even possible that increased levels of oxidatively damaged molecules may be present even if ROS production is normal, i.e. if the clearance of these ROS-damaged molecules is defective (Fig. 3).

Conclusion

Clearly, significant advances in our understanding of the role of mitochondria in ageing have been made and we may obtain even greater insights into this growing field in the years to come. It is very likely that current theories, e.g. mitochondrial theory of

ageing, will have to be revised as the link between mtDNA mutations and ROS production is questioned. ROS are certainly toxic and may damage a variety of cellular component, however, there are also data to suggest that the organism may cope with increased ROS damage without developing premature ageing. The role for mitochondria in the CR response is becoming increasingly interesting and available data are sometimes contradictory and not easy to reconcile. It is likely that mitochondria will remain at the forefront of ageing research also for the next decade.

Conflict of interest statement

No conflict of interest was declared.

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