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Review

Mitochondrial energy metabolism and ageing

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ABSTRACT

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". Ageing is likely a multifactorial process caused by accumulated damage to a variety of cellular components. During the last 20 years, gerontological studies have revealed different molecular pathways involved in the ageing process and pointed out mitochondria as one of the key regulators of longevity. Increasing age in mammals correlates with increased levels of mitochondrial DNA (mtDNA) mutations and a deteriorating respiratory chain function. Experimental evidence in the mouse has linked increased levels of somatic mtDNA mutations to a variety of ageing phenotypes, such as osteoporosis, hair loss, graying of the hair, weight reduction and decreased fertility. A mosaic respiratory chain deficiency in a subset of cells in various tissues, such as heart, skeletal muscle, colonic crypts and neurons, is typically found in aged humans. It has been known for a long time that respiratory chain-deficient cells are more prone to undergo apoptosis and an increased cell loss is therefore likely of importance in the age-associated mitochondrial dysfunction. In this review, we would like to point out the link between the mitochondrial energy balance and ageing, as well as a possible connection between the mitochondrial metabolism and molecular pathways important for the lifespan extension.

1. Introduction: Mitochondrial energy metabolism

Mitochondria have a central role in the energy metabolism. Part of the free energy derived from the oxidation of food is inside mitochondria transformed to ATP, energy currency of the cell. This process depends on oxygen. When oxygen is limited, glycolytic products are metabolized directly in the cytosol by the less efficient anaerobic respiration that is independent of mitochondria.

The mitochondrial ATP production relies on the electron transport chain (ETC), composed of respiratory chain complexes I–IV, which transfer electrons in a stepwise fashion until they finally reduce oxygen to form water. The NADH and FADH2 formed in glycolysis, fatty-acid oxidation and the citric acid cycle are energy-rich molecules that donate electrons to the ETC. Electrons move toward compounds with more positive oxidative potentials and the incremental release of energy during the electron transfer is used to pump protons (H+) into the intramembrane space. Complexes I, III and IV function as H+ pumps that are driven by the free energy of coupled oxidation reactions. During the electron transfer, protons are always pumped from the mitochondrial matrix to the intermembrane space, resulting in a potential of ~150–180 mV. Proton gradient generates a chemiosmotic potential, also known as the proton motive force, which drives the ADP phosphorylation via the ATP synthase (F_oF_1 ATPase — complex V). F_o domain of ATPase couples a proton translocation across the inner mitochondrial membrane with the phosphorylation of ADP to ATP [1]. The rate of mitochondrial respiration depends on the phosphorylation potential expressed as a [ATP]/[ADP] [Pi] ratio across the inner mitochondrial membrane that is regulated by the adenine nucleotide translocase (ANT). In the case of increased cellular energy demand when the phosphorylation potential is decreased and more ADP is available, a respiration rate is increased leading to an increased ATP synthesis. There is usually a tight coupling between the electron transport and the ATP synthesis and an inhibition of ATP synthase will therefore also inhibit the electron transport and cellular respiration. Under certain conditions, protons can re-enter into mitochondrial matrix without contributing to the ATP synthesis and the energy of proton electrochemical gradient will be released as heat [2]. This process, known as proton leak or mitochondrial uncoupling, could be mediated by protonophores (such as FCCP) and uncoupling proteins (UCPs) [3,4]. As a consequence, uncoupling leads to a low ATP production concomitant with high levels of electron transfer and high cellular respiration [2].

2. Mitochondrial theory of ageing

Even though the process of oxidative phosphorylation is efficient, a small percentage of electrons may "leak" from the ETC, particularly from complexes I and III, during normal respiration and prematurely reduce oxygen, forming reactive oxygen species (ROS) [5]. It has

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been shown that other mitochondrial proteins such as the α-glycerophosphate dehydrogenase, the α-ketoglutarate and the pyruvate dehydrogenase have a role in ROS production [6]. Outside mitochondria, ROS can be produced by plasma membrane NADPH oxidases, lipid peroxidation and by some cytosolic enzymes [6]. Nevertheless, ROS produced within mitochondria presents almost 90% of the total ROS produced in the cell. The fact that the mitochondrial electron transport chain is the major ROS production site lead to the suggestion that mitochondria are a prime target for oxidative damage and hence the mitochondrial theory of ageing, a correlate to the free radical theory [7]. This idea is intellectually very appealing, as mitochondria are also the only organelle in animal cells that posses their own DNA, mtDNA, which is localized in the physical proximity to the mitochondrial respiratory chain (MRC). Over the years, substantial evidence has emerged from morphological, bioenergetic, biochemical and genetic studies to lend support to this theory. It was shown that mitochondria become larger and less numerous with age, accumulating vacuoles, cristae abnormalities and intramitochondrial paracrystalline inclusions [8]. Mitochondrial respiratory chain enzyme activities decrease, as well as mitochondrial membrane potential, on which the production of ATP is dependent, while the amount of oxidative damage to proteins and mtDNA increases, with an associated accumulation in the quantity of mtDNA mutations [9]. In addition, age-associated decrease in the MRC capacity was reported in various tissues, such as skeletal muscle [10] and liver [11]. Point mutations and deletions of mtDNA accumulate in a variety of tissues during ageing in humans, monkeys and rodents [12]. These mutations are unevenly distributed and can accumulate clonally in certain cells, causing a mosaic pattern of the respiratory chain deficiency in tissues such as heart, skeletal muscle and brain [13]. In terms of the ageing process, their possible causative effects have been intensely debated because of their low abundance and purely correlative connection with ageing [12].

We recently developed a mouse model that provided the first experimental evidence for a causative link between mtDNA mutations and ageing phenotypes in mammals [14]. We created homozygous knock-in mice that expressed a proofreading deficient form of the nuclear-encoded mitochondrial DNA polymerase (Polg) [14]. The introduced mutation was designed to create a defect in the proofreading function of Polg, leading to the progressive, random accumulation of mtDNA mutations during the course of mitochondrial biogenesis. As the proofreading is efficiently prevented, these mice develop an mtDNA mutator phenotype (mtDNA mutator mice) with a three to fivefold increase in the level of point mutations, as well as increased amounts of linear mtDNA molecules with deletions. The mtDNA mutator mice display a completely normal phenotype at birth and 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, anemia, reduced fertility and heart enlargement [14].

The mitochondrial theory of ageing predicts that levels of mtDNA mutations should increase exponentially as a consequence of a vicious cycle by accelerating the oxidative stress. However, an approximately linear increase of mtDNA mutation levels from midgestation to late adult life was detected in mtDNA mutator mice, implying that there is no vicious cycle [15]. Furthermore, it has been shown that there is no direct connection between increased levels of mtDNA mutations and elevated ROS production and this argues against direct role of oxidative stress in the ageing process [15]. We have detected a moderate increase of mitochondrial mass and a progressive reduction of both respiratory chain enzyme activities and mitochondrial ATP production rates in mtDNA mutator mice, consistent with previous reports in aged wild type animals [14,16]. In addition, we found a general reduction in all inducible respiratory states in both mtDNA mutator heart and liver mitochondria, while the basal respiration rate, limited by the mitochondrial membrane proton leak and not by the respiratory chain, was not altered [17]. Our latest results strongly argue that the observed phenotypes in mtDNA mutator mice are a direct consequence of the accumulation of mtDNA point mutations in protein-coding genes, leading to a decreased assembly of MRC complexes, respiratory chain dysfunction and thus to premature ageing [17].

3. “Uncoupling to survive”

The relationship between energy metabolism and longevity has been suggested by two seemingly opposing theories. According to the “rate of living hypothesis” proposed by Pearl in 1928, there was a direct link of the metabolic output of an organism to its longevity [18]. This is only the modern interpretation of probably the oldest explanation of ageing. Already Aristotle believed that we possess a finite amount of some “vital substance.” When that substance is consumed, we die. In essence, people recognized that things wear out when we use them and if we use them a lot they will not last as long. Some philosophers even argued that each person had only a finite, predetermined number of breaths or heartbeats and that once they were used, death ensued. Until recently many people believed this to be true, especially after it was supported by celebrities like Neil Armstrong, an American astronaut and the first man on the moon, who said: “I believe every human has a finite number of heartbeats. I don't intend to waste any of mine by running around doing exercises”. In the 20th century, scientists proposed a new twist on this old theory: energy consumption limits longevity. In other words, an organism's metabolic rate determines its lifespan [18]. Later, after the discovery of oxidative stress and formulation of the free radical theory of ageing the interpretation was simplified to factors that decrease an organism's metabolic rate would increase the longevity and vice versa. Today the rate of living theory is rejected as being a valid overall explanation for why we and most other species age. Scientists now believe that although the metabolic rate can affect ageing, that doesn't mean that it always does so. Caloric restriction, the only intervention known to extend life in many different species, does so without reducing the animal's metabolic rate. In addition, experimentally boosting an animal's metabolic rate does not always reduce longevity. And even though there is a rough correlation among species between body size, metabolic rate, and longevity, there are many exceptions to this rule. For example, birds typically have a metabolic rate 1.5–2.0 times as high as similar-sized mammals, yet they live on average about three times as long. Besides, a number of recent studies have demonstrated that even within a species, metabolic rate and longevity are not inversely related — a pattern inconsistent with the rate of living theory [19,20].

On the other hand, the “uncoupling to survive” theory proposes that energy metabolism is in a positive relation with longevity [21]. While following MF1 female mice throughout their lifespan, Speakman et al. observed that mice with higher metabolic intensities had higher proton conductance across skeletal muscle inner mitochondrial membrane and lived longer, providing evidence in favor of the “uncoupling to survive theory” [22].

This theory is also based on the notion that inefficiency in the mitochondrial ATP generation may be necessary to reduce ROS generation in the cell [21]. High proton motive force that drives an efficient ATP synthesis comes with an additional cost, the production of ROS. Because ROS production is highly dependent on the proton motive force, proton leak might help to limit the oxidative damage. There are a number of articles suggesting that UCPs could play an important role in this process [23]. UCPs are mitochondrial transporters present in the inner mitochondrial membrane and are found in all mammals. The term “uncoupling protein” was originally used for UCP1, a brown fat specific proton carrier that is activated by fatty acids

and dissipates the proton gradient as heat [23]. UCP2 and UCP3 are recently identified UCP1 homologues but their function in normal cellular physiology is still unclear. UCP2 and UCP3 proteins are found in much less amounts in the inner mitochondrial membrane and might be involved in the proton conductance only upon activation with certain activators (such are ubiquinone, superoxide, reactive alkenals and other alkenal analogues) leading to a conclusion that they are not involved in the adaptive response to cold as UCP1 [2,23]. Their presence in some ectothermic species (i.e. fishes) indicates some other function that these proteins may be enrolled in [24]. In fact up to date, there is no hard evidence that UCP2 or UCP3 has an uncoupling function when endogenously expressed (for review see [2]). All experiments in which an uncoupling effect of ectopically expressed (or entopically overexpressed) UCP2 or UCP3 has been demonstrated are potential artifacts. The same is true for the UCP1 protein, which when ectopically expressed leads to a non-physiological uncoupling [2]. Apparently, the general uncoupling effect of these carriers can be explained by the fact that, when overexpressed, they are not correctly inserted into mitochondrial membranes. Furthermore, an apparent uncoupling effect is not specific only for members of the UCP family; for instance the oxoglutarate carrier, when ectopically expressed, also leads to uncoupling [25].

However, in a number of experiments an ectopic expression of UCPs has been used to increase proton leak in mitochondria, and although this has nothing to do with the physiological role of these proteins, still has an uncoupling effect on the mitochondrial respiration, and therefore is interesting for this review. Mice with UCP1 ectopically expressed in skeletal muscles had increased median survival (but not maximal lifespan), decreased adiposity, increased temperature and metabolic rate as well as an increased activity of SIRT2 (NAD-dependent deacytelase) and AMPK (AMP-activated protein kinase, a sensor of intracellular AMP/ATP ratio in the cells) [26]. Beside increased metabolic rates these mice did not have alteration in food intake, fecal fat or caloric output in feces [26]. Thereby, it has been proposed that an elevated uncoupling activity in skeletal muscles can delay death and age-related diseases like malignancy, atherosclerosis, diabetes and hypertension [26].

A direct influence of the core body temperature (CBT) on longevity was assessed by making transgenic mice that overexpressed the UCP2 protein in hypothalamus [27]. Because the hypothalamus in mice is near the temperature control center, the mice's brain thought the body was overheating and lowered the core temperature. The results show that a drop of 0.6 °C extended the life of the mice by 12 to 20% [27]. Decreased CBT in these mice was in correlation with reduced metabolic activity indicating that the increased longevity is inversely correlated with the metabolic rate as it was proposed by 'rate of living' theory. The lower temperature may slow the rate of oxygen metabolism. The problem is that the lower temperature may also change a number of other systems and processes in the body. At the end, it is not clear why those mice lived longer, only that they did [27].

Important part of the "uncoupling to survive" theory is a direct connection of the increased mitochondrial proton conductance with lowered ROS production [21]. It has been proposed that UCPs have a role in the protection from oxidative damage by lowering a proton motive force thus causing a "mild" uncoupling and the attenuation of superoxide production from electron chain [21]. During "mild" uncoupling, caused by UCPs activation with superoxide and other ROS products derived upon oxidation of membrane phospholipids, ATP is still synthesized, a respiration rate is increased and in parallel a ROS production is decreased [21,28]. It has been proposed that a superoxide induces the uncoupling of mitochondria by interacting with the UCPs, that this process requires fatty acids and can be inhibited with purine nucleotides [28]. Yet it is likely that the fatty-acid induced response is also mediated by some other mitochondrial carrier protein beside UCPs such are the adenine nucleotide translocator or the dicarboxylate carrier [29,30]. In contrast, other studies showed that neither ROS nor oxidative damage has a regulatory role on the UCPs activity and therefore it has been argued against protective role of UCPs from oxidative damage [2]. In addition, some studies indicated that the "mild" uncoupling in pancreatic β cells in the presence of free fatty acids can actually increase the mitochondrial ROS production, lower a glucose-stimulated increase in mitochondrial membrane potential, cellular ATP, cytoplasmic calcium and insulin secretion [31]. Direct role of uncoupling in lowering oxidative damage and ROS production has also been challenged by results showing that, the overexpression of UCP3 protein in mouse skeletal muscle resulting in an increased basal proton conductance, did not decrease the oxidative damage to mitochondria [32].

To summarize, the role of uncoupling in longevity and protection against oxidative damage remains unresolved due to the inconsistency between different studies and general acceptance of this hypothesis will have to await future experimental confirmation.

4. A worm paradox: Respiratory chain dysfunction causes lifespan extension

A significant decrease in the mitochondrial bioenergetic capacity with advancing age has been shown in numerous animal models and recently in a study of human volunteers [33]. A decreased MRC activity during ageing can be due to a decreased protein expression, an enzyme inhibition or a modification upon ageing [34,35]. Oddly enough, several *Caenorhabditis elegans* mutants, defective in MRC functions but also in transport of the ETC substrates, are long lived [36–40]. This class of long-lived mutants is named Mit mutants and they usually carry a loss-of-function or a reduced-in-function alteration in components of the canonical ETC. Most of them exhibit a 20–40% increase in the mean adult life span, but in some cases life extension can be on the order of 300%. One example is a mutant in the *isp-1* gene that encodes a Rieske iron sulphur protein (ISP), a subunit of the mitochondrial complex III. The *isp-1(qm150)* mutant allele corresponds to a missense point mutation, that most likely results in a decreased number of electrons moving down the high affinity arm of the Q-cycle and onto the cytochrome *c* [38]. Surprisingly, this mutant, beside slow embryonic development and decreased mitochondrial respiration with a low oxygen consumption, has significantly prolonged lifespan [38]. *C. elegans clk-1* mutants provide another link between a defective MRC function and prolonged lifespan. These mutants lack an enzyme required for the ubiquinone (coenzyme Q) synthesis that is essential for a complex I-dependent respiration [41]. The *clk-1* mutant worms have slower development, slower rhythmic behavior and an increased lifespan [41]. Although some have reported that *clk-1* mutant animals have normal respiration levels [41], others have proposed that the main mechanism promoting longevity in these worms is a lower metabolic rate leading to a decreased ROS production and oxidative damage [42].

Majority of other models with decreased mitochondrial function that ensure prolonged lifespan, are actually not mutants, but worms with decreased expression of different components of the ETC obtained after RNAi treatments. Most of them have been indentified through genome wide RNAi screens for longevity genes, and they encode for the essential components of mitochondrial respiratory chain complexes [36–38,40]. Although decreased amount of major subunits of the MRC leads to a reduction of oxygen consumption and ATP levels in all different developmental stages, a lifespan extension is achieved only when the RNAi treatment is initiated after hatching. Surprisingly, in animals treated with RNAi against respiratory chain subunits during development, restoring mRNA levels during adulthood did not increase the level of ATP. This suggests that reduced respiratory chain activity during development permanently changes the system so that it continues to limit the ATP production even when the original impediment is removed [37]. Furthermore, a complete ablation of major MRC subunits leads to an embryonic arrest or severe phenotype with sterility and shortened lifespan, and only a moderate

decrease of these proteins has a positive effect on the lifespan. That "threshold" levels of MRC subunits are extremely important for the lifespan extension was demonstrated in a recent, detailed study of different respiratory chain subunits using an RNAi dilution approach [39]. It was shown that within certain "window", a decrease in the amount of single MRC subunit will cause a lifespan prolongation, while any further ablation will result in the lifespan shortening [39]. In agreement, a mild decrease in the level of protein in most cases did not have an effect on the lifespan. However, not all deficiencies of MRC subunits will lead to an increased lifespan, *i.e. mev-1(kn-1))* deletion mutant in a subunit of complex II had shortened lifespan, while a partial deficiency of this protein to different levels had no effect on the lifespan [39].

One proposed explanation for the lifespan extension of long-lived Mit mutants is that these animals have an upregulation of the fermentative malate dismutation, where fumarate is terminally reduced at complex II to succinate [43]. This is an alternative anaerobic metabolic pathway unique for nematodes that is upregulated during a dauer formation and is proposed to lead to lower ROS production. Several other explanations have also been anticipated, such as antioxidative role of ubiquinone and reduced complex I activity (in case of *clk-1* mutant), induction of endogenous protective system against ROS (SOD, catalase, glutathione peroxidase or by triggering SKN-1 dependent responses), but a precise mechanism is still unclear [44]. Most of these explanations still wait for an experimental confirmation, and recent results in other model organisms point toward a more general mechanism, and therefore could not be explained by processes specific for *C. elegans* such as the malate fermentation.

Recently, several mouse models with a decreased MRC activity that leads to a prolonged lifespan have been developed. A homozygous inactivation of *Mclk1*, the mouse ortholog of *clk-1* enzyme, leads to a severe developmental delay and embryonic lethality, whereas a partial deficiency of *Mclk-1* increases the lifespan on average by 31% in mice of three different genetic backgrounds [45]. Likewise, inactivation of *Surf1*, a gene encoding for an assembly factor of complex IV, significantly increases the lifespan in mice [46]. These mice have a mild reduction in the amount of fully assembled complex IV and COX activity that, surprisingly, provide also a broad protection from *in vivo* induced neurodegeneration [46]. On the contrary, *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 encepholomyelopathy denoted as the Leigh syndrome. Similarly, *Surf1* knockdown in the central nervous system in *Drosophila melanogaster* induced longevity whereas a constitutive knockdown of *Surf1* caused the embryonic lethality in flies [47].

Taken together, it seems that a decreased MRC activity could prolong a lifespan in a number of different organisms and that this is not "a worm paradox" any more, but a more general mechanism for lifespan extension that still awaits proper clarification.

5. Link between insulin/IGF-like signaling and mitochondrial respiration

Insulin/IGF (insulin-like growth factor) — like signaling (IIS) stands out as an important evolutionary conserved pathway involved in the longevity determination. The IIS pathway has pleotropic effects on growth, development, metabolic homeostasis, fecundity and the lifespan regulation [48]. Not only that lowered IIS can increase the longevity in fly, worms and mice, but also can improve health at older ages in these organisms [49–51]. Although complexity of the IIS pathway is different in different organisms, it has been shown that it always intersects with JNK (c-Jun N-terminal kinase) and TOR (target of rapamycin) pathways, which are implicated in the regulation of protein synthesis and growth in a response to different stress signals, respectively [48]. However, when it comes down to the influence of IIS pathway on the metabolic rate, things are not as clear. For instance, a lifespan analysis of long-lived flies (*chico*, a mutant in Ins/IGF receptor substrate) did not shown any difference in the metabolic rate or oxygen consumption when compared to the control strains, whereas several studies on the metabolic rate in long-lived *C. elegans daf-2* mutants, (deficient in insulin/IGF receptor), demonstrated a clear reduction in both parameters [20,52]. In mice, a selective disruption of insulin receptor in the adipose tissue (FIRKO mice) extends lifespan for about 18% [53]. These mice are leaner due to a lipodystrophy, but otherwise have normal appearance, food intake and fertility [53]. It has been shown that FIRKO mice had increased oxygen consumption per gram of body weight that most likely corresponds to the increased mass-specific food intake and an increased mitochondrial activity. In parallel, these mice had elevated mitochondrial mtDNA content, a modest increase of mitochondrial size and an increased gene expression for proteins involved in the mitochondrial biogenesis suggesting that increased oxidative metabolism and metabolic rate of the white adipose tissue could contribute to the observed lifespan increase of FIRKO mice and their resistance to metabolic diseases [54]. This model demonstrated an important role of adipose tissue in longevity and indicated a close relationship between the energy metabolism and a lifespan determination. However, not all metabolic changes of the adipose tissue result in lifespan prolongation. Over-expression or ablation of PEPCK-C (phosphoenolpyruvate carboxykinase, a protein involved in gluconeogenesis and glyceroneogenesis), in the white adipose tissue caused obesity or lipodistrophy, respectively, but did not result in a lifespan extension [55]. On the other hand, PEPCK-C overexpression in skeletal muscles increased the oxygen consumption and the mitochondrial biogenesis resulting in an extended lifespan [55].

A study on aged rats showed an increased intra-mitochondrial ROS production and oxidative damage, increased proton leak rates resulting in a depletion of membrane potential and a reduction of ATPase and complex IV activities. Treatment of aged rats with the insulin-like growth factor 1 (IGF1) corrected these parameters indicating that a cytoprotective effect of IGF1 is closely related to the mitochondrial protection [56].

Clearly, the role of mitochondrial energy production in a lifespan extension due to lowered IIS is still a matter of intense debate. We believe that future studies dealing with this topic have also to take into account the difference in the dependence of various model organisms and tissues on the oxidative metabolism.

6. Caloric restriction increases a mitochondrial respiration rate (?)

Caloric restriction (CR) is the only dietary intervention that consistently increases median and maximal lifespan in organisms ranging from yeast to mammals. This dietary regime implies 20–50% restriction of the overall caloric intake of animals on *ad libitum* regime [57]. It is currently not clear if CR also can extend life span in non-human 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 [58]. The CR response thus seems to have been conserved during animal evolution and there is an intense ongoing research to define the underlying molecular pathways [58]. Many hypotheses have been proposed for mechanisms that are responsible for the caloric restriction since the first study demonstrating that the CR can significantly extend mean and maximum lifespan in rats [59]. Initially it has been anticipated that the reduction of nutritive calories would lead to a down regulation of metabolic rate which in turn will lower the ROS production and the oxidative damage during the course of ageing. However, a growing body of evidence in fact suggests an increase in the oxidative metabolism and the metabolic rate as important underlying mechanisms of CR.

Caloric restriction is a regulated process that under certain regimes is dependent on the sirtuins, a family of proteins related to the yeast SIR2 protein (NAD-dependent protein deacetylase). Under moderate

CR conditions (0.5% glucose in contrast to normal 2% glucose), SIR2 mediates aspects of the CR response in budding yeast (*Saccharomyces cerevisiae*) [60]. It has been proposed that CR in the yeast shifts carbon metabolism from glycolysis towards oxidative metabolism involving the tricarboxylic acid cycle (TCA) and the ETC. Moderate CR in yeast leads to a 25% increase in the replicative lifespan and a two-fold increase in the respiration rate [61]. Overexpression of Hap4, a transcription factor that causes a switch from glycolysis to respiration, significantly prolongs the lifespan (35%) of yeast cells grown on glucose medium and also increases the respiration rate [61]. Furthermore, growth at 0.5% glucose fails to extend a replicative lifespan in yeast strains lacking an intact electron transport chain (ETC), suggesting that a metabolic shift towards respiration is necessary for the CR-induced increase of lifespan [61]. However, other studies argue that the respiration and SIRT2 protein are not required for lifespan extension by CR [62,63]. CR at low glucose concentrations (0.05%) significantly increases lifespan in some yeast strains lacking mtDNA (rho^0 cells) [62]. 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 organelles are functional.

Both genetic and environmental CR manipulations have been shown to increase the lifespan in *C. elegans*, including mutations that decrease a feeding rate (*eat* mutants), growth in axenic culture (chemically defined medium without bacteria), and a dilution of the bacterial food source. Initial results indicated that CR does not change metabolic rate in worms grown in reduced food conditions [64]. Similar findings have been reported in *D. melanogaster*, where CR had no effect on the resting metabolic rate [65]. Opposing results have recently been offered by Bishop and Guarente who have shown that a CR mediated lifespan extension in worms require two sensory neurons, whose signals trigger an increase in the respiration rate [66]. This increase is essential for the lifespan extension, as ETC inhibitors (myxothiazol and antimycin) suppressed this effect, without shortening the lifespan of worms fed *ad libitum* [66]. In addition, a specific restriction of intracellular glucose by treatment with 2-deoxy-glucose (DOG — a proposed CR mimetic) also extends lifespan in worms by inducing higher respiration rates and the antioxidant stress response [67].

In mice, CR increases the respiration rate and induces a mitochondrial biogenesis via induction of endothelial nitric oxide synthase (eNOS). [68]. eNOS is responsible for the nitric oxide production in endothelial cells, but is also induced upon CR in other tissues where mediates the mitochondrial biogenesis and induces the SIRT1 (mammalian ortholog of SIR2) gene expression, thus linking CR response to the sirtuin family in mammals [68]. Interestingly, SIRT1 deacetylates and therefore activates the eNOS enzyme, indicating a positive feedback mechanism between SIRT1 and eNOS [69]. An increased mitochondrial function during CR could also be a consequence of the increased SIRT1 activation of PGC-1α, a transcriptional coactivator and major inducer of mitochondrial biogenesis. Similar results have been obtained from an *in vitro* study showing that CR reduces oxidative stress, and upregulates mitochondrial biogenesis via PGC1α activation [70].

Effects demonstrated in mice were recently verified in a study of human volunteers subjected to a short CR regime that resulted in the expected reductions in body weight and blood insulin [71]. Skeletal muscle fibers from these calorie-restricted individuals showed an upregulation of SIRT1, eNOS, TFAM (mitochondrial transcription factor A) and mitochondrial mass, compared to cells from individuals on a normal diet. These findings suggest that a conserved pathway may operate in mammals during CR involving the activation of SIRT1 and eNOS that triggers an increase in the mitochondrial mass and activity. However, not all studies showed an upregulation of mitochondrial respiration and/or mitochondrial biogenesis during CR [72]. It is often difficult to compare results from different studies due to the use of different *in vivo* and *in vitro* model systems as well as different CR regimes.

A number of molecular mechanisms on how mitochondrial upregulation contribute to the beneficial effect of CR have been proposed. Many believe that increased respiration and mitochondrial biogenesis would lead to a reduced oxygen level in mitochondria thus lowering the chance for ROS production from complex I and II or that greatly increasing the mitochondrial surface would spread the charge over the large area thereby preventing the electron stalling and the ROS production [61]. A new twist to this old idea is that an increased mitochondrial respiration would induce low-level ROS production that in turn will act to stimulate antioxidant defense systems of the cell [67]. This proposed preconditioning of mitochondria is called mitohormesis [67]. Other proposed scenarios do not rely on an important role of ROS, but rather point out the importance of keeping mitochondria intact and healthy [61]. In summary, the precise molecular mechanisms of the life-extending actions of caloric restriction still remains unclear, but most likely mitochondrial energy metabolism plays a very important role in this process.

7. Concluding remarks

Studies that link 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, known to prolong lifespan, have been shown to both decrease and increase the ATP production in cells. The molecular mechanism behind this dualism is not known, and undoubtedly more experiments are needed to clarify the role of mitochondrial biogenesis, mitochondrial respiration rate and ROS production in different aspects of ageing. However, mitochondria are today in the scientific spotlight and sure hold promises for the future ageing research. That is certainly enough to make mitochondria a center of our attention.

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