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Mitochondrial reactive oxygen species: Do they extend or shorten animal lifespan?☆

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ABSTRACT

Testing the predictions of the Mitochondrial Free Radical Theory of Ageing (MFRTA) has provided a deep understanding of the role of reactive oxygen species (ROS) and mitochondria in the aging process. However those data, which support MFRTA are in the majority correlative (e.g. increasing oxidative damage with age). In contrast the majority of direct experimental data contradict MFRTA (e.g. changes in ROS levels do not alter longevity as expected). Unfortunately, in the past, ROS measurements have mainly been performed using isolated mitochondria, a method which is prone to experimental artifacts and does not reflect the complexity of the in vivo process. New technology to study different ROS (e.g. superoxide or hydrogen peroxide) in vivo is now available; these new methods combined with state-of-the-art genetic engineering technology will allow a deeper interrogation of, where, when and how free radicals affect aging and pathological processes. In fact data that combine these new approaches, indicate that boosting mitochondrial ROS in lower animals is a way to extend both healthy and maximum lifespan. In this review, I discuss the latest literature focused on the role of mitochondrial ROS in aging, and how these new discoveries are helping to better understand the role of mitochondria in health and disease. This article is part of a Special Issue entitled 'EBEC 2016: 19th European Bioenergetics Conference, Riva del Garda, Italy, July 2–6, 2016', edited by Prof. Paolo Bernardi.

1. Introduction

The Free Radical Theory of Ageing, later updated to the Mitochondrial Free Radical Theory of Ageing (MFRTA) was proposed by Denham Harman in 1956 and 1972 respectively [1,2]. The efforts to test MFRTA have been instrumental in gaining a better understanding of the aging process, moreover our knowledge of how free radicals participate in cellular physiology has been extended far beyond the aging field. Primary support for MFRTA comes from descriptive studies which established that ROS production and oxidative damage accumulate with age [3–6], and from correlative studies showing that ROS levels correlate with lifespan in long-lived animals [7–9] or individuals [10–13]. Additionally, excessive ROS levels have been reported in many age-related and degenerative diseases such as Parkinson's disease (PD) [13], diabetes [14] and cancer [15]. In contrast to this, MFRTA has been repeatedly challenged by experimental data, which has demonstrated that neither alteration of antioxidant levels nor direct manipulation of ROS production alter longevity as predicted by MFRTA [16–18]. Administration of antioxidants has repeatedly failed to extend lifespan in several animal models reviewed in [19]. Furthermore, manipulation of endogenous antioxidant levels also did not support MFRTA [20]. A paradigmatic example is *Caenorhabditis elegans*, where suppression of all superoxide dismutase activity by knocking-out all genes encoding superoxide dismutase enzymes fails to reduce lifespan even by a day, despite significantly increasing sensitivity to oxidative stress [21]. Although, *Drosophila melanogaster* or *Mus musculus* are more sensitive to superoxide levels i.e. knock-out of *Sod2* dramatically shortens lifespan to a few days in both animal species, heterozygous *Sod2* knock-out mice have a normal lifespan despite higher levels of oxidative damage [16]. Direct manipulation of ROS produced by the electron transport chain (ETC) does not alter longevity as expected either. Reducing superoxide leak from ETC does not extend lifespan in fruit flies [18], but even more counterintuitive is the fact that low doses of ROS-generating toxins such as rotenone or paraquat, in spite of having different effects on mitochondrial respiration (i.e. only rotenone is a direct inhibitor of respiratory complex I (CI) [22]), extend lifespan in worms in a ROS-dependent manner [17]. Furthermore, mutations in genes encoding subunits of CI increase ROS and extend lifespan in both worms and flies through a ROS dependent mechanism, independently of their effects on mitochondrial respiration

Abbreviations: DCFDA), 2′,7′-dichlorofluorescin diacetate; FCCP), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; CoQ), Coenzyme Q; CI), Complex I; ETC), electron transport chain; H_2O_2), hydrogen peroxide; HE), hydroethidine; LC), liquid chromatography; MS), mass spectrophotometry; mtDNA), mitochondrial DNA; MFRTA), Mitochondrial Free Radical Theory of Aging; mtROS), mitochondrial Reactive Oxygen Species; mtUPR), mitochondrial Unfolded Protein Response; PD), Parkinson's disease.

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[23,24]. The concept of mitohormesis proposes that boosting ROS levels activates a program of responses to stress that over-activate mechanisms of protection (including antioxidants) which compensate for initial damage caused by ROS [25]. Mitohormesis, fails to explain why overexpression of these same mechanisms of protection alone is not sufficient to extend lifespan or why overexpression of these mechanisms suppresses lifespan extension in those models where ROS have been experimentally increased [17,24]. Nevertheless, as mentioned previously, ROS levels have been shown repeatedly to be altered in aging and age-related diseases. In this review, I intend to discuss the role that ROS play in determining animal lifespan, reviewing the latest findings in the field. In the interest of space, I will mainly focus on the three animal models in which the greatest extension in lifespan has been reported: *C. elegans*, *D. melanogaster* and *M. musculus.* For the same reason, I will not discuss ROS generated outside the mitochondrion, although it is clear that they can also contribute to the onset of aging and age-related disease [26,27]. For example, it has recently been shown that lifespan of the long-lived *clk-1* mutant worm is further extended by increasing mitochondrial ROS levels through knock-out of *sod2* [28]. However, increasing cytosolic ROS levels, by knocking out *sod3* and *sod5*, shortened lifespan of this long-lived mutant. This indicates that ROS can have opposing effects on longevity, depending on whether they are produced within or outside of mitochondria. This highlights the importance of understanding where ROS are generated.

2. ROS are not all the same

Mitochondria are the powerhouses of the cell, producing a considerable share of cellular ATP, as well as many other essential cellular components such as iron–sulfur clusters or pyrimidine nucleotides, that the cell requires for survival. In addition, mitochondria participate in calcium metabolism and play a leading role in the initiation of apoptosis and as such are instrumental in maintaining cellular homeostasis acting as important signaling organelles in different tissues [29]. For example, mitochondrial ROS are essential for the elimination of bacteria by the macrophages [30], induction of differentiation of hematopoietic progenitors in fruit flies [31] or control of insulin release in pancreatic β-cells [32]. Most physiological processes taking place in mitochondria (e.g. ATP generation) are to a greater or lesser extent coupled to mitochondrial respiration, which makes mutations, in genes encoding ETC subunits or those proteins involved in mtDNA maintenance, the most common cause of inherited metabolic disorders [33]. Most oxygen consumption occurs during cellular respiration and as a consequence most of the superoxide produced within the cell is generated by the mitochondrion in the majority of, although not all, cell types [34].

Since mitochondria play a central role in cellular function and metabolism, it is not surprising that decreased mitochondrial performance is a hallmark of aging [35]. In fact, mitochondrial malfunction can have terrible consequences as exemplified by those phenotypes associated with mitochondrial disease i.e. inherited mitochondrial disorders that result in progressive neuropathies and myopathies [33]. Thus, many sporadic and age-related diseases such as PD [36] or diabetes are suspected to have a mitochondrial component [37]. It is possible that age-related mitochondrial loss of function is a consequence and not a cause of aging. A recent intriguing paper supports this hypothesis. Siegfried Hekimi's laboratory has shown that controlled disruption of Coenzyme Q (CoQ) biosynthesis, through knock-out of the *Mclk1* gene, severely affects mitochondrial function and dramatically reduces lifespan [38]. Interestingly, restoration of CoQ levels through administration of 2,4-dihydroxybenzoic acid (an analogue of 4-hydroxybenzoic acid, the natural precursor of CoQ) that is only able to partially rescue the mitochondrial phenotype completely rescued the shortened lifespan of *Mclk1* mutant mice. This result is totally unexpected, as chronic mitochondrial dysfunction should cause the accumulation of irreversible damage and a shortened lifespan, if mitochondrial dysfunction is an underlying cause of aging. Conversely, Hekimi's work suggests [38] that mitochondrial dysfunction *per se* does not cause aging, as replacement of damaged mitochondria with functional mitochondria instantly restored a youthful phenotype. This defies the dominant paradigm that states that chronic mitochondrial dysfunction accelerates aging. It would be interesting to test if this is applicable to other models of mitochondrial dysfunction such as mutations in the mitochondrial polymerase (DNA polymerase γ) that has also been shown to accelerate aging [39] through a reduction in mitochondrial function [40], or if this effect is unique to alterations in CoQ synthesis.

A topic highly debated in the field is the role that mitochondrial ROS play in age related and non-age related pathological processes with a mitochondrial component. Are ROS a cause or a consequence of mitochondrial dysfunction? This is a very important question, which needs to be addressed, since it will affect the treatment of those pathologies. Considering all the available evidence, it is plausible to suggest that ROS can have both positive and negative roles depending on the type of the ROS, when, where and how much is produced. Therefore, we can talk about "Good" and "Bad" ROS. "Good" ROS being low reactivity ROS (i.e. superoxide or hydrogen peroxide (H_2O_2)) produced at specific places, at specific times and in moderate amounts and "Bad" ROS being highly reactive ROS (or low reactive ROS as H_2O_2 or superoxide produced at high concentrations) generated continuously and unspecifically. Experimental evidence suggests that boosting ROS production can contribute to the maintenance of cellular homeostasis and positively affect lifespan when induced correctly, whereas if produced in excess or in an unspecific way, they shorten survival and accelerate the onset of age-related disease.

In my opinion, there are two reasons why the role of ROS in aging and in different diseases is not yet fully understood. Firstly, ROS are usually considered as a single entity and are measured using unspecific probes that are prone to experimental artifacts. For example, 2′,7′-dichlorofluorescin diacetate (the popular DCFDA) reacts non-specifically with many types of free radicals that are produced ubiquitously, thus its use is associated with many caveats as for example its propensity to autoxidize [41]. Each ROS has specific properties that are determined by its intrinsic reactivity and relative abundance. The second issue is that the majority of ROS measurements -in the study of aging and age-related diseases- have been performed *in vitro* using isolated mitochondria or cells in culture. I will discuss the first issue now, and will focus on the second in the following section.

As previously mentioned, it is common to see reference to "ROS" without any mention of which specific ROS is being measured or where they are produced. There are many types of ROS but the three most studied in aging and age-related pathologies are superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (•OH), which are the result of the incomplete reduction of oxygen with one, two and three electrons respectively. It is usually accepted that ROS are mainly produced during oxidative phosphorylation, by mitochondria, as a result of the incomplete reduction of O_2 to superoxide. This is true for the majority of cell types but not all, since in some cells other organelles (e.g. peroxisomes or endoplasmic reticulum) or enzymes (e.g. NADPH oxidases, xanthine oxidase, lipoxygenase, cyclooxygenase, cytochrome p450s or nitric oxide synthase) are the main generators of ROS (reviewed elsewhere [42–44]). In normal conditions, generation of superoxide is not particularly problematic for the cell since it is promptly detoxified to H_2O_2 by superoxide dismutase. In fact, neither superoxide nor H_2O_2 are particularly reactive when maintained at low concentrations and are unable to for example cause mutations to DNA by themselves [45]. However, they can both generate more reactive ROS, which are able to cause macromolecular damage including DNA mutations. The main target of superoxide is the iron–sulfur clusters of proteins such as aconitase or respiratory CI and II, which release free iron as a result of superoxide attack [46,47]. This free iron reacts with superoxide and H_2O_2 to form the extremely toxic. ·OH through the Fenton/Haber-Weiss reactions. In addition, superoxide can also react with nitric oxide to form another highly toxic ROS: peroxynitrite ($OONO^-$). Both ·OH and $OONO^-$ can react with and damage all biological components

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