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The sites and topology of mitochondrial superoxide production

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ABSTRACT

Mitochondrial superoxide production is an important source of reactive oxygen species in cells, and may cause or contribute to ageing and the diseases of ageing. Seven major sites of superoxide production in mammalian mitochondria are known and widely accepted. In descending order of maximum capacity they are the ubiquinone-binding sites in complex I (site IQ) and complex III (site IIIQo), glycerol 3-phosphate dehydrogenase, the flavin in complex I (site IF), the electron transferring flavoprotein:Q oxidoreductase (ETFQOR) of fatty acid beta-oxidation, and pyruvate and 2-oxoglutarate dehydrogenases. None of these sites is fully characterized and for some we only have sketchy information. The topology of the sites is important because it determines whether or not a site will produce superoxide in the mitochondrial matrix and be able to damage mitochondrial DNA. All sites produce superoxide in the matrix; site IIIQo and glycerol 3-phosphate dehydrogenase also produce superoxide to the intermembrane space. The relative contribution of each site to mitochondrial reactive oxygen species generation in the absence of electron transport inhibitors is unknown in isolated mitochondria, in cells or in vivo, and may vary considerably with species, tissue, substrate, energy demand and oxygen tension.

1. Introduction

In the mitochondrial free radical theory of ageing (Harman, 1972), mitochondrial reactive oxygen species (ROS) generation is an inevitable consequence of oxidative ATP production, and the primary cause of macromolecular damage. Some damage is not repaired, causing progressive failure of cellular machinery, ageing-related diseases, and ageing. There is considerable evidence both in favour and against the theory (Beckman and Ames, 1998; Finkel and Holbrook, 2000; Golden et al., 2002; Muller et al., 2007; Sanz et al., 2006). Most authors think that mitochondria-generated oxidative stress is important, particularly in ageing-related diseases, but is not the sole cause of ageing. According to a recent review (Muller et al., 2007), mitochondrial ROS convincingly determines lifespan in fungi (hyphal senescence in *Podospora* and chronological ageing in *Saccharomyces*) and reasonably convincingly in *Caenorhabditis elegans*. The case is tentative in *Drosophila* but inconclusive in mouse and human, and better tests are required.

Seven specific sites that are involved in ROS generation have been defined in isolated mammalian mitochondria using electron transport chain inhibitors (Andreyev et al., 2005; Brand et al., 2004; Jezek and Hlavata, 2005; Murphy, 2009; Raha and Robinson, 2000; Turrens, 2003), but we lack reliable measurements of their rates in the absence of such inhibitors, and there is no consensus on their relative importance. In cells our knowledge of which sites are important is even worse. It is based mostly on measurements using inhibitors of electron transport. Typically, if rotenone (a complex I inhibitor) raises ROS production in cells, endogenous ROS generation is inferred to be from complex I, but this inference is unjustifiable, and new approaches are needed.

2. The mitochondrial free radical theory of ageing

An association between mitochondrial ROS generation and age-related disease is generally accepted, although it is also agreed that the mitochondrial free radical theory's explanation of ageing is incomplete (Beckman and Ames, 1998; Finkel and Holbrook, 2000; Golden et al., 2002; Muller et al., 2007; Sanz et al., 2006). Since age is the primary risk factor for many diseases, understanding the mechanisms of ageing may allow us to significantly reduce the burden of disease and increase human healthspan. It is therefore crucial to fully understand mitochondrial ROS production to assess its role in age-related diseases and ageing, and ultimately to allow rational design of beneficial therapies.

Age-related disease can be caused by overproduction of superoxide, resulting in molecular damage. Mitochondrial superoxide is detoxified to H_2O_2 by superoxide dismutases (matrix Mn–SOD, cytosolic Cu/Zn–SOD), then to O_2 and H_2O by antioxidant defences, including catalase or glutathione peroxidase. However, these antioxidants are imperfect, and superoxide that evades them damages proteins, lipids and DNA. H_2O_2 is relatively unreactive, but in the presence of Fe(III) it forms reactive hydroxyl radicals, initiating

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membrane lipid peroxidation (Halliwell and Gutteridge, 1999). The products of sugar, protein and lipid oxidation cause secondary damage to proteins (Shigenaga et al., 1994; Sohal and Weindruch, 1996). Thus, mitochondrial superoxide causes 'oxidative stress'. The toxicity of matrix superoxide is shown by Mn–SOD knockout mice, which live only 10–20 days even in the presence of antioxidants (Lebovitz et al., 1996; Li et al., 1995). In contrast, cytosolic superoxide is not lethal in Cu/Zn–SOD knockout mice (although they are sensitive to oxidative stress (Ho et al., 1998)) showing that extramitochondrial superoxide is less toxic. Many pathologies are related to oxidative stress, including atherosclerosis, hypertension, ischemia–reperfusion, inflammation, cancer, diabetes, Parkinson's and Alzheimer's disease (Halliwell and Gutteridge, 1999).

In ageing, there is good evidence for the importance of mitochondrial ROS generation (Cadenas and Davies, 2000). Beckman and Ames (1998) discuss 14 lines of evidence for the free radical theory, most implicating mitochondrial ROS. However, many are correlative and do not prove causality. One intriguing inverse correlation, between maximum lifespan and mitochondrial ROS production during reverse electron transport through complex I in different species (Barja et al., 1994; Ku et al., 1993; Lambert et al., 2007), is hard to explain if complex I ROS production does not contribute to ageing. More direct tests involve altering antioxidant defences, but give ambiguous results. Overexpression of Cu/Zn–SOD in *Drosophila* either has no effect (Seto et al., 1990), or increases lifespan (Sun et al., 2002). SOD/catalase mimetics increase lifespan in *C. elegans* (Melov et al., 2000), but only under specific conditions (Keaney and Gems, 2003). Overexpression of antioxidant defences in mice does not generally increase lifespan (Perez et al., 2009). In principle, the best test is to show that decreasing mitochondrial ROS production slows ageing. A striking result from RNAi screens is that knockdown of most mitochondrial electron transport proteins extends life in *C. elegans* (Dillin et al., 2002; Lee et al., 2003). However, because we do not know which sites in the electron transport chain produce the most ROS, it is hard to know how to alter ROS production without also altering ATP synthesis.

3. Mitochondria as a source of ROS

The respiratory chain produces superoxide when single electrons leak to O_2 as electron pairs flow down the chain (Chance et al., 1979). Interest in mitochondrial ROS began more than 40 years ago (Boveris and Chance, 1973; Boveris et al., 1972; Hinkle et al., 1967; Jensen, 1966) and has been well-reviewed (Andreyev et al., 2005; Brand et al., 2004; Jezek and Hlavata, 2005; Murphy, 2009; Raha and Robinson, 2000; Turrens, 2003). There are currently seven separate sites of mammalian mitochondrial ROS production that have been identified and widely accepted (Fig. 1) and these are discussed in more depth below. The sites with the greatest maximum capacities to produce ROS are at complex I (site IQ) and complex III (site IIIQo) (Barja, 1999; Chen et al., 2003; Kudin et al., 2004; Liu et al., 2002; Raha and Robinson, 2000; St-Pierre et al., 2002; Votyakova and Reynolds, 2001), although the other five sites also have significant maximum rates (Andreyev et al., 2005) (Fig. 1). The topology may be vital, given the location of mitochondrial DNA in the matrix and its susceptibility to oxidative damage. Complex I produces superoxide to the matrix, whereas complex III produces it to the matrix and intermembrane space at about equal rates under de-energized conditions (Fig. 1) (Han et al., 2001, 2003; Miwa and Brand, 2005; Miwa et al., 2003; Muller et al., 2004; St-Pierre et al., 2002).

In isolated cells, or in vivo, it is thought that mitochondria produce significant amounts of ROS (Skulachev, 1996), although the evidence is not fully compelling. The main indirect evidence comes from the lifespan-shortening effect of Mn–SOD knockout in mice (Lebovitz et al., 1996; Li et al., 1995). Fluorescent probes such as dichlorodihydrofluorescein or dihydrorhodamine have been used in hundreds of studies to infer mitochondrial ROS production in

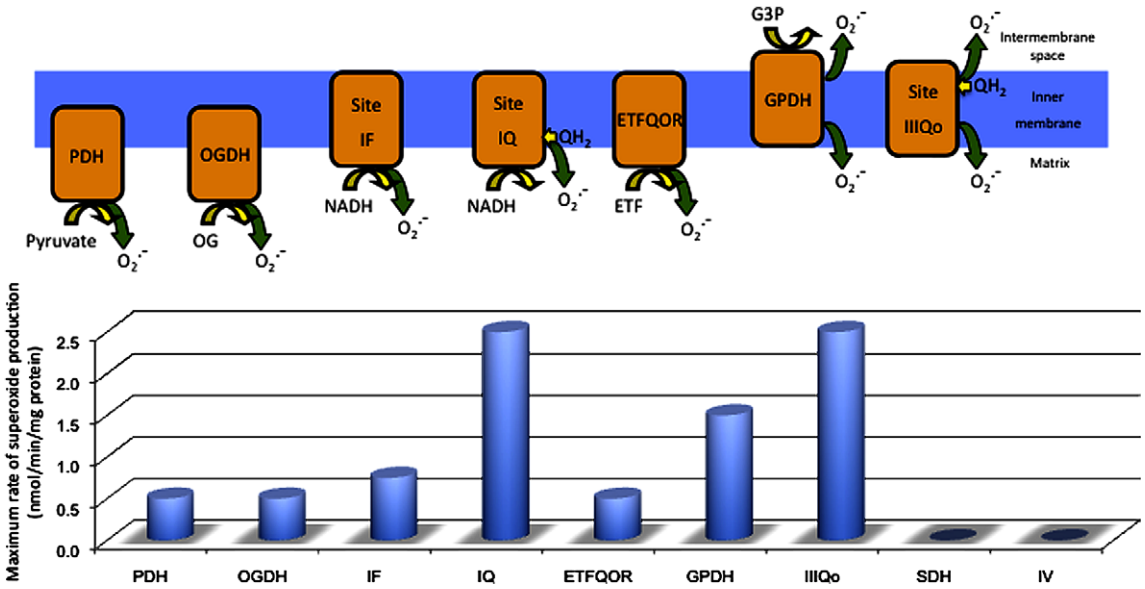

Fig. 1. The sites and topology of mitochondrial superoxide production. The seven identified sites of superoxide production in mitochondria are shown in the upper diagram, with an indication of the location of their substrate-binding sites and the topology of their superoxide production. PDH, pyruvate dehydrogenase; OGDH, 2-oxoglutarate dehydrogenase; site IF, the FMN-containing NADH binding site of complex I; site IQ, ubiquinone reduction site of complex I; ETFQOR, electron transferring flavoprotein ubiquinone oxidoreductase – the entry point of electrons from flavin-linked beta-oxidation of fatty acids via ETF (electron transferring flavoprotein) to ubiquinone in the electron transport chain; GPDH, glycerol 3-phosphate dehydrogenase; site IIIQo, the outer quinone-binding site of the Q-cycle in complex III. PDH and OGDH may also produce hydrogen peroxide directly. The lower panel shows representative values of the maximum superoxide production rate from each site, and from SDH (succinate dehydrogenase; complex II) and IV (complex IV). Rates are for rat skeletal muscle mitochondria, from Lambert et al. (2007, 2008a,b), Lambert and Brand (2004a,b), St-Pierre et al. (2002) and Jason Treberg, Casey Quinlan and Martin D. Brand (unpublished observations), and are greatest from sites IQ and IIIQo, followed by GPDH, then sites IF, EFTQOR, PDH and OGDH. There is insignificant superoxide production from complexes II and IV (or from pool Q and cytochrome *c*, not shown).