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Review

There is no evidence that mitochondria are the main source of reactive oxygen species in mammalian cells

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ABSTRACT

It is often assumed that mitochondria are the main source of reactive oxygen species (ROS) in mammalian cells, but there is no convincing experimental evidence for this in the literature. What evidence there is suggests mitochondria are a significant source for ROS, which may have physiological and pathological effects. But quantitatively, endoplasmic reticulum and peroxisomes have a greater capacity to produce ROS than mitochondria, at least in liver. In most cells and physiological or pathological conditions there is a lack of evidence for or against mitochondria being the main source of cellular ROS. Mitochondria can rapidly degrade ROS and thus are potential sinks for ROS, but whether mitochondria act as net sources or sinks within cells in particular conditions is unknown.

Contents

1. Introduction

Mitochondria are often thought of as an important therapeutic target (in aging and pathologies such as diabetes, neurodegeneration, cancer and cardiovascular disease) in part because they are considered to be the main source of reactive oxygen species (ROS) in the cells. However, the evidence base of this idea that mitochondria are the main source of cellular ROS is obscure, even though the concept is often repeated in the literature. In this brief review, we look for the origin of this idea, and examine the evidence base for it.

2. Cellular sources of reactive oxygen species

ROS include a number of molecular species derived from oxygen that are relatively reactive, but biologically most of them are derived from either superoxide ($O_2^{\cdot-}$) and/or hydrogen peroxide (H_2O_2). So if we are interested in where most ROS come from, in principle we can restrict our attention to superoxide and hydrogen peroxide. In mammalian cells, a number of sources of ROS are known including: i) mitochondria (mainly complex I & III, but also monoamino oxidase, α-ketoglutarate dehydrogenase, glycerol phosphate dehydrogenase, $p66^{shc}$ (Starkov 2008)), ii) endoplasmic reticulum (mainly cytochrome *P*-450 and *b*5 enzymes, diamine oxidase, Ero1 (Gross et al. 2006)), iii) peroxisomes (mainly fatty acid oxidation, D-amino acid oxidase, L-2-hydroxyacid oxidase and urate oxidase

Abbreviations: DPI, diphenylene iodonium; H_2DCF, 2′7′-dichlorofluorescin; MAO, monoamino oxidase; NOX, NADPH oxidase; PHOX, phagocytic NADPH oxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; TTFA, thenoyltri-fluoroacetone.

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(Boveris et al. 1972), iv) cytosol (NO synthases, lipoxygenases and PGH synthase (Kukreja et al. 1986; Roy et al. 1994)), v) plasma membrane (NADPH oxidases, lipoxygenase (O'Donnell and Azzi 1996)) and extracellular space (xanthine oxidase (McNally et al. 2003)).

ROS production, and the relative contribution of different sources, may in principle be different for: a) different ROS, i.e. in this case superoxide and hydrogen peroxide, b) different cell types, c) different species, and d) different physiological and pathological conditions. Various complexities arise from the presence of ROS consumers (enzymes consuming superoxide or hydrogen peroxide) in different compartments. The presence of ROS consumers in the same compartment as ROS producers often means the absolute rate of ROS production is underestimated. However, the net rate is what determines whether a particular compartment exports or imports H_2O_2. Thus, for example, peroxisomes may produce large amounts of H_2O_2 and consume large amounts of H_2O_2 (due to the presence of catalase) but it is the net rate, not the absolute rates, which determines whether it will import or export H_2O_2 to the rest of cell (Mueller et al. 2002). Superoxide is not thought to cross membranes appreciably, because of its charge, (although small amounts may cross in the protonated form or pass via anion transporters), so most superoxide produced within an organelle is going to stay there, prior to consumption. However, most superoxide is assumed to be converted (by SOD) to H_2O_2, which is then free to leave the organelle or be consumed there. Organelles may produce superoxide on the inside or outside, for example the mitochondrial respiratory chain produces superoxide both into the matrix and into the intermembrane space, and the latter may be consumed by SOD in the intermembrane space or escape into the cytosol via porin.

3. Estimating the relative contribution of different ROS sources by measuring rates

The relative contribution of different sources of ROS to total cellular ROS can be estimated in two general ways: (i) measure the absolute rates of ROS production of different sources, and compare them or compare them to total ROS production, or (ii) measure the fractional change in total ROS (or some proxy for this) caused by inhibiting the source of interest. Often the change in some function or dysfunction is measured in response to inhibition of some source, but this is not helpful in estimating the contribution to total ROS.

One of the first quantitative estimations of ROS production by isolated mitochondria and other organelles was provided by Chance and colleagues (Boveris et al. 1972). They tested various respiratory substrates and various metabolic states of isolated liver mitochondrial respiration and found that succinate was the most effective substrate stimulating H_2O_2 production in mitochondria. In State 4 the rate of H_2O_2 production was found to be 0.5 nmol/min/mg protein and this accounted for about 1–2% of the total oxygen consumption by isolated liver mitochondria. The ROS generation rates substantially decreased during transition from State 4 to State 3 and were at the level of 0.06-0.08 nmol/min/mg protein with all tested substrates (succinate, glutamate plus malate, palmitoylcarnitine, octanoate), i.e. 0.018% of the rate of oxygen consumption. Note that this is the source reference of the often quoted finding that mitochondrial ROS production is 1-2% of total oxygen consumption, when in fact this was only for an unphysiological substrate (succinate) in an unphysiological state (State 4) with an unphysiologically high oxygen level.

The study by Boveris (Boveris et al. 1972) also provided the most explicit comparison of H_2O_2 generation rates by other organelles such as peroxisomes, endoplasmic reticulum membranes and cytosolic enzymes. The H_2O_2 production rate (estimated by a peroxidase binding assay measuring H_2O_2 release rather than total production) by intact peroxisomes without added substrates was found to be 0.4 nmol/min/mg protein, which accounts for about 18% of oxygen uptake by peroxisomes. The rate increased after addition of peroxisomal substrates - D-alanine (ROS generation rate was 0.56 nmol/min/mg protein) and uric acid (7.8 nmol/min/mg protein). In the presence of azide to inhibit catalase, the maximal rate of H_2O_2 production was found to be 11 nmol/min/mg protein (about 63% of the total oxygen consumption by peroxisomes) (Boveris et al. 1972). The microsomal fraction of a liver homogenate (consisting mainly of endoplasmic reticulum membranes containing cytochrome b5, NADPH-cytochrome *c* reductase, etc.) generated ROS with a maximal rate of about 1.7 nmol/min/mg protein (using NADPH as a substrate) (Boveris et al. 1972).

To compare the relative contributions of different sources of ROS generation Boveris et al. (1972) calculated the rates of H_2O_2 production in liver homogenates from the average values of specific activities and the amount of protein in different isolated fractions. They estimated that the total rate of H_2O_2 generation by liver homogenates with endogenous substrates was 38 nmol/min/g liver, which accounted for about 10% of the total oxygen consumption of the liver homogenate. Then they calculated that mitochondrial H_2O_2 production was in the range of 4–12 nmol/min/g of liver depending on substrate. The microsomal fraction generated ROS at rates between 3 and 42 nmol/min/g of liver (the latter value was considered to be close to physiological, and was estimated with NADPH as substrate). The rate of ROS generation in peroxisomes was the highest and ranged between 30 and 100 nmol/min/g of liver (the value of 30 was considered as most physiological). And finally the liver cytosolic fraction containing soluble proteins generated H_2O_2 with a rate of 4 nmol/min/g of liver. From these estimates, it was concluded that mitochondria, microsomes, peroxisomes and cytosolic enzymes contribute to the total cellular H_2O_2 production in rat liver by 15%, 45%, 35% and 5%, respectively (Chance et al. 1979). Thus the only study to quantitatively measure the relative ROS production by different cellular sources concluded that mitochondria are a significant ROS source, but not the main source, the main source in liver being microsomes or peroxisomes.

Interestingly, some authors have reported that H_2O_2 production by isolated peroxisomes of rat liver respiring on fatty acids may reach rates as high as 3,500 nmol/min/g liver (Lazarow and De Duve 1976). On the other hand, it was demonstrated that most of H_2O_2 produced in peroxisomes is destroyed by catalase within this organele so that only 11-42% of H_2O_2 is released into cytosol. Even taking into account this, it is obvious from these calculations that mitochondria are far from being the main source of ROS production in liver. And even if we assume that in certain conditions additional mitochondrial enzymes (such as MAO in brain or $p66^{shc}$ in apoptotic cells) may generate ROS, it is unlikely that would increase ROS generation by about 10 folds to reach the rate of peroxisomal ROS production.

A more recent study (Kudin et al. 2008) quantified ROS production by mitochondria and non-mitochondrial sources in brain tissue homogenates. They found that in digitonin-permeabilized homogenates of either rat hippocampus, whole mouse brain or human parahippocampal gyrus there was a roughly 2-fold stimulation of H_2O_2 production by addition of succinate. The rates of H_2O_2 production were found to vary from 90 pmol/min/mg protein in human parahippocampal gyrus homogenates with endogenous substrates up to 825 pmol/min/mg protein in mouse whole brain homogenates supplemented with succinate. In contrast, addition of glucose, which was expected to stimulate ROS production by cytosolic enzymes entrapped in synaptosomes did not increase ROS production in brain homogenates. However, as the authors noted, the contribution of cytoplasmic sources of ROS may have been underestimated due to dilution of cytosolic factors in digitonin-permeabilized homogenates and due to the absence of direct substrates for non-mitochondrial sources of ROS such as NADPH or fatty acids. The study (Kudin et al. 2008) also showed a linear relationship between the rate of oxygen consumption by the homogenates and ROS generation with succinate, and it was estimated that in the presence of added succinate about 1%