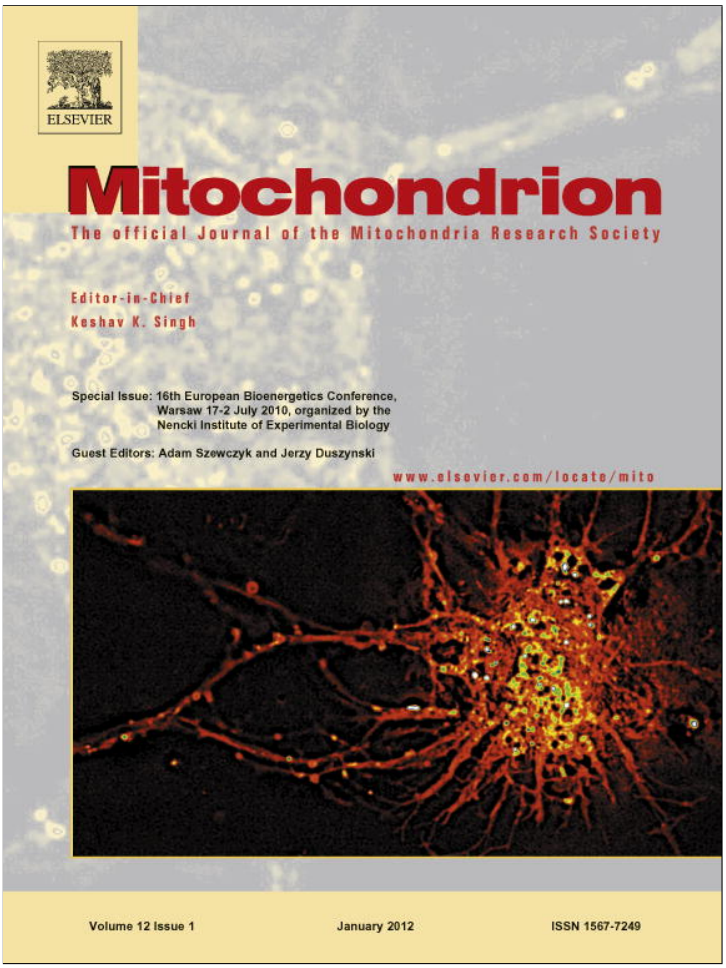

Mitochondrion 12 (2012) 1–4

Contents lists available at ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito

Review

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

Guy C. Brown [a], Vilmante Borutaite [b,*]

[a] Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, UK
[b] Institute for Biomedical Research, Lithuanian University of Health Sciences, Eiveniu str. 4, Kaunas LT-50009, Lithuania

ARTICLE INFO

Article history:
Received 28 October 2010
Received in revised form 10 January 2011
Accepted 1 February 2011
Available online 15 February 2011

Keywords:
Mitochondria
Reactive oxygen species
Respiration

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^-) 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.

* Corresponding author. Tel.: +370 37 302968; fax: +370 37 302959.
E-mail addresses: vilbor@vector.kmu.lt, vilbor@gmail.com (V. Borutaite).

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doi:10.1016/j.mito.2011.02.001

(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%

of the respiratory chain electron flow is redirected to ROS generation. However, succinate is a non-physiological substrate that maximally stimulates mitochondrial ROS production, so this finding is difficult to relate to physiological conditions.

4. Absolute estimates of H_2O_2 production rates

It has been suggested that apparent mitochondrial ROS production is an artefact of ROS probes interacting directly with the respiratory chain, and if this artefact is excluded then there is no measured mitochondrial ROS production (Staniek and Nohl 1999; Staniek and Nohl 2000). However, these authors only assessed some methods of ROS measurement, not including the currently popular method of Amplex Red plus horseradish peroxidase for H_2O_2 measurement. Nevertheless, it holds true that ROS measurement is artefact prone (Wardman 2007).

Brand and colleagues (Treberg et al. 2010) have shown that rat skeletal muscle mitochondria have high rates of H_2O_2 consumption by mitochondrial glutathione peroxidase. And this may result in underestimation of mitochondrial ROS production when it is measured using H_2O_2-detection assays. According to Treberg et al. (2010), the corrected rate of H_2O_2 production by mitochondria in various conditions varies between 0.2 and 2.5 nmol H_2O_2/min/mg protein. Mitochondrial ROS production is tissue dependent (Malinska et al. 2009; Tahara et al. 2009). The highest rates of mitochondrial H_2O_2 production were detected in isolated brain and heart mitochondria, reaching levels of 1–2 nmol/min/mg protein in State 4 with succinate as substrate and accounting for about 3% of total oxygen consumption. In phosphorylating mitochondria (which represent a more physiological state) H_2O_2 production rates dropped to 0.1 nmol/min/mg protein or less in heart, brain, and skeletal muscle (<0.2% of oxygen consumption), 0.3 and 0.4 nmol/min/mg protein in kidney and liver. Starkov (2008) estimated H_2O_2 production by brain mitochondria to be 0.3-3% of respiration rate in State 4, and 0.04-0.2% of respiration rate in State 3 on various substrates. Several other authors have estimated mitochondrial ROS production under physiological conditions to be around 0.1-0.2 % of the total respiratory rate (Rigoulet et al., 2011; St-Pierre et al. 2002; Staniek and Nohl 2000; Tahara et al. 2009).

For comparison, the rates of H_2O_2 production by intact liver peroxisomes have been estimated to be: 90–370 nmol/min/mg on fatty acyl-CoAs, 25 on D-amino acids, 250 on glycolate, and 2640 on urate (Mueller et al. 2002). These peroxisomal rates are up to a thousand times the mitochondrial rates above, but they are expressed per mg of organelle protein, and thus need to be multiplied by the relative amount of organelle protein in cells, to estimate the relative ROS production.

It has been estimated that protein thiol oxidation via protein disulfide isomerise (PDI) and Ero1 in the endoplamic reticulum accounts for 25% of H_2O_2 production in growing cells, but this was based on a theoretical rather than experimental calculation (Gross et al. 2006; Tu and Weissman 2004). The phagocyte NADPH oxidase (PHOX) is known to generate large quantities of superoxide and H_2O_2 accounting for significant fraction (10-90%) of total oxygen consumption of neutrophils, macrophages and microglia when activated (Bal-Price et al. 2002; McBride and Brown 1997; Souza et al. 2002). However, the quantitative contribution of PHOX and other NADPH oxidases to total cellular ROS production in resting or more physiological conditions is less clear. Kakinuma et al. (1977) measured the NADPH-dependent H_2O_2 production of the granular fraction of polymorphonuclear leukocytes (neutrophils) as 0.5 nmol/min/mg in resting conditions and 5.3 nmol/min/mg when phagocytosis was activated by adding heat-killed bacteria to the leukocytes. This H_2O_2 production should correspond to that of the NADPH oxidase, and was shown to be quantitatively similar to the H_2O_2 production of the intact cells, measured extracellularly.

5. Estimating the relative contribution of different ROS sources by inhibition

Some researchers have tried to estimate the contribution of mitochondria to cellular ROS production by measuring some marker of the latter in cells, and then inhibiting the mitochondrial respiratory chain or making cells hypoxic (Dawson et al. 1993; Duranteau et al. 1998; Vanden Hoek et al. 1997). Intracellular H_2O_2 has often been measured with 2′7′-dichlorofluorescin (H_2DCF), which becomes fluorescent 2′7′-dichlorofluorescein after enzyme-catalyzed reaction with H_2O_2 or other oxidants. Dawson et al. (1993) showed that in hepatocytes incubated with H_2DCF, myxothiazol partially inhibited azide- or cyanide-induced H_2DCF oxidation, suggesting that the Q_o site of complex III contributed to azide- and cyanide-induced cellular ROS production. However, myxothiazol alone induced more H_2DCF oxidation than cyanide, and cyanide and azide inhibit a variety of non-mitochondrial enzymes, so it is difficult to conclude anything quantitative from this study. Duranteau et al. (1998) showed that in cardiomyocytes incubated with H_2DCF, hypoxia induced an increase in H_2DCF oxidation, which was partially inhibited by adding both rotenone (to inhibit complex I) and thenoyltri-fluoroacetone (TTFA, to inhibit complex II), suggesting that the hypoxia-induced H_2DCF oxidation was partially mediated by ROS from complex III. However, the effects of rotenone or TTFA alone were not shown in this study and the specificity of TTFA is unclear. Vanden Hoek et al (1997) showed that in cardiomyocytes incubated with H_2DCF, the addition of rotenone, antimycin or cyanide induced an increase in H_2DCF oxidation, but again this is difficult to interpret. All these studies with H_2DCF are now in doubt, because the nature of the cellular catalyst is unclear, and studies with isolated mitochondria have shown that hypoxia does not increase ROS production (Starkov 2008; Wardman 2007). Cytochrome *c* can catalyze H_2DCF oxidation (Burkitt and Wardman 2001), which complicates interpretation when cytochrome *c* changes redox state during hypoxia or respiratory inhibition, and cytochrome *c* is released from mitochondria during cell death. And there is the potential in all these studies, for mitochondrial respiratory inhibition to affect other cellular sources of ROS, e.g. by changes in ATP or NADH, and consequent changes in glycolysis and pH. The interpretation of the effects of rotenone on cellular ROS is further complicated by the finding that it stimulates ROS production of isolated mitochondria when respiring on NAD-linked substrates, but inhibits when respiring on succinate.

Lee and colleagues (Lee et al. 2006) demonstrated (using H_2DCF and mitochondrial ROS marker CMXROS) that in certain conditions (serum withdrawal in cultured 293 T cells) cellular ROS production can be acutely (in 5 min) increased by about 10-folds. This acute phase of ROS production was shown to be partially dependent on mitochondria as a 30% decrease in cellular ROS level was observed when rotenone, an inhibitor of mitochondrial complex I, was added. However, ROS production during later phases (8 hours) was due to NOX1 activation which contributed by about 40% to total cellular ROS production (as detected by silencing of NOX1 with small interfering RNAs). A similar pattern was observed in human keratinocytes exposed to UV or ceramide which caused an increase in intracellular ROS levels as measured with H_2DCF: ROS production was inhibited by ~65-80% with DPI (diphenylene iodonium, a non-selective inhibitor of NADPH oxidase) or by NOX1-siRNR, while cellular ROS production was inhibited only by 15-30% with MitoQ (a mitochondrially-targeted antioxidant) (Valencia and Kochevar 2008). A hypoxia-induced rise of ROS in endothelial cells was found to be almost equally inhibitable by both DPI and mitoQ (Schäfer et al. 2003). These and similar studies (Caraceni et al. 1995) suggest that NOX can be an equal or more significant source of cellular ROS than mitochondria even in non-phagocytic cells. Note however that DPI inhibits most cellular sources of ROS including complex I, and mitoQ does not effectively scavenge superoxide or hydrogen peroxide.

The inhibitor-based method was also used by McNally and colleagues (McNally et al. 2003), who found that shear stress-stimulated ROS production in endothelial cells was not inhibitable by miconazole, rotenone or apocynin (thus excluding cytochrome *P*-450, mitochondrial electron transport chain and NOX as sources of ROS). In contrast, oxypurinol, an inhibitor of xanthine oxidase, significantly decreased ROS levels, leading to the conclusion that the main source of shear stress-induced ROS production was xanthine oxidase.

6. Sources, sinks and gradients

Mitochondria can scavenge cellular superoxide (via intermembrane Cu,Zn-SOD and cytochrome *c*, and matrix Mn-SOD) and H_2O_2 (via intermembrane catalase and matrix peroxidases), and thus have a high capacity to act as cellular sinks for ROS generated outside the mitochondria (Starkov 2008). Rates of extra-mitochondrial H_2O_2 consumption by brain mitochondria have been measured to vary between 0.3 nmol/min/mg for de-energized mitochondria to 6.7 nmol/min/mg for mitochondria respiring on glutamate and malate (Zoccarato et al. 2004) and 8.3 nmol/min/mg for mitochondria respiring on succinate (Drechsel and Patel 2010). Note that these rates of H_2O_2 consumption by energised mitochondria were several times the maximal rates of H_2O_2 production. Moreover, in liver mitochondria the rates of exogenous H_2O_2 removal were found to be much higher than in brain, reaching 43–49 nmol/min/mg and were independent of respiratory substrate (Drechsel and Patel 2010). Thus mitochondria have the capacity to act as net sinks for cellular H_2O_2. Mitochondria can also rapidly consume extra-mitochondrial superoxide, and can even respire significantly on superoxide via cytochrome *c* and cytochrome oxidase (Mailer 1990). However, whether mitochondria act as net sources or sinks of ROS in cells is unknown, and this will determine whether there are gradients of superoxide and hydrogen peroxide to or from mitochondria within the cell. ROS probes targeted to mitochondria have been used to estimate mitochondrial ROS production, but if cellular ROS can enter mitochondria, these targeted probes may also be measuring non-mitochondrial ROS.

7. Conclusions

There is no convincing evidence that mitochondria are the main source of ROS in mammalian cells. What evidence there is suggests mitochondria are a significant source and sink for ROS, but not the main source at least in liver. In most cells and conditions there is a lack of evidence for or against mitochondria being the main source of cellular ROS.

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