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Commentary
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Intracellular generation of reactive oxygen species by mitochondria

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Abstract

Mitochondria have bioenergetic properties that strongly suggest their involvement in the cellular formation of reactive oxygen species (ROS). Apparent confirmation of this process has come from work with isolated mitochondria, which have been shown to produce H_2O_2 from dismutating superoxide radicals. Two different sites were reported to shuttle single electrons to oxygen out of the normal respiratory sequence. However, the mechanisms for ROS formation at these two sites are controversial. Arguments against mitochondrial ROS formation in the living cell are based on the fact that bioenergetic alterations may result from the mechanical removal of mitochondria from their natural environment. Furthermore, the invasive detection methods that are generally used may be inappropriate because of the possible interaction of the detection system with mitochondrial constituents. The use of non-invasive detection methods has proved that ROS formation does not occur unless changes in the physical state of the membrane are established. The aim of this commentary is to discuss critically the arguments in favor of mitochondria as the main intracellular source of ROS. The *pros* and *cons* of working with isolated mitochondria, as well as the detection methodology are carefully analyzed to judge whether or not the above assumption is correct. The conclusion that mitochondria are the main ROS generators in the cell contradicts the fact that ROS release was not observed. However, if electron flow from ubiquinol to the bc_1 complex is hindered due to changes in lipid fluidity, single electrons may transfer to dioxygen and produce H_2O_2 via superoxide radicals.

Keywords: Mitochondria; Reactive oxygen species; Superoxide radical; Hydrogen peroxide; Phospholipid membrane

Since the detection of the catalytic function of superoxide dismutase, scientists have focused much interest on the substrate for this enzyme, the superoxide radical anion ($O_2^{\bullet -}$). The physicochemical properties of molecular oxygen require the stepwise transfer of single electrons. If one electron is added to the outer orbital of oxygen, the superoxide radical anion is formed. Since it is clear that the first reduction product of oxygen is the substrate for superoxide dismutase, it is of interest to identify the biological reductant that provides single electrons for one-electron reduction of oxygen. A variety of enzymatic and non-enzymatic biomolecules have been reported to reduce molecular oxygen to superoxide radicals [1–4]. The recognition that reactive oxygen species (ROS) possess cell-signaling functions [5,6] implies that there must be a permanent radical source, which operates under physiological conditions.

More than 90% of total cellular oxygen is reduced to water stepwise via electron carriers of the mitochondrial respiratory chain. Most of the electron carriers involved undergo one-electron redox shuttles and the majority of these electron carriers are thermodynamically capable of reducing oxygen to superoxide radicals (see Scheme 1). In fact isolated mitochondria have been shown to generate $O_2^{\bullet -}$ in the presence of antimycin A, which inhibits regular electron transfer [7]. This observation clearly demonstrates the principle that mitochondria are capable of shuttling electrons to dioxygen. They have, therefore, been considered as the main superoxide radical source in the cell. This assumption, however, is not accepted unequivocally [8,9]. The concept of H_2O_2 generation from $O_2^{\bullet -}$, whose synthesis is induced by antimycin A, was first introduced by Chance and co-workers [2] as a model system for mitochondrial ROS formation. In vivo production of ROS from respiring mitochondria cannot be concluded from these experiments, because antimycin A is a

Abbreviations: ROS, reactive oxygen species; $O_2^{\bullet -}$, superoxide radical anion

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product of a streptomyces species which is not present in mammals [10].

On the other hand, it was also demonstrated by Chance and co-workers [2] that isolated mitochondria also generate $O_2^{\bullet-}$-derived H_2O_2 in the absence of this respiratory inhibitor. The presence of superoxide dismutase in the matrix of mitochondria rapidly converts $O_2^{\bullet-}$ to H_2O_2. Chance and his colleagues observed some H_2O_2 production under conditions of high membrane potential [2], which is established when its energy is not used for ATP synthesis (=state IV respiration) [11]. This concept was adopted by a variety of research groups who reported that a high mitochondrial membrane potential is the trigger of $O_2^{\bullet-}$-derived H_2O_2 release [12–16].

There are also some articles, which provide evidence that a low membrane potential initiates H_2O_2 generation [17]. For example, aging is reported to stimulate H_2O_2 formation while the membrane potential declines [18].

Two different sources of the mitochondrial respiratory chain are considered to contribute to the univalent reduction of dioxygen. The identity of the first site (complex I) was found by following H_2O_2 formation in the presence or absence of various complex I inhibitors. This source was independent of the membrane potential [7,14,19]. This is in contrast to the second site which was only active when the membrane potential was high, for instance, under conditions of state IV respiration [12,14,16]. Kadenbach and Arnold [15] have recently reported that cytochrome oxidase, which is the terminal electron acceptor of the respiratory chain, has various binding centers for ATP. Phosphorylation and dephosphorylation are regulated by hormones [20]; dephosphorylation inhibits energy-linked respiration, which is associated with a dramatic increase of the mitochondrial membrane potential [15]. These authors believe that dephosphorylation of cytochrome oxidase is the trigger for mitochondrial superoxide generation [13]. We have isolated mitochondria according to Ref. [8]. Although we measured an extremely high membrane potential (around 230 mV under state IV respiration) [21], we were unable to observe any ROS formation [8]. Mechanical removal of mitochondria from their natural surroundings in the cell can be expected to affect a variety of bioenergetic parameters that control electron transfer pathways to oxygen. The inevitable contamination of intact mitochondria with mitochondrial fragments may be a source of artifacts because the regular electron transfer is out of control in these fragments. Other problems may arise as a result of the use of inappropriate methods for the detection of $O_2^{\bullet-}$ or H_2O_2. This may also explain conflicting results in the literature, which describe both the capability and the inability of isolated mitochondria to generate ROS.

These drawbacks were subject of some recent reports [8,22]. Unfortunately, there is no clear published evidence that proves the transferability of results from in vitro experiments with isolated mitochondria to the behaviour of mitochondria in the living cell. However, these data describe conditions under which mitochondria become a source of reactive oxygen species [23,24] and thus may also be valid for mitochondrial respiration in the intact cell.

1. Conditions triggering H_2O_2 formation in isolated mitochondria

The first report on H_2O_2 generation as by-product of regular respiration was published around 30 years ago by Chance and co-workers [2] (Table 1). They found that in the absence of ADP, mitochondria release H_2O_2 that is derived from superoxide radicals. The lack of ADP in isolated mitochondria preserves the membrane potential at the highest level. When ADP is added, the membrane potential is used for ATP synthesis and H_2O_2 generation is suppressed. This observation inspired other scientists to check whether or not the membrane potential is essential for mitochondrial H_2O_2 formation. They found that the membrane potential is required for reversed electron flow from succinate, which supplies reducing equivalents via complex II to complex I [14]. H_2O_2 was not observed when the reversed electron flow to complex I was inhibited by the presence of rotenone. The electron donor that reduces oxygen to superoxide radicals was, therefore,

Table 1
Mitochondrial H_2O_2 formation

Conditions	H_2O_2 increase		References
	Complex I	Complex III	
State IV respiration	–	↑	Boveris et al. [2]
Increase of membrane potential	↑	↑	Korshunov et al. [36]
Reversed electron flow	↑	↑	Liu [16]
Proton-motive force	↑	↑	Korshunov et al. [12]
Ethanol	↑	–	Bailey et al. [37]
Arachidonic acid (uncoupling)	↑	↑	Cocco et al. [24]
Aging	↑	↑	Herrero and Barja [38]
Ischemia/reperfusion	↑	–	Turrens et al. [39]

concluded to be an iron–sulfur center (N2) of complex I [7,19]. According to the earlier observation [2], H_2O_2 formation declined to zero when the membrane potential was used up for ATP synthesis. Since rotenone was not sufficient for total suppression of H_2O_2, it was concluded that a second source of superoxide radicals exists on the oxidant site of complex I. This other reductant of oxygen was found to be a ubisemiquinone radical, which interacts with the Rieske iron–sulfur–protein and cytochrome b of complex III [25]. Both electron carriers are involved in the stepwise oxidation of ubiquinol. Ubisemiquinone was also identified as the superoxide radical source when antimycin A inhibits regular electron flow. This particular redox shuttle is not built in the presence of myxothiazol, which is another inhibitor of the respiratory chain. Thus, myxothiazol is used as a tool to identify the reductant in mitochondria responsible of superoxide radical formation.

2. Efforts to demonstrate mitochondrial ROS formation in cell suspensions

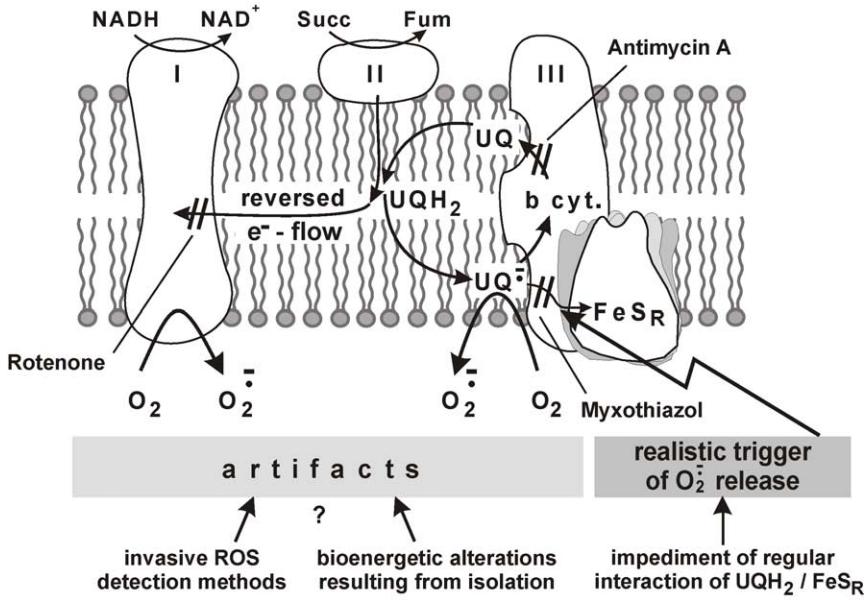
The objection to using isolated mitochondria as a model for intracellular radical formation prompted some researchers to study the ability of mitochondria in suspended cells to reduce oxygen to H_2O_2 via superoxide radicals [26–28]. The measurement of intracellular H_2O_2 is generally performed with 2′7′-dichlorofluorescin, which becomes fluorescent (2′7′-dichlorofluorescein) after enzyme-catalyzed reaction with H_2O_2. Loading of 2′7′-dichlorofluorescin into cells requires the diacetate derivative since the parent compound is impermeable to cell membranes. Endogenous esterases hydrolyze this derivative and release the non-fluorescent 2′7′-dichlorofluorescin. The accumulation procedure needs around 30 min at 37 °C in order to accelerate enzymatic hydrolysis. Detection of the highly fluorescent 2′7′-dichlorofluorescein, which indicates H_2O_2 formation, requires excitation at 490 nm, whilst the emission is measured at 520 nm [26]. However, absorption of light at these wavelengths by cellular constituents cannot be excluded. Furthermore, horseradish peroxidase, which is a plant enzyme, is necessary for the catalytic oxidation of 2′7′-dichlorofluorescin. However, horseradish peroxidase cannot cross the cell wall. Cytochrome c has been reported to catalyze dichlorofluorescin oxidation [29]. In addition, dichlorofluorescin itself has been found to be a source of free radicals [30]. The assignment of mitochondria as the site of H_2O_2 generation is, therefore, uncertain since many other sources in the cell have to be considered. There are also significant extramitochondrial sources of H_2O_2 from a variety of oxidases, such as peroxisomal oxidases [31], plasma membrane NADH oxidases [32] or xanthine oxidase associated with endothelial cells [33]. Thus, the increases or decreases in H_2O_2 levels produced using selective inhibitors are not necessarily associated with mitochondria.

3. Arguments against mitochondrial ROS formation

The main evidence for the in vivo generation of ROS through mitochondria is the observation that under certain conditions isolated mitochondria become a source of H_2O_2 [7]. However, this hydrogen peroxide formation was observed under non-physiological conditions, for instance oxygen tension normally applied in incubation buffers exceeds cellular oxygen tension by a factor of 4 (air-saturated buffer) to 20 (buffer saturated with 100% oxygen). Also substrates supplying mitochondria with reducing equivalents were far beyond physiological concentrations. Furthermore, it is unknown in what direction bioenergetic parameters change when mitochondria are mechanically removed from their natural surrounding in the cell. According to Kadenbach and co-workers the membrane potential of isolated mitochondria drastically increases due to calcium-activated dephosphorylation of cytochrome oxidase [13]. It has also been shown that detection methods that applied routinely to the measurement of $O_2^{\bullet -}$ or H_2O_2 may give false positive or negative results due to an interaction with electron carriers of the respiratory chain [8,34].

4. Selective cellular conditions triggering mitochondrial ROS formation

The presence of ROS-metabolizing enzymes in mitochondria reveals the risk of ROS formation as a result of a single electron deviation from the respiratory chain. This may happen under pathophysiological rather than physiological conditions. For instance, decompartmentalization of calcium during ischemia may activate membrane-destructive enzymes such as phospholipase A_2. It was recently demonstrated that the transfer of reducing equivalents from ubiquinol to the respective oxidants in complex III is extremely sensitive to the nature and physical state of surrounding phospholipids [23,35]. Fluidity changes in any direction result in an impairment of the regular electron flux associated with the loss of single electrons via autoxidation of ubisemiquinone [23]. Fig. 1 summarizes the results from spin labelling experiments, which indicate a decrease in fluidity after loading cholesterol into the inner membrane and an increase in fluidity in the presence of erucic acid. Both affect the flow rate of reducing equivalents from ubiquinol to the Rieske iron–sulfur protein in a way, which accumulates ubisemiquinones over stabilizing binding capacities. As a consequence, ubisemiquinone undergoes autoxidation and gives rise to ROS production. Accumulation of cholesterol in mitochondrial membranes, which may result from hypercholesterolemia, is an example in this respect.



Scheme 1. Critical aspects of mitochondrial ROS-generation. (UQ, ubiquinone; $UQ^{\bullet -}$, ubisemiquinone; Succ, succinate; Fum, fumarate; b cyt., b-cytochromes).

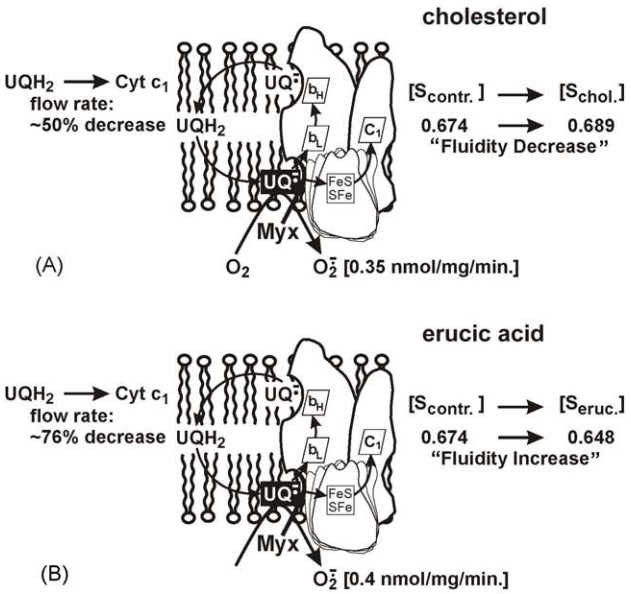


Fig. 1. The effect of physical state alterations in mitochondria following cholesterol (A) and erucic acid (B) insertion. Experimental details are given in [23].

5. Take home scheme

Previous work which claims to prove that ROS generation occurs as a byproduct of regular respiration may in fact show bioenergetic artifacts as a result of working with isolated mitochondria or through the use of invasive detection systems and may not represent mitochondrial activity in the cell. ROS formation occurs when the flow rate of reducing equivalents from ubiquinol (UQH_2) to the Rieske iron–sulfur protein (FeS_R) is impeded (see Scheme 1).

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