Cell respiration and formation of reactive oxygen species: facts and artefacts

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Abstract

It is generally taken as an established fact that mitochondrial respiration is associated with the generation of small amounts of ROS (reactive oxygen species). There are many arguments supporting this side activity. A major argument is the particular physico-chemical configuration of dioxygen, which prevents the transfer of a pair of electrons. Instead, oxygen is reduced by the successive transfer of single electrons, necessarily leading to intermediates with odd electrons. The high rate of turnover of oxygen in the respiratory chain in combination with the existence of single-electron carriers supports the concept of mitochondria as the major cellular ROS generator. Experimental evidence on the ability of mitochondria to generate ROS was, however, based essentially on in vitro experiments with isolated mitochondria. A variety of structural and functional alterations associated with the removal of mitochondria from the cell, as well as the routinely applied ROS detection methods, may lead to artefactual deviation of odd electrons to dioxygen. We therefore checked these correlations in view of ROS formation, including the often reported effect of the membrane potential on the establishment of a redox couple with oxygen out of sequence. For this purpose we developed novel methods to prove the authenticity of mitochondria for ROS generation in the living cell. Based on our experiments, we can exclude spontaneous release of ROS from mitochondria. However, we describe conditions under which mitochondria can be transformed to mild ROS generators. The site of single-electron deviation to dioxygen was found to be ubiquinol interacting with the Rieske iron-sulphur protein and low-potential cytochrome b of the bc₁ complex.

Introduction

Following the detection of superoxide dismutase as a constituent of all aerobic organisms, it became clear that superoxide radicals (O₂•−) are formed in living systems. The bioenergetic properties of mitochondria in combination with their high rate of turnover of O₂ qualify these organelles as an active source of ROS (reactive oxygen species). The assumption that mitochondria are the major intracellular source of ROS was, however, based essentially on in vitro experiments with isolated mitochondria. The application of results obtained in isolated mitochondria to the living cell may, however, be incorrect. Artefacts due to the preparation procedure or inadequate detection systems for ROS may lead to false-positive results.

One of the most critical electron transfer steps in the respiratory chain is electron bifurcation from UQH₂ (ubiquinol) to the cytochrome bc₁ complex. Recent studies elucidated that electron bifurcation from UQH₂ to the bc₁ complex requires the free mobility of the head domain of the Rieske iron–sulphur protein. On the other hand, it has long been known that inhibition of electron bifurcation by antimycin A causes leakage of single electrons to O₂, which results in the release of O₂•−. These findings led us to study whether hindrance of the interaction of UQH₂ with the bc₁ complex is the regulator of single-electron diversion to O₂. Impediment of electron bifurcation was observed following alterations of the physical state of membrane phospholipids in which the bc₁ complex is inserted. Irrespective of whether the fluidity of the membrane was elevated or decreased, electron flow rates to the Rieske iron–sulphur protein were drastically decreased. Concomitantly, O₂•− were released from these mitochondria, strongly suggesting the involvement of the UQH₂/bc₁ redox couple in this process.

Results and discussion

We have developed a sensitive method which allows determination of the percentage of fragmented mitochondria adhering to the intact mitochondrial bulk [1] (Figure 1). This is an important issue with regard the transferability of data obtained with isolated mitochondria. In fragmented mitochondria, both bioenergetic parameters and the control of regular electron transfer suggested to trigger O₂•− formation are completely lost.

Whereas intact mitochondria generate ATP from ADP imported via the adenine-nucleotide carrier (which is sensitive to carboxyatractyloside), fragmented mitochondria hydrolyse ATP and release Pᵢ. The percentage of fragmented mitochondrial particles adhering intact mitochondria was determined in the following way. ATP was added to the mitochondrial suspension in the presence of uncouplers (carbonyl cyanide m-chlorophenylhydrazone or 2,4-dinitrophenol)

Key words: ATPase activity, bc₁ complex, glutathione, hydrogen peroxide, mitochondria, superoxide radical.

Abbreviations used: ROS, reactive oxygen species; UQH₂, ubiquinol.

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and Pi was measured. The amount of Pi released by ATPase-catalysed hydrolysis was taken as 100% (control). The same experiment was performed in the presence of carboxyatractyloside or oligomycin, which both prevent ATP hydrolysis, but by different mechanisms. Since none of these inhibitors is effective in fragmented mitochondria, the percentage of the latter was calculated by deduction from the former value.

Depending on the isolation procedure, the amount of damaged mitochondria ranges between 4% and 12%. Mitochondrial preparations used in our experiments contained approx. 4% damaged mitochondria. Using these rather clean preparations, we were unable to observe \( \text{O}_2^{•−} \)-derived \( \text{H}_2\text{O}_2 \) formation under any conditions reported previously to trigger oxygen radical generation. However, \( \text{H}_2\text{O}_2 \) release was clearly seen in antimycin A-inhibited mitochondria. In contrast with earlier studies reported in the literature, we applied a novel non-invasive method for \( \text{H}_2\text{O}_2 \) detection that excluded artefactual results [2]. Although the latter method was 3–6 times more sensitive than the conventional methods, \( \text{H}_2\text{O}_2 \) formation was not observed (unless antimycin A was present).

It was reported previously that \( \text{H}_2\text{O}_2 \) is produced under state IV conditions, where the mitochondrial membrane potential reaches a maximum, whereas the falling membrane potential after the addition of ADP (state III) inhibits \( \text{H}_2\text{O}_2 \) release. Our data show that, irrespective of the bioenergetic state and substrates used, \( \text{H}_2\text{O}_2 \) release did not occur (Table 1). The values represent the amount of \( \text{H}_2\text{O}_2 \) accumulated during 3 min in the extramitochondrial space, which was separated from mitochondria by subsequent centrifugation prior to \( \text{H}_2\text{O}_2 \) determination. The small negative deflection from zero observed under state III conditions can be discounted.

The inability of mitochondria to operate as a permanent ROS source was also confirmed by another experiment in which glutathione ratios (GSH/GSSG) were followed under conditions reported elsewhere to trigger ROS formation. The sensitivity of this method was documented through antimycin A-induced ROS formation (Figure 2). GSH in the mitochondrial matrix can be considered as a sensitive indirect indicator of mitochondrial ROS generation [3]. The presence of matrix-bound superoxide dismutase [4] and catalase [5] reveals that mitochondrial respiration is prepared to cope with ROS in mitochondria. Earlier

### Table 1 | \( \text{H}_2\text{O}_2 \) release from intact rat heart mitochondria determined non-invasively with scopoletin and horseradish peroxidase

<table>
<thead>
<tr>
<th>Conditions</th>
<th>( \text{O}_2^{•−} )-derived ( \text{H}_2\text{O}_2 ) (nmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I substrates (glutamate+malate)</td>
<td></td>
</tr>
<tr>
<td>State IV</td>
<td>0.007 ± 0.017</td>
</tr>
<tr>
<td>State III</td>
<td>−0.051 ± 0.024</td>
</tr>
<tr>
<td>+Antimycin A</td>
<td>1.313 ± 0.403</td>
</tr>
<tr>
<td>Complex II substrate (succinate)</td>
<td></td>
</tr>
<tr>
<td>State IV</td>
<td>0.051 ± 0.062</td>
</tr>
<tr>
<td>State III</td>
<td>0.016 ± 0.006</td>
</tr>
<tr>
<td>+Antimycin A</td>
<td>6.628 ± 1.081</td>
</tr>
</tbody>
</table>
Rat heart mitochondria (0.5 mg of protein/ml) were incubated at 25°C in an air-saturated medium containing 0.3 M sucrose, 20 mM triethanolamine, 1 mM diethylenetriaminepenta-acetic acid, 4 mM Pi (pH 7.4), 0.5 mg/ml BSA and 5 mM glutamate+5 mM malate or 10 mM succinate+2 µg/ml rotenone respectively (control). Other additions were 760 µM ADP, 2 µg/ml antimycin A (AA) or 10 µM H₂O₂. After 5 min the reaction was stopped by the addition of 2 µg/ml myxothiazol and the probes were frozen immediately in liquid nitrogen and stored therein. GSH and GSSG were determined the next day by an HPLC method according to Cereser et al. [11]. Data represent means ± S.E.M. of six independent mitochondrial preparations.

Figure 2 | Oxidation of mitochondrial GSH as an indicator of ROS formation

For more details, see Gille and Nohl [8]. Cyt, cytochrome; Myx, myxothiazol; S, order parameter; contr., control; chol., cholesterol; eruc., erucic acid.

Figure 3 | Effects of physical state alterations in mitochondria following insertion of cholesterol or erucic acid
experiments with mitochondria from senescent rats have shown that changes in the physical state of the mitochondrial inner membrane correlate with the release of ROS [6]. The same observation was made when, instead of decreased membrane fluidity observed as a function of age, the fluidity was increased following incorporation of toluene [7].

Unexpectedly, we found that both an increase and a decrease in the order parameters of phospholipid membranes resulted in myxothiazol-sensitive $\text{O}_2^{**}$ formation (Figure 3). Therefore we focused our interest on the UQH$_2$/bc$_1$ redox couple, where myxothiazol interferes. Flow rates from UQH$_2$ to the first oxidant, the Rieske iron–sulphur protein, were found to be down-regulated when order parameters were both increased (cholesterol accumulation) and decreased (erucic acid insertion) [8]. It is well established that an underanged interaction between UQH$_2$ and the Rieske iron–sulphur protein requires free spatial movement of the latter oscillating between the UQH$_2$ binding domain and cytochrome c$_1$ [9].

Any change in hydrophobic/hydrophilic interactions affects the activity of all respiratory complexes. The UQH$_2$/bc$_1$ redox couple is, however, particularly sensitive to conformational restriction, since this redox pair is the most highly regulated redox couple of all respiratory compounds. This susceptible electron-transfer complex deviates single electrons to dioxygen out of sequence when regular redox exchange is hindered. We have work in progress to support this concept.

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References

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