Superoxide: Production and Destruction

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Using mitochondria-targeted molecules to study mitochondrial radical production and its consequences

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Abstract

The production of ROS (reactive oxygen species) by the mitochondrial respiratory chain contributes to a range of pathologies, including neurodegenerative diseases, ischaemia/reperfusion injury and aging. There are also indications that mitochondrial ROS production plays a role in damage response and signal transduction pathways. To unravel the role of mitochondrial ROS production in these processes, we have developed a range of mitochondria-targeted probe molecules. Covalent attachment of a lipophilic cation leads to their accumulation into mitochondria, driven by the membrane potential. Molecules developed so far include antioxidants designed to intercept mitochondrial ROS and reagents that specifically label mitochondrial thiol proteins. Here we outline how mitochondrial ROS formation and its consequences can be investigated using these probes.

Introduction

During metabolism, electrons from NADH and FADH₂ are passed down the mitochondrial respiratory chain to drive ATP synthesis by oxidative phosphorylation (Figure 1). As the electrons move down the potential energy gradient to oxygen, the redox energy is conserved by pumping protons across the inner membrane to build up a ΔµH⁺ (proton electrochemical potential gradient). This gradient, composed of a substantial Δψ (membrane potential) and a smaller ΔpH (pH gradient), is used by the ATP synthase to make ATP.

The mitochondrial respiratory chain is also the major site of ROS (reactive oxygen species) generation within the cell. Superoxide is a by-product of normal respiration through the one-electron reduction of molecular oxygen (Figure 1) [1,2], and it damages iron–sulphur-centre-containing enzymes such as aconitase [3]. Superoxide can also react with nitric oxide to form the damaging oxidant peroxynitrite [4], and this is likely to be significant in vivo, as nitric oxide diffuses easily into mitochondria and may also be produced there [5–7]. The mitochondrial enzyme MnSOD (manganese superoxide dismutase) converts superoxide into hydrogen peroxide, which in the presence of ferrous or cuprous ions forms the highly reactive hydroxyl radical that damages all classes of biomolecule. The availability of free iron and copper within mitochondria is uncertain, although the reaction of superoxide with the iron–sulphur centre in aconitase releases ferrous iron [3]. Consequently, mitochondrial superoxide production initiates a range of damaging reactions through the formation of superoxide, hydrogen peroxide, ferrous iron, hydroxyl radical and peroxynitrite, all of which can damage lipids, proteins and nucleic acids [8]. The proximity of ROS generation makes mitochondrial function particularly susceptible to...
Mitochondrial radical production and oxidative damage

The mitochondrial respiratory chain (top) catalyses electron movement from NADH and FADH₂ to oxygen. This leads to the pumping of protons across the mitochondrial inner membrane to establish a ΔΨm (negative inside); only the ΔΨm component of ΔµH⁺ is shown. ΔµH⁺ is used to drive ATP synthesis by the F₁F₀-ATP synthase. The exchange of ATP and ADP across the inner membrane is catalysed by the adenine nucleotide transporter (ANT), and the movement of Pᵢ is catalysed by the phosphate carrier (PC; top left). The respiratory chain also produces superoxide (O₂•⁻), which can damage iron-sulphur proteins such as aconitase, thereby ejecting ferrous iron. Superoxide also reacts with nitric oxide (NO) to form peroxynitrite (ONOO⁻). In the presence of ferrous iron, hydrogen peroxide forms the very reactive hydroxyl radical (•OH).

Both peroxynitrite and hydroxyl radical can cause extensive oxidative damage, including lipid peroxidation (bottom right). The defences against oxidative damage (bottom left) include MnSOD, and the hydrogen peroxide it produces is degraded by glutathione peroxidase (GPX) and peroxiredoxin III (PrxIII). Glutathione (GSH) is regenerated from glutathione disulphide (GSSG) by the action of glutathione reductase (GR), and the NADPH for this is supplied in part by a transhydrogenase (TH). Lipid peroxidation is prevented by the action of vitamin E and coenzyme Q within the phospholipid bilayer.

Mitochondrial antioxidant defences

Mitochondria have a range of defences against oxidative damage (Figure 1). The antioxidant enzyme MnSOD converts superoxide into hydrogen peroxide [9]. The mitochondrial isoform of glutathione peroxidase and the thioredoxin-dependent enzyme peroxiredoxin III both detoxify hydrogen peroxide [10]; alternatively, hydrogen peroxide can diffuse from the mitochondria into the cytoplasm, where it may act as a redox signal [11,12]. The mitochondrial glutathione pool is distinct from that in the cytosol and is maintained in its reduced state by a mitochondrial isoform of glutathione reductase [10]. This enzyme requires NADPH, which is produced within mitochondria by the NADP⁺-dependent isocitrate dehydrogenase and through a ΔµH⁺-dependent transhydrogenase [10]. Within the mitochondrial phospholipid bilayer, the fat-soluble antioxidants vitamin E and coenzyme Q prevent lipid peroxidation, while coenzyme Q also recycles vitamin E and is itself regenerated by the respiratory chain [13]. The mitochondrial isoform of phospholipid hydroperoxide glutathione peroxidase degrades lipid peroxides within the mitochondrial inner membrane [10].

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Sources of mitochondrial ROS

That mitochondrial ROS production occurs *in vivo* is suggested by the fact that mice lacking MnSOD die within a few days of birth [14], while those lacking the cytosolic isoform, CuZnSOD, survive [15]. Further evidence of mitochondrial ROS production under normal conditions includes the efflux of hydrogen peroxide from intact mitochondria and from perfused organs, suggesting that mitochondria produce superoxide *in vivo*, which is then converted into hydrogen peroxide [2]. There is also evidence that, under certain conditions, mitochondrial DNA and protein accumulate greater oxidative damage *in vivo* than the rest of the cell [16].

Complex III produces large amounts of superoxide when inhibited by antimycin, which stabilizes a ubiquinone radical at ubiquinol binding site o [1]. This ubiquinone radical transfers an electron to oxygen to form superoxide on the outside of the mitochondrial inner membrane [1,17]. Complex I produces superoxide from NADH when it is inhibited by rotenone, and the mechanism is Δψ-independent [18,19]. Complex I also generates superoxide from ubiquinol when there is a sufficiently large Δψ to drive reverse electron transport through Complex I in intact mitochondria [17,20]. In this case superoxide is produced on the matrix side of the inner membrane, and its generation is inhibited by rotenone or an uncoupler [17]. The maximum rate of superoxide production by antimycin-inhibited complex III is generally greater than that by complex I, which has led some to assume that the situation *in vivo* is similar. However, in the absence of antimycin, superoxide production by complex III is minimal [17], and it seems probable that *in vivo* complex I is the major source of superoxide through reverse electron transport [17,18,20], and possibly also from forward electron transport [19].

In addition to respiratory complexes, many other enzymes associated with mitochondria can also produce superoxide or hydrogen peroxide under certain conditions, so the current tacit assumption that only complexes I and III produce significant amounts of ROS may have to be reassessed. Even so, some conclusions about ROS formation by the respiratory chain are possible [17]. Mitochondrial ROS production is increased by high levels of reduced respiratory electron carriers, particularly the coenzyme Q pool, and by a large Δψ. These conditions favour superoxide production from complex I by enhancing reverse electron transport, and may also act by increasing the lifetime of the ubiquinone radical at the o site of complex III. Furthermore, as the rates of the non-enzymic reactions of oxygen with radical intermediates to form superoxide are proportional to the local oxygen concentration, an elevated oxygen concentration will also promote superoxide production [21]. All of the conditions that increase superoxide production occur when mitochondria are oxidizing respiratory substrates but not making ATP (state 4). In contrast, when ATP is being formed by mitochondria (state 3), the lower Δψ, increased oxidation of electron carrier pools and decreased local oxygen concentration will decrease superoxide production.

**Targeting antioxidants to mitochondria**

To deliver antioxidants selectively to mitochondria, we covalently attach them to the triphenylphosphonium cation through an alkyl chain (Figure 2) [23,24]. The delocalized
positive charge of these lipophilic cations enables them to permeate lipid bilayers easily and to accumulate several-hundred-fold within mitochondria, due to the large mitochondrial membrane potential ($\Delta \psi_{m}$; $-150$ to $-170 \text{ mV}$, negative inside; Figure 2). The plasma membrane potential ($\Delta \psi_{p}$; $-30$ to $-60 \text{ mV}$, negative inside; Figure 2) also drives their accumulation from the extracellular fluid into cells, from where they are further concentrated within mitochondria.

As the natural antioxidants vitamin E and coenzyme Q are thought to protect mitochondria from oxidative damage in vivo, mitochondria-targeted derivatives of these molecules were developed first [MitoVit E (mitochondria-targeted derivative of $\alpha$-tocopherol) and MitoQ (mitochondria-targeted ubiquinone) respectively; Figure 2]. In addition, we have recently developed MitoPBN, a mitochondria-targeted derivative of the spin trap PBN (phenyl-$t$-butylnitronate) (Figure 2). Experiments in vitro showed that MitoVit E and MitoQ were rapidly and selectively accumulated by isolated mitochondria, and by mitochondria within isolated cells [25–27]. Importantly, the accumulation of these antioxidants by mitochondria protected them from oxidative damage far more effectively than untargeted antioxidants, suggesting that the accumulation of antioxidants within mitochondria does increase their efficacy. Most interestingly, these compounds were several-hundred-fold more effective at preventing cell death in fibroblasts from Friedreich’s ataxia patients than were untargeted antioxidants [28]. As cell death in this disease model is due to endogenous mitochondrial oxidative damage [29], this suggests that it is the accumulation of these antioxidants by mitochondria within cells that makes them far more effective than untargeted antioxidants. Mitochondria-targeted antioxidants have also been shown to modulate the role of mitochondrial ROS production in redox signalling pathways [30,31] and telomere shortening [32].

**Mitochondria-targeted molecules in vivo**

If these mitochondria-targeted molecules are to be used as probes of mitochondrial ROS production in vivo, or to have therapeutic potential, they must be taken up selectively by mitochondria in vivo. As alkyltriphenylphosphonium cations pass easily through lipid bilayers by non-carrier-mediated transport, they should be taken up by the mitochondria of all tissues, in contrast with hydrophilic compounds, which rely on the tissue-specific expression of carriers for uptake [33]. To test this, mice were fed mitochondria-targeted antioxidants for several weeks, and this led to stable steady-state concentrations within all tissues assessed, including the brain, heart, liver and kidneys [34]. Uptake was reversible, as shown by the rapid clearance of the simple lipophilic cation methyltriphenylphosphonium from all organs when oral administration stopped [34]. That these compounds can enter the bloodstream and distribute to tissues in their intact, active form was shown by solvent extraction of the brain, heart and liver of mice fed MitoVit E, followed by MS [34].

These data are consistent with the following pharmacokinetic model. After absorption from the gut into the bloodstream, orally administered mitochondria-targeted antioxidants are taken up into all tissues by non-mediated movement through the lipid bilayer of the plasma membrane, assisted by $\Delta \psi_{p}$. From the cytosol, most of the lipophilic cations are taken up into mitochondria, driven by the large $\Delta \psi$. After several days’ feeding, the cation concentration within mitochondria comes to a steady-state distribution with circulating blood levels. At this point the mitochondrial concentration will be several-hundred-fold higher than that in the bloodstream. As the mitochondrial pool of compound is in dynamic equilibrium, once feeding stops the accumulated cations will re-equilibrate back into the bloodstream and be excreted relatively rapidly. Work is ongoing to see if these targeted antioxidants may be of use in preventing oxidative damage to mitochondria in mouse models of mitochondrial oxidative damage.

**Targeting thiol probes to mitochondria**

Changes to the redox state of mitochondrial thiol proteins are likely to be of significance in the response of mitochondria to oxidative stress [10,35]. To explore this possibility, we developed mitochondria-targeted thiol reagents which comprise the triphenylphosphonium cation attached to a thiol-reactive moiety [36]. These compounds, 4-thiobutytriphenylphosphonium [37,38] and 4-iodobutyltriphenylphosphonium [39], bind selectively to mitochondrial thiol proteins, enabling their detection using antiserum against triphenylphosphonium [39]. This labelling is dramatically affected by redox alterations to thiols, enabling redox-active thiol proteins to be assessed [39]. So far this procedure has been used to localize redox-active thiols on complex I [38,39].

**Conclusion**

Although the development of mitochondria-targeted reagents is at an early stage, they have already proven to be useful tools in manipulating mitochondrial ROS production in isolated mitochondria and in cells. Preliminary data suggest that it may also be possible to extend this approach to in vivo situations. However, much still needs to be determined about the basic chemistry and interactions with mitochondria of the small number of probes developed to date. In addition, a wide range of more specific antioxidants and ROS-reactive probes is under development. The hope is that the use of these molecules will help extend current work on elucidating the nature and significance of particular ROS in mitochondrial oxidative damage and redox signalling.

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References


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