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Review article

Mitochondrial generation of superoxide and hydrogen peroxide as the source of mitochondrial redox signaling

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ABSTRACT

This review examines the generation of reactive oxygen species by mammalian mitochondria, and the status of different sites of production in redox signaling and pathology. Eleven distinct mitochondrial sites associated with substrate oxidation and oxidative phosphorylation leak electrons to oxygen to produce superoxide or hydrogen peroxide: oxoacid dehydrogenase complexes that feed electrons to NAD^+; respiratory complexes I and III, and dehydrogenases, including complex II, that use ubiquinone as acceptor. The topologies, capacities, and substrate dependences of each site have recently clarified. Complex III and mitochondrial glycerol 3-phosphate dehydrogenase generate superoxide to the external side of the mitochondrial inner membrane as well as the matrix, the other sites generate superoxide and/or hydrogen peroxide exclusively in the matrix. These different site-specific topologies are important for redox signaling. The net rate of superoxide or hydrogen peroxide generation depends on the substrates present and the antioxidant systems active in the matrix and cytosol. The rate at each site can now be measured in complex substrate mixtures. In skeletal muscle mitochondria in media mimicking muscle cytosol at rest, four sites dominate, two in complex I and one each in complexes II and III. Specific suppressors of two sites have been identified, the outer ubiquinone-binding site in complex III (site III_{Qo}) and the site in complex I active during reverse electron transport (site I_Q). These suppressors prevent superoxide/hydrogen peroxide production from a specific site without affecting oxidative phosphorylation, making them excellent tools to investigate the status of the sites in redox signaling, and to suppress the sites to prevent pathologies. They allow the cellular roles of mitochondrial superoxide/hydrogen peroxide production to be investigated without catastrophic confounding bioenergetic effects. They show that sites III_{Qo} and I_Q are active in cells and have important roles in redox signaling (e.g. hypoxic signaling and ER-stress) and in causing oxidative damage in a variety of biological contexts.

1. Introduction

Superoxide and hydrogen peroxide formation by isolated mitochondria were first recognized and explored in the 1970s [1–5]. Mitochondrial production of these species and their various roles in signaling and disease have been studied in depth ever since [6–33]. We now know that mammalian mitochondria can generate superoxide and/or hydrogen peroxide from at least 11 different sites associated with substrate catabolism and the electron transport chain, and that these different sites have very distinct properties. Superoxide and/or hydrogen peroxide can be generated from the major sites at high rates both in the mitochondrial matrix and on the cytosolic side of the mitochondrial inner membrane. Mitochondrial and cytosolic scavenging of superoxide and hydrogen peroxide are also very powerful, so four sets of dynamic balances between production and consumption regulate the levels of superoxide and hydrogen peroxide in the mitochondrial matrix and the cytosol. These balances allow small changes in production or consumption to alter steady-state levels of superoxide and hydrogen peroxide in each compartment and initiate different cellular signaling pathways. When levels of superoxide and hydrogen peroxide rise too high, the molecular damage they cause becomes too great for the cell to repair, triggering a range of different pathologies.

In this review I discuss the different mitochondrial sites of superoxide and hydrogen peroxide production, including their topologies, properties and capacities; highlight recent studies that show their absolute and relative importance *ex vivo*; describe selective suppressors of two major sites than can be used to probe the status of the sites in cells and tissues, and comment on the status of the mitochondrial sites in redox signaling and pathology.

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2. Sources and sinks of mitochondrial superoxide and hydrogen peroxide

Electrons can leak prematurely to oxygen from electron carriers associated with substrate catabolism and the mitochondrial electron transport chain. If they leak singly they generate superoxide radical; if they leak in pairs they generate hydrogen peroxide (and if they are transferred effectively four at a time, as in complex IV, they harmlessly form water and drive oxidative phosphorylation). The rate at which a particular mitochondrial site leaks electrons to oxygen depends on the concentration of the reduced electron donor and the rate constant for the leak reaction. Surprisingly, in isolated mitochondria where substrate concentrations are clamped, the rate appears to be effectively independent of oxygen concentration in the physiological range, until it slows at very low oxygen concentrations approaching the K_M of complex IV for oxygen [34,35]. The increase in superoxide production from complex III that signals hypoxia to the HIF-1α signaling system in cells [36] is probably caused not by direct mitochondrial effects of decreased oxygen tension, but by indirectly changed metabolite concentrations that deliver more electrons to the electron transport chain and lead to higher electron leak to oxygen.

The rate of electron leak to generate superoxide or hydrogen peroxide does not depend on respiration rate or the rate of electron flow in the electron transport chain, except insofar as altered flow into or out of a particular site alters the concentration of the reduced electron donor in that site, i.e., its redox state. Thus, inhibitors of electron transport lower respiration rate, but will either raise or lower the rate of superoxide/hydrogen peroxide production at a given site, depending on whether they act predominantly downstream (leading to reduction of the site and increased electron leak) or upstream (leading to oxidation of the site and decreased electron leak). Similarly, increases in respiration rate can cause either an increase in production of superoxide/hydrogen peroxide if the increase in respiration rate is driven by increased substrate supply (so the electron transport chain becomes more reduced), or a decrease if it is driven by increased ATP demand or proton leak (so the electron transport chain becomes more oxidized). For this reason, knowledge of the percentage of electron flow that is diverted to superoxide/hydrogen peroxide is not particularly useful, although it is often highlighted in the literature. The value in isolated mitochondria is not 1–2% as frequently reported, see [37,38]. With low concentrations of conventional substrates [39–41] or in a medium mimicking the substrate concentrations in the cytosol of skeletal muscle at rest [42] about 0.1–0.5% of the electrons passing down the respiratory chain leak prematurely to oxygen. In medium mimicking muscle exercise, where faster respiration is driven by a mixture of increased substrate supply and increased ATP demand and the rate of superoxide/hydrogen peroxide production diminishes while the rate of respiration increases, this value is an order of magnitude lower, about 0.01–0.03% [42]. Similarly, the rate of electron leak does not depend directly on the mitochondrial membrane potential or protonmotive force, except insofar as altered protonmotive force will affect electron transport and alter the concentration of the reduced electron donor in a site. Thus, inhibitors of electron transport can either raise or lower the rate of superoxide/hydrogen peroxide production, depending on whether they act predominantly upstream or downstream of a site, even though they lower protonmotive force in either case.

For these reasons, unspecified “mitochondrial dysfunction” may either raise or lower the rate of mitochondrial superoxide/hydrogen peroxide production and the levels of superoxide and hydrogen peroxide, depending on the cause of the dysfunction, and no general statement that mitochondrial dysfunction leads to increased superoxide/hydrogen peroxide production or levels can be made [43]. If the dysfunction involves inhibition of electron transport at a particular site it will lead to reduction of upstream sites in the electron transport chain and increased superoxide/hydrogen peroxide production by those sites, and oxidation and decreased production at downstream sites. Depending on the sites active in the non-dysfunctional control, the net effect of such an inhibition may be an increase or a decrease in total superoxide/hydrogen peroxide production rate. If the dysfunction uncouples oxidative phosphorylation, all sites will tend to become oxidized, decreasing total production rate. However, if dysfunction also decreases the glutathione reduction level in the mitochondrial matrix, antioxidant defenses may be compromised, and net levels of superoxide and hydrogen peroxide may rise. It is best not to assume that mitochondrial dysfunction generally leads to increased levels of superoxide and hydrogen peroxide, or to oxidative stress. Instead, a straightforward bioenergetic analysis of the redox levels in different parts of the electron transport chain will allow better-quality predictions to be made for any particular mitochondrial dysfunction.

There are antioxidant defense systems in both mitochondria and cytosol that consume superoxide or hydrogen peroxide, and may be just as important as production rate in setting the steady state or transient levels of these species in the two compartments [15,17,21,30,44]. Under some conditions in which hydrogen peroxide is produced in the cytosol, by NADPH oxidases for example, mitochondria may become net consumers rather than producers [45]. The focus in the present review is on mitochondrial production of superoxide and hydrogen peroxide, so it is often appropriate to prevent or correct for the activity of the antioxidant defenses to allow better comparisons between sites with different topologies (and therefore working against defenses in different compartments of the cell), or between different conditions in which differences in the strength of the antioxidant defenses are a potential confounder. When measuring external hydrogen peroxide production by isolated mitochondria, manganese-dependent superoxide dismutase (MnSOD) converts superoxide essentially quantitatively to hydrogen peroxide in the mitochondrial matrix, and the greatest losses in the overall signal caused by antioxidant defenses occur through glutathione-dependent hydrogen peroxide-degrading processes in the matrix catalyzed by glutathione peroxidases and the peroxiredoxin and thioredoxin systems. These processes can be greatly attenuated by depleting matrix glutathione before assay by pretreatment of the mitochondria with 1-chloro-2,4-dinitrobenzene (CDNB) [46–48] (which may [49] or may not [50] also inhibit thioredoxin and thereby alter peroxide degradation by peroxiredoxin 3), or by mathematical correction using empirical equations to correct rates in non-pretreated mitochondria to those that would be observed after treatment [42,46–48,51–56]. Such treatment obviously disturbs the redox environment of the matrix and could mask redox control of particular sites, however, hydrogen peroxide production from all matrix-facing sites responds similarly to CDNB pretreatment (but not to acute treatment) [46], suggesting that such redox control can often be disregarded. Overall, the quantitative benefits of correction for matrix peroxidase activity outweigh the potential disadvantages in many cases, and such correction has been applied to the analysis of different sites reviewed below. Of course, once the characteristics of the producers have been worked out, it is essential to consider the consumers as well to get a full picture of the strength of mitochondrial redox signaling through transient and steady-state superoxide and hydrogen peroxide levels in the matrix and cytosol.

3. The capacities and properties of the 11 characterized sites of mitochondrial superoxide and hydrogen peroxide production

To date, 11 sites of production of superoxide and/or hydrogen peroxide linked to substrate catabolism, electron transport and