Review:

Using Isolated Mitochondria to Investigate Mitochondrial Hydrogen Peroxide Metabolism

Jason R. Treberg

Department of Biological Sciences,

University of Manitoba

Winnipeg, MB, R3T 2N2

Canada

Email: jason.treberg@umanitoba.ca

Phone: 204 474 8122

Abstract

Mitochondria are recognized as centrally important to cellular reactive oxygen species (ROS), both as a potential source and due to their substantial antioxidant capacity. While much of the initial ROS formed by mitochondria is superoxide, this is rapidly converted to hydrogen peroxide (H_2O_2) which more readily crosses membranes making H_2O_2 important in both redox signalling mechanisms and conditions of oxidative stress. Here I outline our studies on mitochondrial H_2O_2 metabolism with a focus on some of the challenges and strategies involved with developing an integrated model of mitochondria being intrinsic regulators of H_2O_2. This view of mitochondria as regulators of H_2O_2 goes beyond the simpler contention of them being net producers or consumers. Moreover, the integration of both consumption and production can then be tied to a putative mechanism linking energy sensing at the level of the mitochondrial protonmotive force. This mechanism would provide a means of mitochondria communicating their energetic status the extramitochondrial compartment via local H_2O_2 concentrations. I conclude by explaining how these concepts developed using rodent muscle as a model have high relevance and applicability to comparative studies.

Key words: reactive oxygen species (ROS), CDNB, auranofin, peroxidase

1. Introduction

This paper illustrates how in vitro experiments were used to describe a role for mitochondria in cellular reactive oxygen species (ROS) that is not often articulated; mitochondria have the capacity to be intrinsic regulators of H_2O_2 concentrations. This idea of mitochondria as regulators of H_2O_2 was derived using isolated mitochondria, which are an excellent example of a reductionist technique with both strengths and weaknesses as a model system. My strategy is to include both a primer on the use of mitochondrial preparations as well as specific examples of issues we faced while developing techniques to address H_2O_2 metabolism by mitochondria. I will expand with some specific examples that we encountered because these lessons are often not shared outside laboratory or collaborative research groups. My intention is to use these details to help demystify the process of problem-solving with assay design and development. However, before getting into those details, some background is needed for context.

1.1. Reductionist approaches can be valuable to explore complex systems

One reason the reductionist approach is appealing is that a simplified system, that retains fundamental mechanisms of interest, can be more readily manipulated or controlled than the more complex whole (Treberg et al., 2020). In the case of mitochondria, a reduced system can be used for direct tests of function not plausible with intact cells. An end goal of using these reductionist approaches is to develop generalizable mechanisms that can then be applied to more complex systems. I choose this particular work because through these reductionist mitochondrial experiments we have developed rationale that can now inform studies using more complex systems.

1.2. Why hydrogen peroxide?

Hydrogen peroxide is a ROS of central importance to cellular signalling and a prominent intermediate in cellular redox regulation (reviewed in Sies and Jones, 2020). There are important differences in the expected concentration of H_2O_2 depending on the tissue/cell type (reviewed in Sies, 2017) and substantial cellular level concentration gradients, with most evidence suggesting a large inwardly directed gradient from the extracellular fluid to the intracellular space (Fig. 1). Hydrogen peroxide is a small molecule with no net charge and can diffuse across membranes, either by passive diffusion through the phospholipid bilayer or perhaps more importantly by facilitated diffusion via aquaporins (reviewed in Bienert and Chaumont, 2014). This membrane permeability allows H_2O_2 to act as a signal that can traverse cellular compartments. While it is possible that H_2O_2 may act directly as a means of initiating redox sensitive molecular switches (Sies and Jones, 2020), the concentrations needed for this are unphysiologically high. Additionally, the reaction of H_2O_2 with proteins does not fully fit the criteria that might be expected for reversible redox regulation of protein activity (for instance, see Shelton et al., 2005). Therefore, it should also be considered that H_2O_2 can act via a more subtle effect on the local redox active metabolites, such as the glutathione pool, which can be a sensitive regulatory mechanism for cellular communication via protein S-glutathionylation and regulated further by the glutaredoxin system (Mailloux and Treberg, 2016).

1.3. Mitochondrial H_2O_2 metabolism: general overview

Hydrogen peroxide is a central component of mitochondrial ROS metabolism, with mitochondria being identified as potential sources of cellular H_2O_2 for many decades (Jensen, 1966; Boveris et al., 1972). Many mitochondrial enzyme complexes have electron exchange sites where, under the right circumstances, oxygen may have access to electron exchange sites. If oxygen can access the site of electron exchange then electrons can leak to oxygen 'prematurely' rather than being transferred within the protein or to an electron carrier (Andreyev et al., 2005; Brand, 2016; Treberg et al., 2018b). What controls the rate of ROS formation at any specific 'site' of electron leak to oxygen can be a mixture of factors that may be unique to the particular site; however, in the simplest form the hypothesis is that increasing the electron availability at a site of electron leak, either by high supply of electrons or inhibited flow of electrons out of the site, will increase the rate of electron leak.

While superoxide is the primary ROS formed by many major sites of mitochondrial electron leak (reviewed in Brand, 2016), superoxide has a pKa of about 4.9, meaning superoxide is predominantly in the charged form at physiological pH for most animal cellular compartments. The charged nature and short half-life within cells makes superoxide a less likely means of signalling from the intramitochondrial space to the rest of the cell, with the exception of superoxide from complex III which does reach the intermembrane space (Muller et al., 2004; Bleier et al., 2015; Pagacz et al., 2020). The superoxide formed is rapidly dismutated to H_2O_2 either spontaneously or by superoxide dismutases (SOD) and the vast majority of mitochondrially produced superoxide and H_2O_2 is directed towards the matrix (Fig. 2). While superoxide anions do not readily cross membranes, H_2O_2 can transit from the mitochondrial matrix to the extramitochondrial space and vice-versa, making H_2O_2 an important intermediate in how mitochondria may influence cellular redox imbalances and oxidative stress. This bidirectional movement is one reason why mitochondrial H_2O_2 metabolism should be considered in the context of an integrated whole.

One way to begin assessing mitochondrial ROS metabolism as a whole is to examine how mitochondrial energetics controls electron leak. The earliest demonstration, to my knowledge, that electron leak increases as a function of the protonmotive force (or membrane potential) was before 1997 (reviewed in Lui 1997). As shown for several substrate combinations, as long as electron supply is not limiting, high protonmotive force or high mitochondrial membrane potential, which is often use as a proxy for the protonmotive force, leads to higher rates of electron leak (Korshunov et al., 1997; Lui 1997; Starkov and Fiskum, 2003; Starkov et al., 2004). As the protonmotive force increases, there tends to be more electrons residing in the electron carrier pools (NAD, cytochromes, ubiquinone) and a more reduced state is maintained at the redox centres (for example see Quinlan et al. 2011; Treberg et al. 2011; Quinlan et al., 2012a; 2012b; 2013) where electrons can leak to oxygen before reaching complex IV (Fig. 3). This interrelationship between the protonmotive force and rates of electron leak is why increased ATP turnover in cells is expected to decrease the amount of ROS formed. Increasing ATP demand leads to increasing ADP availability, which decreases the mitochondrial protonmotive force while increasing electron flux through the ETS. As such, rather than the idea that increased energy demand in the cell will necessarily come with a burden of increased ROS exposure,

elevated ATP demand would normally fit a decline in electron leak in mitochondria. Moreover, the link between electron leak rates and the protonmotive force can be further demonstrated because using uncouplers yields a relationship similar, although not always identical, to that found with the addition of ADP (for example see Korshunov et al., 1997, Treberg et al., 2019). Thus, in general the protonmotive force is a major determinant of the mitochondrial electron leak that leads to producing H_2O_2.

Along with the many sources of electron leak there are also multiple candidate sinks for H_2O_2 in mitochondria (Fig. 2). The NADPH-dependent glutathione (GSH) peroxidase and thioredoxin-dependent peroxiredoxins along with catalase are the most prominent in the mitochondrial matrix (reviewed in Andreyev et al., 2005). Matrix level consumers are of particular importance because the matrix is where most of the mitochondrial H_2O_2 production also takes place. In the systems we study, striated muscle in vertebrates, catalase seems to be a minor contributor (Phung et al., 1994; Munro et al., 2016) and will not be elaborated on in this section.

The NADPH-dependent systems of H_2O_2 consumption rely on a supply of electrons from carbon substrates, much like respiration and oxidative phosphorylation (Figs. 2, 3). The divergence of function between nicotinamide cofactors in the mitochondrial matrix is important to appreciate. In this context, NADPH in the matrix is predominantly for antioxidant purposes while NADH is a major electron supply for respiration and thus ROS formation (Fig 3). To emphasize that point, consider mitochondrial nicotinamide nucleotide transhydrogenase (NNT), which maintains the NADP-pool of cofactors disproportionately reduced at the cost of NADH and dissipation of the protonmotive force (Hoek and Rydström, 1988; Jackson, 2003). Here NNT is a clear case where natural selection has led to a functional link between maintaining the peroxidase systems active by coupling NADPH formation to respiratory substrate supply and generation of the protonmotive force, the latter also being a contributor to mitochondria needing an antioxidant system in the first place. To this end, it is remarkable that a very poorly oxidized substrate like malate can energize mitochondria sufficiently to maintain very high H_2O_2 consumption capacity (Banh and Treberg, 2013), almost as though the antioxidant system is primed in advance of the mitochondrion reaching a state where high rates of ROS formation are likely.

1.4. The source or sink argument

The earliest demonstration that energized mitochondria can consume extramitochondrial H_2O_2 that I am aware of was a study of monamine oxidase in brain mitochondria (Sandri et al., 1990); however, as early as 2003 the mitochondrial capacity to be a net source, or sink, of ROS via H_2O_2 has been debated (Zoccarato et al., 2003). Specifically, Zoccarato et al. (2003) concluded that brain mitochondria, "behave as an intracellular Ca^{2+}-controlled peroxide sink". One very strong point in favour of the net sink argument is that most energized mitochondria have vastly higher capacity to consume H_2O_2 than they have capacity to produce it (for example Zoccarato et al., 2003: Drechsel and Patel, 2010; Munro et al., 2016; Kamunde et al., 2018). But that greater capacity to consume H_2O_2 is substrate limited, with rates typically not showing saturation kinetics for H_2O_2 even at micromolar concentrations (Drechsel and Patel, 2010; Banh and Treberg, 2013; Munro et al., 2016). The low affinity of these scavenging systems makes using micromolar concentrations of H_2O_2 problematic to resolving if mitochondria are net sources or

sink because these concentrations are quite superphysiological for the intracellular compartment (Sies, 2017). If the antioxidant systems are high-capacity but with too low affinity for H_2O_2 then it is possible the mitochondria are de facto net producers for the cell regardless of the maximal capacity to consume this ROS.

While the source versus sink debate has been an important challenge to the conventional knowledge that mitochondria 'are the major source of ROS for the cell' (Brown and Borutaite, 2012), it also assumes a false dichotomy of either one or the other role. That mitochondria may act as intrinsic regulators of H_2O_2 concentrations is an important perspective to address because of the rather disparate roles H_2O_2 has which range from regular physiological cellular signalling to pathophysiological mitochondrial dysfunction (reviewed in Sies, 2017; Sies and Jones, 2020). In the following sections I will outline several general aspects regarding mitochondrial studies and segue to how we transitioned from trying to address the source/sink argument to developing our currently proposed model linking mitochondrial energy sensing via communicating the internal state (the protonmotive force) to the extramitochondrial space with H_2O_2 as a signalling molecule.

2. The experimental challenges: define the question to pick a study system

When coming up with a strategy to answer biological questions or hypotheses much of the success, or stumbling, can be due to the experimental design and techniques. A generalized flow diagram of our usual decision making on selecting what type of mitochondrial preparation is given in Fig. 4. Our work on mitochondrial H_2O_2 metabolism began by trying to address the source or sink question. To do so we choose to design experiments that could probe multiple aspects of mitochondrial energetics in concert with measurements of H_2O_2 metabolism. In the following sections I will outline some of the decisions we had to make and the rationale applied starting from the choice of experimental material, assay conditions including unwanted or unanticipated responses of mitochondria to assay constituents, followed by the core problem: monitoring a mobile metabolite (H_2O_2) that is both produced and consumed in a compartment that our assays systems are excluded from (Fig.5).

2.1. Compartmentalization matters: the problematic plasma membrane

The selective permeability of the plasma membrane exerts a great deal of control over the intracellular environment. For mitochondrial experiments many molecules that are used to manipulate mitochondrial processes simply do not transport across the plasma membrane of most animal cells rapidly enough to be practical for experimental manipulation and control. However, strategies for mitochondrial preparations are designed to maintain mitochondrial functionality while giving the experimentalist control over what conditions the mitochondria are exposed or challenged with. This is why mitochondrial preparations are often favoured as an intermediate between isolated membrane fragments or enzyme complexes and the more complex intact cell. Extensive review of mitochondrial preparations is beyond the scope of the current work but the interested reader can find a comprehensive assessment here (Gnaiger et al., 2019).

Now, it is fair to question, why did we not design experiments to address our questions using intact cells? There are many probes, both traditional straightforward cell-permeant options and the more involved genetic strategies, that can produce signals reporting intracellular H_2O_2

concentration (reviewed in Kalyanaraman et al., 2012; Bilan and Belousov, 2016). While several approaches for estimating intracellular H_2O_2, or other ROS, are prone to false positives or ambiguous results, many strategies can show more reliable specificity to particular reactive species or less propensity towards promiscuous reactions to non-ROS (reviewed in Kalyanaraman et al., 2012; Bilan and Belousov, 2016). But even the best approaches for intact cells are often either semi-quantitative (many are based on ratio-metric changes in fluorescence for example) or the probes react too slowly to give real-time kinetics. This means that means cell-based approaches may be excellent for answering questions about the steady states; however, for the kinetic assays that we currently rely on for linking rates to mitochondrial energetics, real-time estimates of product formation or consumption are needed. The need to be able to directly relate different kinetic and steady-state results simultaneously made the available cell-based assays less practical for our needs than isolated mitochondrial preparations.

2.2. Choosing the mitochondrial preparation that suits your needs

Deciding on the source of a mitochondrial preparation requires consideration of practicality and tissue- or species-specific metabolic traits. While it can be tempting to assume that what has worked in one species will work in another, that is not always the case. For example, electron leak from mitochondrial glycerol-3-phosphate dehydrogenase (GPDH) can be studied in rodent muscle (Orr et al., 2013), but the capacity to oxidize glycerol-3-phosphate appears to be absent in some fish muscle (Moyes al., 1989) making such species a poor choice to study electron leak by GPDH. This illustrates how vital it is to understand the nuances of metabolic organization in your study species before planning experiments.

2.2.1. Choosing the source of your mitochondria

To begin with, the source of mitochondria needs to be decided. Because the majority of the work we would be using from the literature was focused on rodents, and more specifically rats, that made selecting a species relatively straightforward. We needed a supply of mitochondria where we could already know how to manipulate the rates of electron leak, from different sites, and know why the rates were changing (altered protonmotive force, blockage of electron flow because of adding a site-specific inhibitor etc.). Changing the model system would require characterizing the control of each of the 12+ sites of electron leak, independently and then in combination (see Brand, 2016 for review). Our comparative studies suggest muscle mitochondria may show differences in absolute and relative capacities for electron leak from different sites across taxa even when the effects of temperature are considered (Banh et al., 2016; Weins et al., 2017; Treberg et al., 2018).

While developing the work described below, we also needed large quantities of well coupled mitochondria that would have relatively low confounding influence from catalase. Unlike other H_2O_2 consumers that occur in the matrix, catalase does not require electrons from NADPH and therefore acts independent of the mitochondrial energetic status. While liver can provide large amounts of mitochondria, it would not suffice because of the high catalase activity associated with liver mitochondrial preparations (Dreschel and Patel 2010). Skeletal muscle and heart ventricle both can provide large amounts of mitochondria per gram of tissue, that are low in catalase. But with skeletal muscle being much larger than the heart tissue, muscle became the

source of mitochondria of general choice. This does raise a concern about mitochondrial subpopulations, both across fibre types (fast and slow twitch) as well as within the cell (subsarcolemmal and intermyofibrillar), which is noted in the next section.

2.2.2. Choosing the mitochondrial preparation for your question

Although most mitochondrial studies are conducted at supraphysiological oxygen concentrations, the observed diffusion limitation for oxygen in permeabilized muscle fibres (for example Pesta and Gnaiger, 2012) has been a substantial concern for our studies. There were already emerging concerns that diffusion limitation of H_2O_2 out of the mitochondrion may be skewing our results or interpretations (Treberg et al., 2010). Had we chosen permeabilized fibres we would be adding further control of the mitochondrial energetics to oxygen supply, which requires supersaturation of the medium to overcome (Pesta and Gnaiger, 2012), However, hyperoxia is also known to increase the rate of H_2O_2 formation (Jaimeson et al., 1986), which made isolated mitochondria a straightforward choice. Since beginning this work the importance of oxygen availability on the observed rates of electron leak has become more clearly demonstrated (for example see Treberg et al., 2018a; Li Puma et al., 2020). We now design experiments so that oxygen levels are not depleted more than ~ 50% of saturation to minimize confounding influence of oxygen availability on H_2O_2 formation. Moreover, the extensive characterization of both individual sites of electron leak (reviewed in Brand, 2016) and complex mixtures of substrates (Quinlan et al., 2012b; Quinlan et al., 2013; Goncalves et al., 2015) by Brand and colleagues with isolated rat skeletal muscle provided important background characterization.

Using isolated mitochondria also brought some analytical advantages over more complex biological mixes, such as homogenates and permeabilized tissue. While most of our assays and techniques can be used with other types of mitochondrial preparations, isolated mitochondria may lower the influences of non-specific binding for some effectors we use in many assays, like those using spectrofluorometry. Having a simpler makeup by removing much of the other cellular constituents, isolated mitochondria may also have less off-target reactions that need to be corrected for and may have better resolving power due to lower noise or other spectral interference. The disruption of mitochondrion-mitochondrion and mitochondrion-other-cell-constituent interactions may artificially enhance ROS formation or otherwise complicate direct comparison to mitochondria in situ, which makes the use of isolate mitochondria debated (Picard et al., 2010; 2011); however, the simplicity of isolated mitochondria combined with the large quantities that can be collected from a mixed skeletal muscle sample won out over other options like permeabilized fibres or homogenates.

Finally, a cautionary detail. Isolating mitochondria will homogenize the entire population of mitochondria of the tissue, cells or organ used; meaning that information on mitochondrial subpopulations will be lost by the isolation procedure. In our work, we use a mix of muscles that contain differing levels of fast, slow and mixed muscle fibre types and employ a protease to liberate intermyofibrillar mitochondria during isolation. Both fibre type (Pande and Blanchaer, 1971; Jackman and Willis, 1996; Leary et al, 2003; Anderson and Neufer, 2006) and location within muscle cells (Servais et al., 2003; Ferreira et al., 2010) can influence mitochondrial metabolic properties. Our need to have sufficient quantity of isolated mitochondria, while

minimizing animal use, led to this 'homogenization' of mitochondrial types becoming a necessary compromise but at the cost of blending these different mitochondrial populations into a single pool.

3. Considerations for the isolated mitochondria toolbox

One of the advantages of using isolated mitochondrial preparations is that many assays can be conducted on the same individual preparation of isolated mitochondria. The capacity to combine multiple measurements made simultaneously on the same homogenous mitochondrial preparation can be a particularly powerful tool, explained and illustrated elsewhere (described in Treberg et al, 2018a). We often combine measurements of oxygen consumption, H_2O_2 efflux and mitochondrial membrane potential, the latter two by fluorometry as a simultaneous experiment. With our instrumental set up, which has two temperature-controlled chambers running in parallel with simultaneous fluorometry readings, we can use the rate of oxygen consumption to confirm the mitochondria in each chamber are acting 'comparably' with each other (see Treberg et al., 2018a for example traces).

While extensive review of pitfalls and challenges in designing experiments with mitochondrial preparations is beyond the scope of the current work, we have raised several issues including the need to sometimes change or test different isolation procedures (Banh et al. 2016), the need to address mitochondrial function decline post-isolation, issues over artifacts caused by media (for example some constituents consume H_2O_2), issues on how membrane potential dyes and cations are inhibitors of mitochondrial function, species-specific differences for sensitivity for inhibition (for examples see Treberg et al., 2018a) and some of the considerations for comparing rates of H_2O_2 metabolism in comparative studies (Treberg et al., 2018b).

3.1. Some words of caution: data do not lie, but we can misinterpret them

Before getting into the nuances of mitochondrial H_2O_2 metabolism measurements, it is important to stress some cautions that should be applied to mitochondrial experiments. This will not be comprehensive and is based on issues we have encountered in our work that, to me, are not always acknowledge sufficiently in the field. For example, testing for off-target effects of effector molecules added to mitochondrial preparations is frequently not addressed. However, rather than singling out other's work, I will rely on specific problems we have encountered during our work on H_2O_2 metabolism to illustrate specific problems and how reached our solutions. But in each case, the problems described could lead to misinterpretations.

3.2. Validating a chemical agent's affect on mitochondrial energetics

Oxygen consumption is a common choice to evaluate if a reagent interferes with mitochondrial energetics. Unfortunately, relying solely on respiration as an indicator of mitochondrial energetics can be misleading. For example, we found while trying to saturate mitochondria with an equimolar mixture of glutamate, malate and succinate that it appeared we were reaching saturation (see Fig 6 in Treberg et al. 2018a); however, by monitoring membrane potential simultaneously it could be shown the increase in respiration with high levels of substrate coincided with a drop in membrane potential. Presumably the highest substate level tested led to substrate or counter ion cycling or some other process that is functionally consistent with uncoupling electron transport and maintenance of the protonmotive force which led to increased

respiratory flux (Treberg et al., 2018a). The salient point is that if only respiration was being monitored, we could have concluded we were approaching substrate saturation when we were instead following an artificially induced increase in respiratory flux. We were saved from this error by monitoring multiple aspects of mitochondrial energetics. For that reason, I would urge the utmost caution before assuming pharmacological agents are specific, benign or free of off-target effects based on oxygen consumption alone.

For our studies on mitochondrial H_2O_2 metabolism we now rely on a multi-metric protocol for initial testing of pharmacological agents (see Banh and Treberg 2013, Munro et al., 2016 for examples). To evaluate off-target effects of a new agent we test the effect on respiration rate in the non-phosphorylating and phosphorylating states, generally followed by a pharmacologically clamped non-phosphorylating state. While a maximal rate of respiration (uncoupled state) can also be useful, we do not regularly use this state in our experiments and therefore usually do not include it. To better understand what mechanisms may be causing inhibition, or elevation, of respiration we also measure membrane potential as a proxy of the protonmotive force and routinely measure the NAD(P)H autofluorescence to test if respiratory flux is impeded sufficiently that electrons are accumulating upstream of complex I. Accumulation of electrons in the NAD-pool would be expected to increase the electron leak from the many NAD-dependent sites of ROS formation (Brand 2016; Treberg et al., 2018b).

3.3. Validations are not always transferable

Emphasized elsewhere (Treberg et al. 2018a), but worth reiterating here, there can be species-specific differences in the sensitivity to chemical agents. For example, in re-evaluation of mitochondrial H_2O_2 metabolism in the long-lived naked molerat compared to the short-lived mouse, Daniel Munro found that the standard concentration of several agents (including Amplex Ultrared and auranofin) we had validated for use with rat mitochondria, were inhibitory with mouse mitochondria (Munro et al. 2019). For that reason, it is worth considering testing agents on new species or in comparative studies when possible.

4. Lessons Learned Studying Mitochondrial H_2O_2 Metabolism

4.1. Which respiratory state(s) are best for studying H_2O_2 metabolism in mitochondria?

The rate of mitochondrial electron leak is a function of mitochondrial energetics and thus the 'respiratory state' the mitochondria are in. Respiratory states that cause high rates of electron flux (phosphorylating or uncoupled) typically lead to lower rates of electron leak than under control conditions when the respiratory substrate concentration is high but electron flux is constrained by forcing protons against the high protonic potential across the inner membrane (non-phosphorylating). This pattern is expected because electron flux is coupled to proton or charge transport. As the protonmotive force increases the force required to 'push' proton transport out against the existing protonmotive force also increases (Fig. 3). This leads to a build up of electrons upstream of energy conserving sites (especially relevant to this work, Complexes I and III), which can be demonstrated by monitoring the reduction state of the electron carriers that supply these complexes or redox centres within the enzyme complexes (Quinlan et al., 2011; 2012a; 2012b; Treberg et al., 2011). The increased residency of electrons on these redox active sites shows why the protonmotive force is linked to the observed rate of electron leak, even when

different substrates for electron supply are used (for examples see Starkov et al., 2004; Goncalves et al., 2013; Brand, 2016). As already noted, most of this work is on rodents and although the absolute rate of electron leak can vary because of respiratory substrate choice the overall patterns seem relatively consistent between what are high producing and low producing conditions when compared across species (Banh et al., 2016; Wiens et al., 2017; Treberg et al., 2018b; Munro et al., 2019).

Non-phosphorylating respiratory states have high protonmotive force and generally higher rates of H_2O_2 efflux than when the same respiratory substrates are used under phosphorylating or in uncoupled conditions. For this reason, we generally use the non-phosphorylating state as our initial condition to characterize electron leak. Starting by manipulating the non-phosphorylating state allows for testing the influence of altering the protonmotive force, or other aspects of the system, while initially maximizing the H_2O_2 efflux 'signal'.

4.2. A core problem - compartmentalization

The compartmentalization that is crucial to mitochondrial energetics becomes a major problem with assays of mitochondrial H_2O_2 metabolism. The majority of the metabolic activity is within the mitochondrial space, but the membrane barriers that create this enclosed space also exclude our detection systems. For instance, real-time measurements of H_2O_2 efflux using destructive peroxidase-based assays rely on maintaining as steep of an outwardly directed diffusion gradient as possible by consuming all H_2O_2 that exists the mitochondrion (Fig 4). But the enzymes needed for detection do not cross the mitochondrial membranes therefore only the product that escapes from mitochondria can be detected (Miwa et al, 2016). Similarly, using an electrochemical sensor or other means of monitoring the disappearance of H_2O_2 assesses only the H_2O_2 that remains outside of the mitochondria. This spatial separation between detection and metabolism could lead to incorrect estimates if the processes within the mitochondria are interfering, biasing or in other ways misleading about what is actually taking place within the mitochondria.

4.3. Measuring electron leak rate using H2O2 efflux

We and many others have settled on using the rate of H_2O_2 efflux from mitochondria as the best choice for estimates of the electron leak rate. The assay is based on excess horseradish peroxidase being added to the medium along with adequate amounts of some substrate that can react with the peroxidase to provide a signal that can be monitored kinetically via absorbance or fluorescence. This assay is destructive in the sense that any H_2O_2 that escapes the mitochondrion should be consumed by the exogenous peroxidase near instantly. If the exogenous peroxidase is not in excess then the assay will underestimate rates because of elevated intramitochondrial consumption of H_2O_2 competing with the detection system (Treberg et al. 2018a). These kinetic assays can be sensitive enough to make detailed titrations feasible. But at the same time, even under ideal conditions, H_2O_2 efflux only measures the electron leak that evades the mitochondrial antioxidant systems. Another important consideration is that the H_2O_2 efflux rate might be seen as the amount of ROS the mitochondria would force the cell to deal with. But this is misleading because the rate of product formation using a destructive detection method is a measurement of unidirectional flux, but this in not a direct measure of how much H_2O_2 accumulates around mitochondria (Fig 5 and 7). In other words, in the absence of the detection

system, mitochondria may have net production rates far lower than the observed efflux rate because much of the H_2O_2 produced is prevented from accumulating because the mitochondria also consume H_2O_2 (Munro and Treberg, 2017). But to know if the H_2O_2 efflux assay is useful for resolving the source/sink argument, critical and quantitative assessment of any underestimate in the H_2O_2 efflux assay became essential.

4.4. Measuring consumption of H_2O_2 by mitochondria

We, and others, have relied on two core H_2O_2 consumption assays. One is based on repeated subsampling of aliquots from an incubation where the reaction is stopped and the remaining H_2O_2 is measured fluorometrically by a destructive peroxidase-based assay. This type of assay has been used with isolated mitochondria, permeabilized fibres and intact cells (Anderson and Neufer, 2006; Wagner et al., 2013; Munro et al., 2016). Our alternative assay uses an electrochemical sensor (we have used Innovative Instruments HP-250 for this purpose) which does destroy some H_2O_2 during detection but this rate is small relative to the biological H_2O_2 consumption in our assays, therefore I see this as a non-destructive approach in comparison to the peroxidase-based endpoint style assays.

We found that de-energized skeletal muscle mitochondria had very low rates of H_2O_2 consumption, consistent with limited catalase being present (Phung et al., 1994; Banh et al., 2013; Munro et al., 2016). Addition of respiratory substrate caused a marked increase in the rate of H_2O_2 disappearance in most cases, with the very high ROS producing condition of succinate without rotenone being an exception (Munro et al., 2016, Tamanna et al., 2020). The rate of disappearance was noted to be non-linear with H_2O_2 concentration (see Banh and Treberg, 2013), but the full importance of that, along with the link to succinate alone having low apparent rates of H_2O_2 consumption, was not fully appreciated by me at the time. Also important, conditions that led to fairly low protonmotive force and low respiration, such as using malate as the sole respiratory substrate, were sufficient to energize the H_2O_2 consumers in our model system such that consumption could be an order of magnitude greater than the production, measured as efflux, under the same conditions (Banh and Treberg 2013; Treberg et al., 2015; Munro et al. 2016).

It was also noted that energized skeletal muscle mitochondria, under conditions where we could measure H_2O_2 efflux using the destructive fluorescence assay, appeared to consume exogenous H_2O_2 down to levels that were not detectable above background noise and sensor drift (Banh and Treberg 2013). This became a crucial observation to explain, how could the mitochondria be demonstrable 'producers' of H_2O_2 but at the same time consume essentially all exogenous H_2O_2 to below the limits of detection? We did not have an answer at the time but did have two important confirmations (Banh and Treberg. 2013); i) consumption could be greater than production and ii) H_2O_2 did not appear to accumulate from energized mitochondria at least not under conditions where relatively low rates of H_2O_2 efflux could be measured.

5. Resolving what is going on inside

To begin addressing the source-sink problem we needed to answer several core questions. First, how reliable were estimates of production? At the time we knew the efflux assay underestimated H_2O_2 formation, but the magnitude of how much was unclear. Demonstrating that the

consumption capacity in skeletal muscle mitochondria was much higher than rates of H_2O_2 efflux did make the concern that efflux underestimates rates of electron leak quite appropriate. Second, what are the relative contributions from the main routes of consumption? Third, how do we reconcile both production and consumption systems into an integrated whole? The first two of these questions I will address followed by how we have begun answering the third, including the transition from the binary source-sink notion towards a more integrative regulatory capacity of mitochondrial systems.

5.1. Assessing the magnitude of underestimation of production rates

We knew that the major sinks for H_2O_2 in mitochondria that may cause underestimations of electron leak rates, based on extramitochondrial detection, were catalase, the GSH-peroxidase system and the thioredoxin-dependent peroxiredoxin system. The source of mitochondria was explicitly chosen to minimize the influence of catalase (Phung et al., 1994), but it was not clear how much of a concern the matrix thiol-based H_2O_2 consumers were for interpretation. Compromising either system requires inhibiting or removing enzyme activity or removal of essential metabolic intermediates. Inhibition of specific proteins may seem like a preferred starting point for compromising H_2O_2 consumption systems. However, finding suitable conditions can prove to be more complex, which is illustrated in the initial attempts to compromise the GSH-peroxidase system, which took place before we began addressing the source/sink question.

5.1.1 The first big step: compromising the GSH-peroxidase system

We have tested several inhibitors of the GSH-peroxidase system, including inhibitors of glutathione reductase which resupplies GSH for peroxidation (Munro et al., 2016); however, the best solution we have been able to find is based on using a glutathione-S-transferase substrate to remove most of the GSH from the mitochondria prior to experimentation. The idea of using compounds like 1-chloro-2,4-dinitrobenzene (CDNB, but also abbreviated DNCB for 2,4-dinitrochlorobenzene) to remove the influence of GSH on mitochondrial H_2O_2 metabolism was not new when we began that work. The strategy developed (Treberg et al. 2010) was based on work from the Kaplowitz group (Han et al., 2003), which demonstrated using CDNB to deplete intramitochondrial GSH that the observed rate of H_2O_2 efflux was a function of the matrix GSH content (Han et al., 2003). Han et al. (2003) explained in their carefully designed study that CDNB is not without potential problems. For instance, there was substantial evidence that caution needed to be used because of potential artifactual elevation of electron leak rates by CDNB (Zoccarato et al, 1988; 1990; Liu et al. 2002). It was with these import cautions that we set out to convince ourselves that the GSH-depletion could be done while minimizing concerns about false positives due to off-target effects of CDNB, such as possibly modifying proteins leading to artifactual electron leak.

5.1.2. CDNB pretreatment – avoiding the false-positive trap

The strategy we used to mitigate the influence of the GSH-dependent peroxidase system warrants elaboration and acts as an illustration of testing a process where the potential for artefacts is high. We had to reconcile two conflicting possibilities, CDNB may either unmask that H_2O_2 efflux was truly under-reporting electron leak rates because of matrix consumption or

CDNB may aberrantly elevate apparent rates due to off-target artefacts. To determine if the CDNB pretreatment was appropriate, a series of experiments were designed to confirm that CDNB pretreatment would not artificially elevate mitochondrial H_2O_2 efflux.

The rationale behind testing if the CDNB pretreatment assay led to artifacts was based on the a priori assumption that matrix H_2O_2 consumption was in competition with the extramitochondrial detection. In the simplest terms, all intramitochondrial sources of electron leak contribute to a common intramitochondrial pool of H_2O_2 and the endogenous consumers of H_2O_2 would have first access to that intramitochondrial pool. As H_2O_2 is formed, the established concentration gradient will cause diffusion to the medium, where H_2O_2 is clamped effectively at zero due to the destructive detection system. If these assumptions were correct, by depleting GSH and thus decreasing the competition between endogenous H_2O_2 consumers and the exogenous detection system, we should see higher rates of H_2O_2 appearance compared to control mitochondria. I will note that we showed the H_2O_2 consumption was inhibited (Treberg et al., 2010), but that was a misinterpretation of the consumption in the de-energized state. We have since confirmed that CDNB pretreatment does decrease the GSH-dependent H_2O_2 consumption in the energized state in rat muscle mitochondria (Munro et al., 2016).

However, simply measuring increased H_2O_2 appearance does not exclude a false positive. To address this problem we devised a strategy of constructing multiple independent 'titrations' of H_2O_2 efflux rates. If the technique only compromised matrix-consumers that were truly in competition with our extramitochondrial detection system then all titrations should fall on a common line. Alternatively, aberrations from the line would indicate that a particular assay condition may not be usable for the CDNB-pretreatment. The initial test was based on manipulating substrate and inhibitor additions to alter which specific sites of electron leak were major contributors (see Treberg et al, 2010 for further details). These assays tested multiple conditions of respiratory substrate (malate, glutamate plus malate, or succinate) in the absence or presence of respiratory inhibitors (rotenone, antimycin A, stigmatellin). These different assay conditions would lead to some conditions dominated by ubiquinone-dependent electron leak, while others would be almost exclusively coming from NAD-linked sites of leak and yet others would have a combination of ubiquinone- and NAD-dependent sites. The combination of these data indicated that the increase in H_2O_2 efflux due to CDNB pretreatment followed what seemed to be a common pattern, the dotted line in Figure 6, irrespective of what specific sites were active. Only showing a pattern based on many different sites under different inhibitor conditions did leave the possibility of a serendipitous relationship leading to a spurious correlation. For that reason we also titrated specific sites of superoxide production including i) titration of site IQ in energized mitochondria and ii) titration of site IIIQo in de-energized mitochondria, all patterns fit a comparable pattern (see Fig. 2 for sites and effects of inhibitors and Fig. 6 for results). That these different titrations under such different conditions fit a similar line as what was found using many different sites is not a trivial point. We had demonstrated that the effect of CDNB pretreatment was common regardless if a single site of production was varied or a variety of different sites were used to generate a range of H_2O_2 production. This means that the CDNB-pretreatment was validated for those specific substrate combinations but it should not be assumed to be free of artefacts in cases where alternative respiratory substrates are used.

5.1.1.2. Why not add CDNB directly to mitochondria?

The CDNB pretreatment approach is laborious, leads to potential loss of mitochondria and requires large amounts of starting material only to open the potential for greater variation due to the additional processing steps. All of this will intrinsically come with a risk that further manipulation may lead to low quality mitochondria. For these reasons the idea of simply adding CDNB to the mitochondria, titrating so that enough is added to consume the GSH but not cause artifacts, should seem appealing. However, we have avoided this simple solution for many reasons, the most substantial being the potential for false or artifactual increases in electron leak independent of the GSH-scavenging system. Even low micromolar concentrations of CDNB cause an activation of NADH-dependent electron leak in membrane fragments that should have no functional GSH-peroxidase system (see Munro et al., 2016 for further details), which indicates a danger of false positives from titrating CDNB with active mitochondria even at micromolar concentrations of CDNB.

5.1.1.3. CDNB and thioredoxin reductase

An additional consideration about CDNB involves the thioredoxin-dependent H_2O_2 consumption system. It is known that some forms of thioredoxin reductase can be inactivated with CDNB, and this does appear to be the case for mitochondrial thioredoxin reductase as shown with the newly released MitoCDNB (Booty et al., 2019). This potential duality of CDNB pretreatment might tempt the researcher to think this strategy can target both the GSH and thioredoxin-dependent systems, an assumption I have also erroneously made in the past. However, based on the effect auranofin has on CDNB-pretreated mitochondria (Munro et al, 2016), the thioredoxin-dependent contribution to mitochondrial H_2O_2 consumption remains active after CDNB-pretreatment. Importantly, the auranofin-dependent H_2O_2 metabolism by isolated skeletal muscle mitochondria shows similar capacity in CDNB-pretreated mitochondria as that seen in those not pretreated with CDNB (Treberg et al., 2015; Munro et al. 2016), indicating CDNB-pretreatment does not appear to substantially interfere with the endogenous mitochondrial peroxiredoxin system's capacity to consume H_2O_2. While speculation, perhaps for CDNB to inactivate thioredoxin reductase there is a need for enzyme catalytic turnover. If so, this would explain why MitoCDNB inhibits thioredoxin reductase in live cells (Booty et al. 2019) but CDNB does not inhibit thioredoxin reductase in the pretreatment approach we developed because the mitochondria are not energized when exposed to CDNB.

5.1.2. Auranofin: too good to be true?

After the complexity of compromising the GSH-dependent peroxidase system, the thioredoxin-dependent system was straightforward to inhibit (Munro et al., 2016). Our first approach on compromising the thioredoxin-dependent H_2O_2 consumption pathway was auranofin, an inhibitor of thioredoxin reductase, which would prevent the peroxiredoxins from being maintained in the active state after peroxide consumption. Auranofin was found to work maximally at concentrations of 1 µM and in the case of rat skeletal muscle mitochondrial this did not alter mitochondrial energetics (Munro et al. 2016). Some caution does need to be urged due to the species-specific nature of how much auranofin mitochondria tolerate (Munro et al., 2019) and calcium levels must be kept low to avoid possible permeability transition pore opening with this compound (Rigobello et al., 2003). However, beyond those cautions, auranofin has been an

effective and invaluable tool that we find works well in mitochondria from other rodents (Munro et al., 2019) as well as from fish muscle mitochondria (Treberg, Munro, Tamanna, Wiens unpublished observations). Using auranofin it can be seen that the thioredoxin-dependent system rivals the GSH-dependent system in rat skeletal muscle mitochondria with regards to the magnitude in which the H_2O_2 efflux assay underestimates electron leak (Munro et al., 2016). But the relative contributions of the peroxiredoxin and GSH-dependent systems does vary by species, with the exceptionally long-lived naked molerat disproportionately relying on the GSH-dependent system relative to mice (Munro et al., 2019) or rats (Munro et al., 2016).

5.2. Understanding mitochondrial H_2O_2 consumption capacity

We and others have shown that mitochondrial H_2O_2 consumption can reach rates far greater than the rate of H_2O_2 efflux seen in the absence of respiratory inhibitors. In the case of mitochondria with low catalase, like brain, skeletal muscle and heart, the rate of H_2O_2 consumption requires respiratory substrate to be maximized (Deschel et al., 2010; Banh and Treberg, 2013; Starkov et al., 2014; Kamunde et al., 2018). The rationale is that the peroxidases require NADPH to maintain activity and reduction of $NADP^+$ requires a supply of electrons. The ultimate supply of these electrons is from matrix dehydrogenases, although the nicotinamide nucleotide transhydrogenase (NNT) can also contribute via transfer of electrons from NADH to NADPH (Fig. 2).

We find that respiratory substrate choice influences the observed rate of H_2O_2 consumption but have argued these observed, or apparent, rates are underestimates and actual rates of consumption may be relatively high and stable across these conditions (Banh and Treberg 2013; Munro et al., 2016; Munro and Treberg, 2017; Treberg et al., 2018b). How do we explain this? One core concept of our model is that it appears even poor respiratory substrates like malate alone can drive consumption at near maximal rates, likely via provision of NADPH via NNT and possibly NADP-dependent malic enzyme. During early characterization of the consumption of H_2O_2 we found with malate as the sole respiratory substrate the exogenous H_2O_2 was depleted to below detection (Banh and Treberg, 2013). This was a clue to the developing model of mitochondria as regulators of H_2O_2.

5.3. A kinetic model of H_2O_2 production and consumption

While we were characterizing the relative H_2O_2 production and consumption capacities in rat skeletal muscle, a vitally important paper came out that reconciled how to describe both systems acting as an integrated whole (Starkov et al., 2014). Using isolated brain mitochondria, Starkov and colleagues (Starkov et al., 2014) noted that the disappearance of exogenously added H_2O_2 with energized mitochondria appeared to have a concentration dependency, similar to a typical first-order reaction rate. They also showed, using methods similar to that shown in Fig. 6, that energized mitochondria accumulated H_2O_2 in the medium only under conditions of high rates of electron leak but the accumulation plateaued over time. To reconcile the accumulation of H_2O_2 around mitochondria to some steady-state level, the authors constructed a simple first-order rate equation allowed for solving for both the rate of production and consumption (Starkov et al. 2014). Since the energized mitochondria were the only source of H_2O_2 for isolated mitochondria the rate of H_2O_2 consumption should increase as H_2O_2 accumulates in the medium thereby

increasing the rate of H_2O_2 consumption until a steady-state concentration is established where production and consumption rates are now equal. The core equations of this model, as modified for our purposes (see Treberg et al., 2015; 2019) are:

$$[H_2O_2]_t = (V^P/k) \cdot (1-e^{-k.pr.t}) \quad \text{(Eq. 1)}$$

Where t = time (s), pr = mitochondrial protein concentration in $g \cdot l^{-1}$, V^P= the rate of actual H_2O_2 production in nmol $H_2O_2 \cdot g\ protein^{-1} \cdot s^{-1}$and k = the first order rate constant for mitochondrial H_2O_2 consumption in $s^{-1} \cdot g\ protein^{-1}$ l. Note, we include mitochondrial protein concentration in our equations while those in Starkov et al. (2014) do not, but both are equivalent approaches, we just explicitly incorporate the protein value in our formula. As $t \rightarrow \infty$ the $[H_2O_2]_{ss}$ can be estimated because Eq. 1 simplifies to:

$$[H_2O_2]_{ss} = V^P/k \quad \text{(Eq. 2)}$$

These concepts combined to reconcile how the rate of production could be related to the rate constant for H_2O_2 consumption to determine the steady-state concentration of H_2O_2 that a particular assay condition would reach as time approaches infinity (see Starkov et al., 2014 for further explanation). This was the first model of mitochondria as candidate regulators of steady-state H_2O_2 concentrations that I am aware of that was simple enough to be both amenable to straightforward empirical testing while also not requiring filling in substantial analytical gaps by computation or inference from other more complex kinetic models.

5.4. Recognizing the difference between apparent and actual rates

Starkov and colleagues' work (Starkov et al., 2014) provided the model needed to reconcile H_2O_2 metabolism in mitochondria as an integrated whole, but the fits based on the first-order kinetics were not always all that good in our experience as shown elsewhere (Treberg et al. 2015). They were sufficient, but not excellent. Our work on quantitating the relative underestimation from H_2O_2 efflux assays hinted on why; were the observed rates accurate representations of the actual true rates? For context, if H_2O_2 is added to energized mitochondria then the consumers within the mitochondria have two sources to consume, the H_2O_2 exogenously added and the H_2O_2 endogenously produced; however, our assays only detect the changes outside of the mitochondrion (section 4.2 and Fig. 4).

We designed experiments to evaluate if the observed rates of production or consumption were consistent with an underestimation due to limitations in exchange between the matrix and medium leading to preferential consumption of intramitochondrial H_2O_2. By inhibiting H_2O_2 consumption with auranofin and manipulating the rate of production by the choice of respiratory substrates added we could demonstrate that indeed mitochondrial H_2O_2 metabolism displayed traits consistent with diffusion as a barrier between extramitochondrial detection and intramitochondrial metabolism (Treberg et al., 2015). We at that point could demonstrate how the observed rates, which we call the apparent rates, could be used in combination with the principles from Starkov and colleague's work (Starkov et al., 2014) to estimate the actual rates of production and consumption (for further details see Treberg et al. 2015; Munro and Treberg 2017). We concluded:

i) the mitochondrial inner membrane does confound interpretation when maintained intact, making the measurements outside of mitochondria intrinsic underestimates;
ii) the concept that energized mitochondria could act as regulators of steady state H_2O_2 concentrations (illustrated in Starkov et al, 2014) was supported using mitochondria from another tissue;
iii) when the only supply of H_2O_2 in the assay is endogenous production from energized mitochondria, the H_2O_2 consumers do not appear to be limited by NADPH supply but the rate of consumption is constrained by H_2O_2 availability, meaning that low rates of production lead to low levels of steady-state H_2O_2 being maintained (often lower than can be detected using the methods in these studies), and
iv) as noted previously, the steady-state concentration of H_2O_2 will be set by the balance between the rate of production and the consumption capacity (Starkov et al., 2014; Treberg et al. 2015).

5.5 Reconciling apparent and actual rates; how bad are the measurements we can make?

With systems that allowed us to manipulate both the GSH-dependent and the thioredoxin-dependent consumption pathways, independently of each other, we could finally assess how accurate, or misleading, measurements of H_2O_2 in the medium reflects actual intramitochondrial rates. It appears that for all energized conditions we tested the respiration dependent consumers would be operating maximally other than being substrate limited for H_2O_2 (Treberg et al., 2015). For that reason, we treat the consumers as generally near-maximal in their influence on observed rates with energized mitochondria, keeping in mind that they are substrate limited at the level of H_2O_2 availability. But how much did the matrix consumers influence measurements of electron leak?

5.5.1. The H_2O_2 efflux assay underestimates more with low rates of formation

Using a combination of CDNB-pretreatment and auranofin we were able to show, for the first time, the rates of H_2O_2 efflux from isolated mitochondria were not only qualitative underestimations, we could approximate how much of an underestimation they were. It is important to note that our strategy to compromise both thiol-linked peroxidase systems appears to be near complete because experiments of net H_2O_2 metabolism, based on net appearance or consumption, yielded rates similar to those measured via the H_2O_2 efflux assay (Munro et al., 2016). In other words, the H_2O_2 efflux rates from CDNB-pretreated mitochondria also exposed to auranofin was approximating the 'actual' rate of net production in these isolated mitochondria. The degree of underestimation was an inverse function of the rate of production, meaning very high rates of electron leak were less prone to underestimation than low rates (Munro et al., 2016). While this should be expected, and was shown independently for both CDNB-pretreatment (Treberg et al., 2010) and auranofin (Treberg et al., 2015), here with both techniques combined we found for low rates of production the observed rate of H_2O_2 efflux may represent as little as 20% of the actual rate of electron leak (Munro et al., 2016), with low rates, in the range that might be anticipated when ATP is actively being regenerated (mitochondria are in the phosphorylating state), being the most prone to potential underestimation.

5.5.2. Taking advantage of competition to estimate H_2O_2 consumption

Estimates of maximal H_2O_2 consumption by isolated mitochondria is confounded when respiratory substate is required to maximize peroxidases because the H_2O_2 produced endogenously is localized with the consumers and therefore is likely to be preferentially consumed over the extramitochondrial H_2O_2. By manipulating assay conditions to modify the apparent rate constant for H_2O_2 consumption we were able to plot the apparent consumption kinetic constant relative to the apparent rate of production (Treberg et al, 2015). Using this style of plot, and assuming the actual rate constant is not varying over the experimental manipulations, allows for estimating the actual value by extrapolation to where production is zero (the y-axis intercept). We recognized the same logic could be applied to estimates of H_2O_2 consumption rates, which are somewhat more intuitive for some than rate constants (Munro and Treberg, 2017). These experiments not only demonstrate the competition between endogenous processes and H_2O_2 dynamics outside of the mitochondrion, the influence of auranofin confirms that the system does behave as though the steady state H_2O_2 concentration is controlled by the balance between the consumption and production. Thus, in the scenario where mitochondria are H_2O_2 regulators, rather than net sources or sinks, it becomes clear that the modification of either production or consumption dynamics should intrinsically alter the steady-state concentration of H_2O_2 the mitochondria would regulate towards.

5.6 The latest step forward – linking H_2O_2 balance to energy sensing

To date, there have been very few demonstrations of how mitochondria can act to regulate H_2O_2 concentrations towards a steady state and they all required very high rates of electron leak, for example from mitochondria with very high contributions from site IQ ROS formation, or the use of inhibitors of matrix H_2O_2 consumption (Starkov et al, 2014; Treberg et al., 2015). However, there had not been the clear demonstration that the steady-state H_2O_2 concentration responded to mitochondrial energetics. There was a need to explicitly show that mitochondrial energetics could be linked to extramitochondrial signalling via H_2O_2 concentration. To do so, we needed a system that could be manipulated sufficiently but not rely on the very unstable and possibly unphysiologically high rate of production seen with succinate alone, which declines rapidly post-isolation. We found that addition of rotenone with succinate as the respiratory substrate gave stable enough dynamics for approximating applying our kinetic estimates using the appearance of H_2O_2 in the medium over time; however, to get sufficient H_2O_2 accumulation we needed to inhibit consumption partially with auranofin (Treberg et al., 2019). Indeed, we could show how the achieved steady-state H_2O_2 concentration was a function of auranofin added, with 1µM approaching a maximum of approximately 300 nM H_2O_2, which is likely much higher than physiological levels. However, this level is still less than the concentration of H_2O_2 we found succinate could achieve in the absence of rotenone (Treberg et al., 2015).

We first tested the influence of altered energetics on the apparent rate of H_2O_2 production. This involved measuring mitochondrial respiration in parallel with membrane potential using fluorescent dye quenching (tetramethylrhodamine methyl-ester, TMRM) or the destructive H_2O_2 efflux assay (Treberg et al., 2019). As expected, by adding the complex II competitive inhibitor

malonate, there was a parallel decline in both the respiration rate and the apparent rate of electron leak as the membrane potential declined (Fig. 7). We also found that addition of ADP or a chemical uncoupler (Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone) lead to increases in respiratory flux that appear to parallel each other when plotted relative to the estimate of membrane potential (Fig. 7). However, the rate of H_2O_2 efflux decreased similarly regardless if the mitochondria were partially depolarized with ADP or FCCP and, importantly, this relationship was very similar to the pattern seen between H_2O_2 efflux and membrane potential with the malonate titration (Treberg et al. 2019). Because the apparent rate of electron leak was strongly related to the estimate of membrane potential, regardless if the manipulation was a respiratory inhibitor, ADP or uncoupler addition (Fig. 7B), the rate of production appears to be largely a function of the membrane potential, and therefore the protonmotive force.

From the above, we conclude that mitochondria have the features required to regulate the H_2O_2 concentration around them. But we still needed to empirically demonstrate that regulation could act to convey the inner mitochondrial state (protonmotive force, or at least the membrane potential component of it) to the extramitochondrial space via H_2O_2 as a mobile secondary messenger. Manipulation of the protonmotive force with malonate, in the presence of auranofin, resulted in two key results: i) the steady state concentration of H_2O_2 was a function of the established membrane potential and ii) based on curve fitting estimates of the actual rate of production and the rate constant for consumption, this was entirely due to declining production with the consumption being unaltered as we decreased the respiratory flux and subsequent protonmotive force that would be established (Treberg et al., 2019). We could now show that mitochondria can maintain H_2O_2 consumption while also altering the steady-state H_2O_2 concentration via changes in production, the latter of which responds to changes in the membrane potential, which is the majority of the protonmotive force under these conditions.

6. Context and Next Steps

Isolated rat skeletal muscle mitochondria have the characteristics needed to allow them to use local H_2O_2 concentrations as a means of communicating their energetic status, membrane potential or protonmotive force, to the extramitochondrial space. Although more confirmation in other model systems is still needed, the framework of how we have demonstrated these processes should provide some guidance on addressing if similar results occur in mitochondria from other tissues and species. But our results do provide some additional opportunities.

6.1 Why a second mitochondrial energy sensing signal?

Adenosine monophosphate-activated protein kinase (AMPK) signalling is a major integrator of cellular energy sensing via changes in the cellular adenylate potential (reviewed in Long and Zierath, 2006; Lage et al., 2008). As the cytoplasm shifts to a lower adenylate charge (lower ATP/ADP), the cell can form AMP from ADP, which activates the AMPK cascade. In skeletal muscle AMPK signalling increases fuel uptake or oxidation and can increase mitochondrial biogenesis (reviewed in Long and Zierath, 2006). However, mitochondria have other roles than just maintaining cellular ATP supply and mitochondria may need to signal their internal energization level via an alternative to the AMPK signalling system. It is my position that mitochondria could use H_2O_2 concentrations as an alternative means of communicating their

internal energetic state (their current protonmotive force), as argued elsewhere (Mailloux and Treberg, 2017), independent of the cellular adenylate pool dynamics.

6.2. Comparative contexts

We have argued that muscle mitochondria from ectotherms may be disproportionately prone to electron leak compared to endotherms (Treberg et al., 2018b) and this may have relevance to how sensitive any H_2O_2 based signalling mechanisms. For instance, while rat skeletal muscle mitochondria do not accumulate detectable amounts of H_2O_2 at physiological temperatures with low production conditions like glutamate plus malate in a non-phosphorylating state (Treberg et al., 2015), we have found that red muscle mitochondria from multiple teleost species readily accumulate H_2O_2 under similar assay conditions (Treberg and Tammana, unpublished observations). As such, this would support the contention that ectotherm muscle mitochondria maintain a much higher steady-state H_2O_2 concentration than skeletal muscle mitochondria in rodents, which may relate to temperature effects or fundamental differences in the set points for H_2O_2 signalling across species. However, that is speculation on my part, with much more work on this area needing to be done.

Likewise, the H_2O_2 consumption in isolated mitochondria may have somewhat lower thermal sensitivity relative to the rate of electron leak (Treberg et al., 2018b) which means for animals experiencing changing internal body temperatures, either as ectotherms or in endotherms during torpor bouts, the relative contributions of production and consumption may require further or differential regulation. While the experiments to establish steady-state conditions of H_2O_2 may be beyond what many comparative studies are able to conduct we have argued, and used, the ratio of production and consumption rates, termed the oxidant index or ratio, as a means of comparing across species and in particular different assay temperatures (Treberg et al., 2018b; Munro et al, 2019). Similarly, we recently concluded that methionine restriction, which increases life span in rodents, does not appear to alter mitochondrial H_2O_2 balance in skeletal muscle because neither production nor consumption capacity is altered (Tamanna et al., 2020).

6.3. Moving past isolated mitochondria

If mitochondria can act as localized regulators of cellular H_2O_2 concentrations, then can the values from our isolated mitochondria experiments be extrapolated to mitochondria within living cells? I would say no, because of the very different levels of oxygen, substrate supply and control along with the recognized changes associated with isolated mitochondria in comparison to those in situ within permeabilized cells (Picard et al., 2010; 2011). But that does not mean our findings have no value for experimentalists using intact cells. The underlying principles we have assessed, such as the impediment the protonmotive force is to electron flow and thus electron leak, should be generalizable and, if so, they can inform experiments well suited to the steady-state conditions using intact cells. For instance, small and sustained decreases in protonmotive force should be accompanied with cellular response that could be linked to changing H_2O_2 concentrations, which can be monitored in live cells (Kalyanaraman et al., 2012; Bilan and Belousov, 2016).

Declaration of competing interested

None to declare

Acknowledgements

My research and that by my lab group members which contributed to the work summarized here was funded by Discovery Grants from NSERC Canada, the Canada Research Chairs program, the University of Manitoba (Faculty of Science), a post-doctoral fellowship to D. Munro (from FRQ-NT) and several NSERC and University of Manitoba Faculty of Science Undergraduate Summer Research Assistantships.

References

Anderson, E.J., Neufer, P.D., 2006. Type II skeletal myofibers possess unique properties that potentiate mitochondrial H2O2 generation. Am. J. Physiol. Cell Physiol., 290, C844-C851.

Andreyev, A. Y., Kushnareva, Y. E., Starkov, A. A., 2005. Mitochondrial metabolism of reactive oxygen species. Biochemistry (Moscow). 70, 200-214.

Ballantyne, J.S., 1997. Jaws: the inside story. The metabolism of elasmobranch fishes. Comp. Biochem. Physiol., 118B, 703-742.

Ballantyne, J. S., 2004. Mitochondria: aerobic and anaerobic design—lessons from molluscs and fishes. Comp. Biochem. Physiol. 139B, 461-467.

Banh, S., Treberg, J. R., 2013. The pH sensitivity of H2O2 metabolism in skeletal muscle mitochondria. FEBS lett. 587, 1799-1804.

Banh, S., Wiens, L., Sotiri, E., Treberg, J. R., 2016. Mitochondrial reactive oxygen species production by fish muscle mitochondria: potential role in acute heat-induced oxidative stress. Comp. Biochem. Physiol. 191B, 99-107.

Bleier, L., Wittig, I., Heide, H., Steger, M., Brandt, U., Dröse, S., 2015. Generator-specific targets of mitochondrial reactive oxygen species. Free Rad. Biol. Med. 78, 1-10.

Bienert, G. P., Chaumont, F., 2014. Aquaporin-facilitated transmembrane diffusion of hydrogen peroxide. Biochim. Biophys. Acta -General Subjects. 1840, 1596-1604.

Bilan, D. S., & Belousov, V. V. (2016). HyPer family probes: state of the art. *Antioxidants & redox signaling*, *24*(13), 731-751.

Booty, L.M., Gawel, J.M., Cvetko, F., Caldwell, S.T., Hall, A.R., Mulvey, J.F., James, A.M., Hinchy, E.C., Prime, T.A., Arndt, S. and Beninca, C., 2019. Selective disruption of mitochondrial thiol redox state in cells and in vivo. *Cell chemical biology*, *26*(3), pp.449-461.

Boveris, A., Oshino, N., & Chance, B. (1972). The cellular production of hydrogen peroxide. *Biochemical Journal*, *128*(3), 617-630.

Brand, M. D. (2016). Mitochondrial generation of superoxide and hydrogen peroxide as the source of mitochondrial redox signaling. *Free Radical Biology and Medicine*, *100*, 14-31.

Brown, G. C., & Borutaite, V. (2012). There is no evidence that mitochondria are the main source of reactive oxygen species in mammalian cells. *Mitochondrion*, *12*(1), 1-4.

Divakaruni, A. S., & Brand, M. D. (2011). The regulation and physiology of mitochondrial proton leak. *Physiology*, *26*(3), 192-205.

Drechsel, D. A., & Patel, M. (2010). Respiration-dependent H2O2 removal in brain mitochondria via the thioredoxin/peroxiredoxin system. *Journal of Biological Chemistry*, *285*(36), 27850-27858.

Driedzic, W. R., & Hart, T. (1984). Relationship between exogenous fuel availability and performance by teleost and elasmobranch hearts. *Journal of Comparative Physiology B*, *154*(6), 593-599.

Ferreira, R., Vitorino, R., Alves, R.M., Appell, H.J., Powers, S.K., Duarte, J.A. and Amado, F., 2010. Subsarcolemmal and intermyofibrillar mitochondria proteome differences disclose functional specializations in skeletal muscle. *Proteomics*, *10*(17), pp.3142-3154.

Goncalves, R. L., Quinlan, C. L., Perevoshchikova, I. V., Hey-Mogensen, M., & Brand, M. D. (2015). Sites of superoxide and hydrogen peroxide production by muscle mitochondria assessed ex vivo under conditions mimicking rest and exercise. *The Journal of biological chemistry*, *290*(1), 209-227.

Gnaiger, E., Åsander Frostner, E., Abdul Karim, N., Abumrad, N. A., Acuna-Castroviejo, D., Adiele, R. C., et al. (2019). *Mitochondrial respiratory states and rate*. MitoEAGLE Task Group, COST Action CA15203 MitoEAGLE.

Hoek, J. B., & Rydström, J. (1988). Physiological roles of nicotinamide nucleotide transhydrogenase. *Biochemical Journal*, *254*(1), 1-10.

Jackman, M.R. and Willis, W.T., 1996. Characteristics of mitochondria isolated from type I and type IIb skeletal muscle. *American Journal of Physiology-Cell Physiology*, *270*(2), pp.C673-C678.

Jackson, J. B. (2003). Proton translocation by transhydrogenase. *FEBS letters*, *545*(1), 18-24.

Jamieson, D., Chance, B., Cadenas, E., & Boveris, A. (1986). The relation of free radical production to hyperoxia. *Annual review of physiology*, *48*(1), 703-719.

Jensen, P. K. (1966). Antimycin-insensitive oxidation of succinate and reduced nicotinamide-adenine dinucleotide in electron-transport particles II. Steroid effects. *Biochimica et Biophysica Acta (BBA)-Enzymology and Biological Oxidation*, *122*(2), 167-174.

Kalyanaraman, B., Darley-Usmar, V., Davies, K.J., Dennery, P.A., Forman, H.J., Grisham, M.B., Mann, G.E., Moore, K., Roberts II, L.J. and Ischiropoulos, H., 2012. Measuring reactive

oxygen and nitrogen species with fluorescent probes: challenges and limitations. *Free radical biology and medicine*, *52*(1), pp.1-6.

Kamunde, C., Sharaf, M., & MacDonald, N. (2018). H2O2 metabolism in liver and heart mitochondria: Low emitting-high scavenging and high emitting-low scavenging systems. *Free Radical Biology and Medicine*, *124*, 135-148.

Korshunov, S. S., Skulachev, V. P., & Starkov, A. A. (1997). High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS letters*, *416*(1), 15-18.

Kushnareva, Y., Murphy, A. N., & Andreyev, A. (2002). Complex I-mediated reactive oxygen species generation: modulation by cytochrome c and NAD (P)+ oxidation–reduction state. *Biochemical Journal*, *368*(2), 545-553.

Lage, R., Diéguez, C., Vidal-Puig, A. and López, M., 2008. AMPK: a metabolic gauge regulating whole-body energy homeostasis. *Trends in molecular medicine*, *14*(12), pp.539-549.

Leary, S. C., Lyons, C. N., Rosenberger, A. G., Ballantyne, J. S., Stillman, J., & Moyes, C. D. (2003). Fiber-type differences in muscle mitochondrial profiles. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *285*(4), R817-R826.

Li Puma, L. C., Hedges, M., Heckman, J. M., Mathias, A. B., Engstrom, M. R., Brown, A. B., & Chicco, A. J. (2020). Experimental oxygen concentration influences rates of mitochondrial hydrogen peroxide release from cardiac and skeletal muscle preparations. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *318*(5), R972-R980.

Liu, S. S. (1997). Generating, partitioning, targeting and functioning of superoxide in mitochondria. *Bioscience reports*, *17*(3), 259-272.

Long, Y.C. and Zierath, J.R., 2006. AMP-activated protein kinase signaling in metabolic regulation. *The Journal of clinical investigation*, *116*(7), pp.1776-1783.

Mailloux, R. J., & Treberg, J. R. (2016). Protein S-glutathionlyation links energy metabolism to redox signaling in mitochondria. *Redox Biology*, *8*, 110-118.

Miwa, S., Treumann, A., Bell, A., Vistoli, G., Nelson, G., Hay, S. and von Zglinicki, T., 2016. Carboxylesterase converts Amplex red to resorufin: Implications for mitochondrial H2O2 release assays. *Free Radical Biology & Medicine*, *90*, p.173-183.

Moyes, C. D., Buck, L. T., Hochachka, P. W., & Suarez, R. K. (1989). Oxidative properties of carp red and white muscle. *Journal of Experimental Biology*, *143*(1), 321-331.

Muller, F. L., Liu, Y., & Van Remmen, H. (2004). Complex III releases superoxide to both sides of the inner mitochondrial membrane. *The Journal of Biological Chemistry*, *279*(47), 49064-49073.

Munro, D., Baldy, C., Pamenter, M.E. and Treberg, J.R., 2019. The exceptional longevity of the naked mole-rat may be explained by mitochondrial antioxidant defenses. *Aging Cell*, *18*(3), p.e12916.

Pagacz, J., Broniec, A., Wolska, M., Osyczka, A., & Borek, A. (2020). ROS signaling capacity of cytochrome bc1: opposing effects of adaptive and pathogenic mitochondrial mutations. *Free Radical Biology and Medicine*.

Pande, S. V., & Blanchaer, M. C. (1971). Carbohydrate and fat in energy metabolism of red and white muscle. *American Journal of Physiology-Legacy Content*, *220*(2), 549-553.

Pesta, D., & Gnaiger, E. (2012). High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. In *Mitochondrial bioenergetics* (pp. 25-58). Humana Press.

Phung, C.D., Ezieme J.A. , Turrens J.F., 1994. Hydrogen peroxide metabolism in skeletal muscle mitochondria. Arch. Biochem. Biophys. 315, 479–482.

Picard, M., Ritchie, D., Wright, K.J., Romestaing, C., Thomas, M.M., Rowan, S.L., Taivassalo, T. and Hepple, R.T., 2010. Mitochondrial functional impairment with aging is exaggerated in isolated mitochondria compared to permeabilized myofibers. *Aging cell*, *9*(6), pp.1032-1046.

Picard, M., Taivassalo, T., Ritchie, D., Wright, K. J., Thomas, M. M., Romestaing, C., & Hepple, R. T. (2011). Mitochondrial structure and function are disrupted by standard isolation methods. *PloS one*, *6*(3), e18317.

Quinlan, C. L., Gerencser, A. A., Treberg, J. R., & Brand, M. D. (2011). The Mechanism of Superoxide Production by the Antimycin-inhibited Mitochondrial Q-cycle. *The Journal of Biological Chemistry*, *286*(36), 31361.

Quinlan, C. L., Orr, A. L., Perevoshchikova, I. V., Treberg, J. R., Ackrell, B. A., & Brand, M. D. (2012a). Mitochondrial complex II can generate reactive oxygen species at high rates in both the forward and reverse reactions.

Quinlan, C., Treberg, J., Perevoshchikova, I., Orr, A., & Brand, M. (2012b). Native rates of superoxide production from multiple sites in isolated mitochondria measured using endogenous reporters. *Free Radical Biology and Medicine*, *53*(9), 1807-1817.

Quinlan, C. L., Perevoshchikova, I. V., Hey-Mogensen, M., Orr, A. L., & Brand, M. D. (2013). Sites of reactive oxygen species generation by mitochondria oxidizing different substrates. *Redox biology*, *1*(1), 304-312.

Rigobello, M. P., Scutari, G., Boscolo, R., & Bindoli, A. (2002). Induction of mitochondrial permeability transition by auranofin, a Gold(I)-phosphine derivative. British Journal of Pharmacology, 136, 1162–1168.

Sandri, G., Panfili, E., & Ernster, L. (1990). Hydrogen peroxide production by monoamine oxidase in isolated rat-brain mitochondria: its effect on glutathione levels and Ca2+ efflux. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1035*(3), 300-305.

Servais, S., Couturier, K., Koubi, H., Rouanet, J.L., Desplanches, D., Sornay-Mayet, M.H., Sempore, B., Lavoie, J.M. and Favier, R., 2003. Effect of voluntary exercise on H2O2 release by subsarcolemmal and intermyofibrillar mitochondria. *Free Radical Biology and Medicine*, *35*(1), pp.24-32.

Shelton, M. D., Chock, P. B., & Mieyal, J. J. (2005). Glutaredoxin: role in reversible protein s-glutathionylation and regulation of redox signal transduction and protein translocation. *Antioxidants & redox signaling*, *7*(3-4), 348-366.

Sies, H. (2017). Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: Oxidative eustress. *Redox Biology*, *11*, 613-619.

Sies, H., & Jones, D. P. (2020). Reactive oxygen species (ROS) as pleiotropic physiological signalling agents. *Nature Reviews Molecular Cell Biology*, 1-21.

Speers-Roesch, B. and Treberg, J.R., 2010. The unusual energy metabolism of elasmobranch fishes. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *155*(4), pp.417-434.

Starkov, A. A., & Fiskum, G. (2003). Regulation of brain mitochondrial H2O2 production by membrane potential and NAD (P) H redox state. *Journal of neurochemistry*, *86*(5), 1101-1107.

Starkov, A. A., Fiskum, G., Chinopoulos, C., Lorenzo, B. J., Browne, S. E., Patel, M. S., & Beal, M. F. (2004). Mitochondrial α-ketoglutarate dehydrogenase complex generates reactive oxygen species. *Journal of Neuroscience*, *24*(36), 7779-7788.

Tamanna, N., Munro, D., Kroeker, K., Banh, S. and Treberg, J.R., 2020. The effect of short-term methionine restriction on hydrogen peroxide metabolism in Fischer-344 rat skeletal muscle mitochondria. *Mitochondrion*, *55*, pp.1-7.

Treberg, J. R., Martyniuk, C. J., Moyes, C. D., 2020. Getting the most out of reductionist approaches in comparative biochemistry and physiology. Comp. Biochem. Physiol. 250B, 110483.

Treberg, J. R., Quinlan, C. L., & Brand, M. D. (2011). Evidence for two sites of superoxide production by mitochondrial NADH-ubiquinone oxidoreductase (complex I). *Journal of Biological Chemistry*, *286*(31), 27103-27110.

Treberg, J. R., Braun, K., Zacharias, P., & Kroeker, K. (2018a). Multidimensional mitochondrial energetics: Application to the study of electron leak and hydrogen peroxide metabolism.

Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, *224*, 121-128.

Treberg, J. R., Munro, D., Jastroch, M., Quijada-Rodriguez, A. R., Kutschke, M., & Wiens, L. (2018b). Comparing electron leak in vertebrate muscle mitochondria. *Integrative and comparative biology*, *58*(3), 495-505.

Wagner, B.A., Witmer, J.R., van't Erve, T.J. and Buettner, G.R., 2013. An assay for the rate of removal of extracellular hydrogen peroxide by cells. *Redox biology*, *1*(1), pp.210-217.

Wiens, L., Banh, S., Sotiri, E., Jastroch, M., Block, B. A., Brand, M. D., & Treberg, J. R. (2017). Comparison of mitochondrial reactive oxygen species production of ectothermic and endothermic fish muscle. *Frontiers in Physiology*, *8*, 704.

Zoccarato, F., Cavallini, L., & Alexandre, A. (2004). Respiration-dependent removal of exogenous H2O2 in brain mitochondria Inhibition BY Ca2+. *Journal of Biological Chemistry*, *279*(6), 4166-4174.

Figure Legends

Figure 1. General compartmentalization of hydrogen peroxide metabolism in a hypothetical animal cell. Arrows indicate potential for transport and metabolism between compartments. Note the expected high inward diffusion gradient for cells in vivo (based on Sies, 2017). The shaded box highlights the focus of this review, which is the role mitochondria may have in overall cellular H_2O_2 metabolism. Peroxisomes, mitochondria and the endoplasmic reticulum all have both source(s) and sinks for H_2O_2.

Figure 2. Important sites of electron leak, routes of H_2O_2 consumption and specific effects of inhibitor strategies discussed in this review. Intramitochondrial superoxide/ H_2O_2 metabolism related to assay conditions. Substrates used, and some of their products, shown as well as relevant enzymes while several metabolites and transport pathways are omitted for clarity. Dotted lines indicate multiple pathways to form α-ketoglutarate. Sites of ROS production relevant to the current experiments shown based on (Quinlan et al., 2012b, Quinlan et al., 2013). Major sites of superoxide/ H_2O_2 production in this study are indicated and include the flavin of complex I (Site I_F), the high superoxide producing condition (Site I_Q) from complex I, that is inhibited by rotenone, when oxidizing succinate. The outer ubiquinone binding site of complex III (Site $IIIQ_o$) is another major site of electron leak, but it releases superoxide to both the matrix and intermembrane space and can be selectively inhibited with stigmatellin (Treberg et al., 2010). The α-ketoglutarate dehydrogenase (αKGDH) complex produces ROS exclusively in the matrix but predominantly procures H_2O_2 instead of superoxide. Note, although complex II may produce ROS, because of the high succinate and malate concentrations used it is assumed to be a negligible contributor in this study (Quinlan et al., 2012a). Image for sites of production modified from Banh et al., 2016 (used with permission).

Matrix H_2O_2 can be metabolized primarily via one of two NADPH-dependent pathways although in mitochondria for non-muscle tissues catalase (not shown) may also contribute; thioredoxin dependent pathway which requires peroxiredoxins (PRx) and thioredoxin reductase (TR) with

thioredoxin (TR) as the intermediate, and the glutathione-dependent pathway which uses glutathione peroxidases (GPx) and glutathione reductase (GR) with glutathione as the intermediate.

NADPH is replenished via matrix dehydrogenases as well as being held generally much more reduced than the NADH-pool because of the action of nicotinamide nucleotide transhydrogenase (NNT), which reduces $NADP^+$ to NADPH at the cost of NADH and the protonmotive force.

Figure 3. Illustration of electron flow from substrate oxidation through energy conserving electron transport complexes and supply of NADPH for matrix H_2O_2 consumers. Focus is on Complex I (CI) and Complex III (CIII) because they are the main points where all net electron flux must pass through from the NAD-isopotential and Ubiquinone(Q)-isopotential sites of electron leak respectively (see Brand 2016 for review). The coupling of electron flow with generation of the protonmotive force (Δp) causes a build up of electrons upstream of energy conserving complexes as the Δp increases because contribution to the Δp becomes increasingly unfavourable. NADPH is also supplied to consume H_2O_2 either directly from NADP-dependent dehydrogenases or indirectly via the nicotinamide nucleotide transhydrogenase (NNT) which uses the Δp to drive the hydride transfer of electrons from NADH to $NADP^+$. As the Δp increases our findings support the contention that the NADPH supply to peroxidases increases in advance of the Δp driven increase in electron leak rates.

Figure 4. Example of decision-making process we use for selecting a mitochondrial preparation

Figure 5. Mitochondrial H_2O_2 metabolism in relation to detection systems.

Figure 6. Comparison of rates of H_2O_2 production from different inhibitor-defined sites by control and CDNB-pretreated mitochondria. Site I_Q: succinate was 5 mM. This rate was more than 60% sensitive to rotenone, indicating that it was predominantly from site I_Q. Superoxide derived from site I_Q was titrated down with submaximal to maximal concentrations of rotenone (0–4.6 μm). Site $IIIQ_o$: succinate (5 mM), rotenone (4μm) and antimycin A (AA) at 100 nM were present. This rate was almost fully sensitive to stigmatellin, indicating that it was predominantly from site $IIIQ_o$. The rate of matrix-directed superoxide (triangles) was calculated as described in the text, and was titrated down with stigmatellin from 0 to 200 nM. The dashed line indicates a 1:1 relationship; the dotted line shows the hyperbola derived from multiple sites of production (for comparison). Data are the mean±SEM for three independent experiments. Error bars that are not visible are obscured by the symbol. Reprinted from Treberg et al., FEBS Journal, 277 (13), 2766-2778, Copyright (2010), with permission from Elsevier.

Figure 7. Assays for accumulation of H_2O_2 in mitochondrial respiration medium. Mitochondria are added, at 0.2 mg protein ml^{-1}, to cuvettes with linking enzymes already present and fluorescence increase is monitored over time. Increases in fluorescence is a function of H_2O_2 outside of the mitochondrion. * indicate the addition of Amplex Ultrared, which completes the H_2O_2 consuming fluorescence detection system. Mitochondria incubated with rotenone (4 μM) and then energized with succinate (5 mM) have measurable rates of electron leak (combined

superoxide and H_2O_2 production), measured as H_2O_2 efflux, but do not accumulate measureable H_2O_2 in the medium (**A.**). Inclusion of 1 µM auranofin (**B.**) leads to an increase in apparent electron leak rate and a time-dependent accumulation of H_2O_2 in the medium (indicated by the arrows). The appearance of H_2O_2 is a function of auranofin concentration (**C.**) Fitting data to Eq. (1) determines V^P and k which can then be used to calculate $[H_2O_2]_{ss}$ (**C.** *inset*). Data are mean±SEM for n = 3–4 independent experiments. For clarity the SEM is omitted in the *inset* figure. Reprinted from Treberg et al., Redox Biology, 20, 483-488, Copyright (2019), with permission from Elsevier.

Figure 8. Relationships between membrane potential (ΔΨ) and mitochondrial respiration (A.) or electron leak (B.). Isolated rat skeletal muscle mitochondria (0.2 mg/ml) and all were assayed with 5 mM succinate and 4 µM rotenone plus 1 µM auranofin (State 2). Other conditions include, addition of 0.5 mM ADP (State 3) followed sequentially by 1 µg/ml oligomycin (State 4o), and two step-wise additions of FCCP (Sate 4o + FCCP). In a separate series of experiments, mitochondria were assayed under State 2 condition with either 0, 0.5, 1.0 or 5.0 mM malonate (Grey symbols). Data are mean±SEM (n=4-5). If error bars are not visible they are obscured by the symbol. Reprinted from Treberg et al., Redox Biology, 20, 483-488, Copyright (2019), with permission from Elsevier.

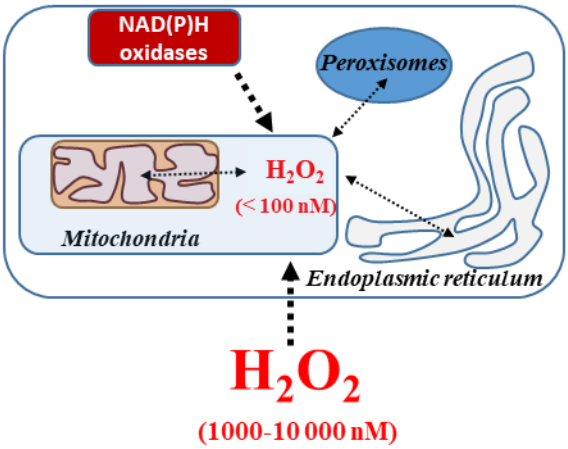
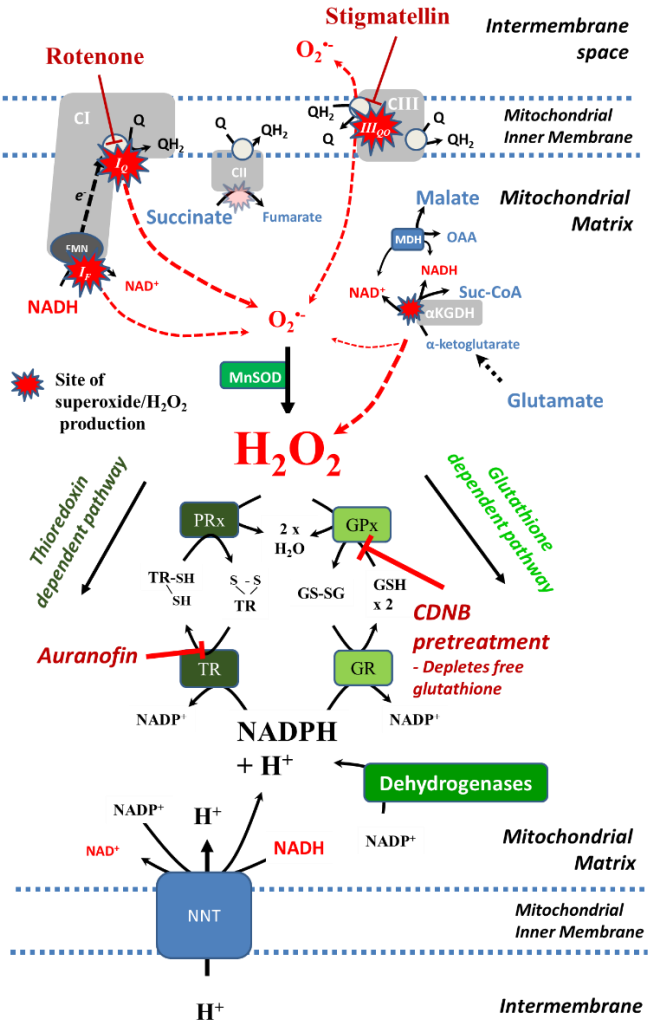

NAD(P)H oxidases
Peroxisomes
H_2O_2
(< 100 nM)
Mitochondria
Endoplasmic reticulum
H_2O_2
(1000-10 000 nM)

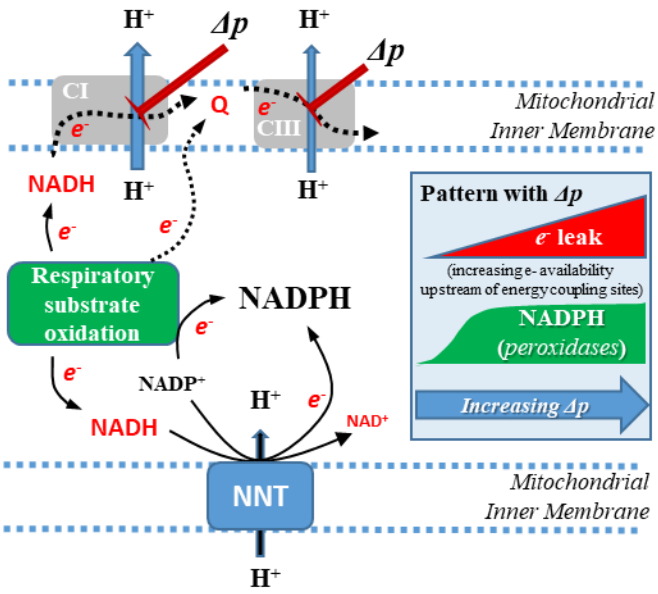
Figure 1



1120

1121

1122 Figure 2



1123

1124 Figure 3

1125

1126

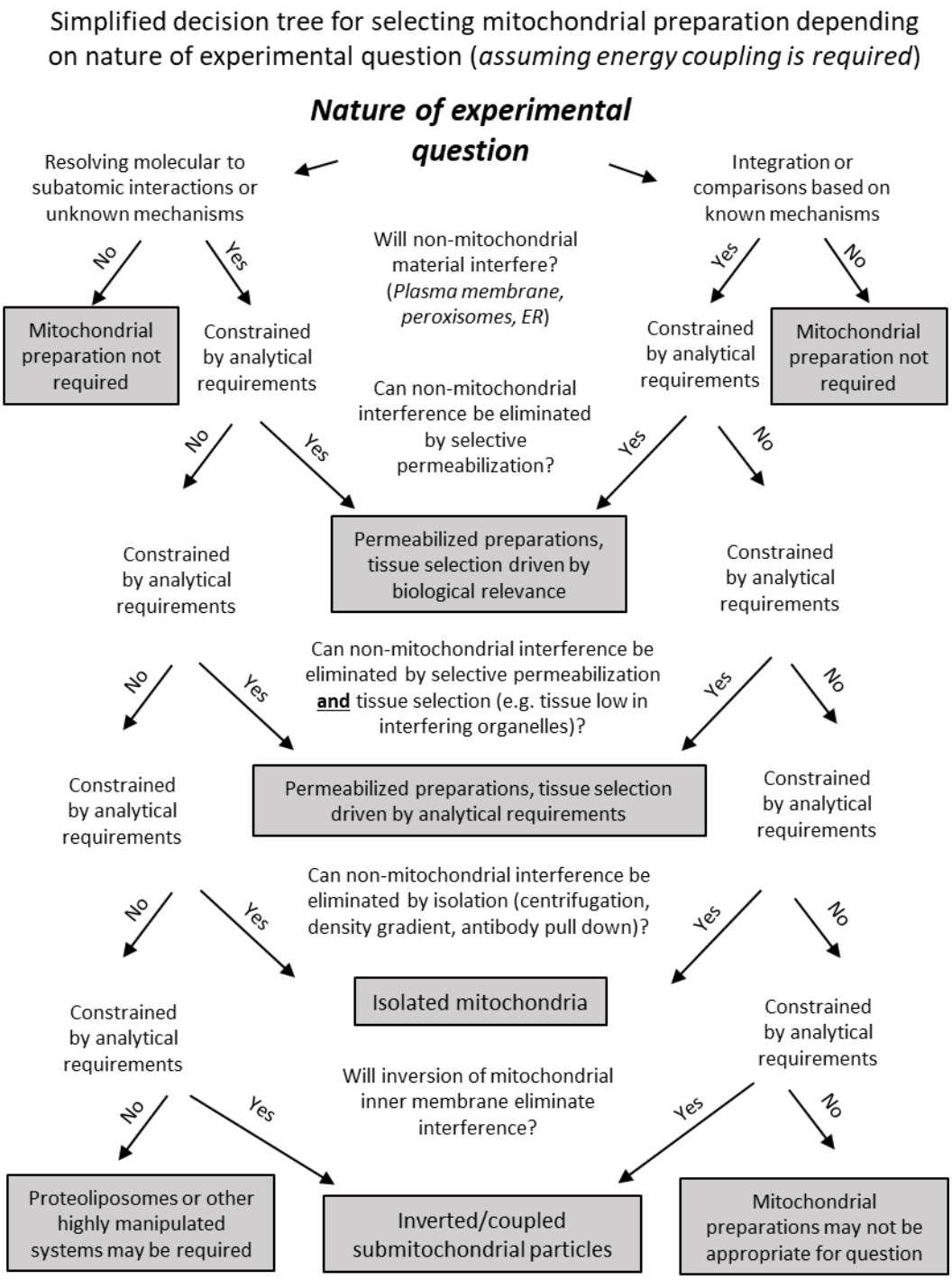
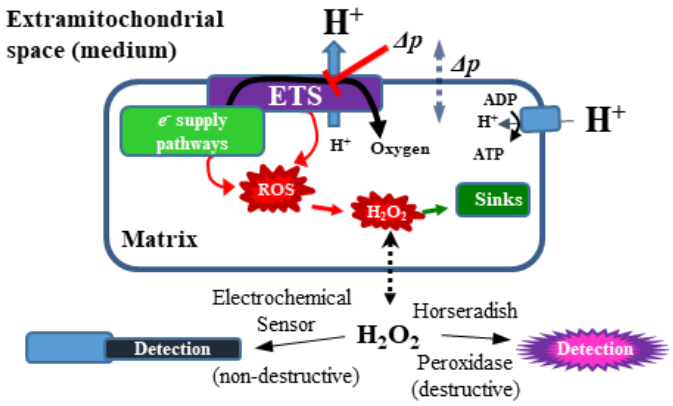

Simplified decision tree for selecting mitochondrial preparation depending on nature of experimental question (*assuming energy coupling is required*)
Nature of experimental question
Resolving molecular to subatomic interactions or unknown mechanisms
Integration or comparisons based on known mechanisms
No
Yes
Yes
No
Will non-mitochondrial material interfere? (*Plasma membrane, peroxisomes, ER*)
Mitochondrial preparation not required
Constrained by analytical requirements
Constrained by analytical requirements
Mitochondrial preparation not required
No
Yes
Yes
No
Can non-mitochondrial interference be eliminated by selective permeabilization?
Constrained by analytical requirements
Permeabilized preparations, tissue selection driven by biological relevance
Constrained by analytical requirements
No
Yes
Yes
No
Can non-mitochondrial interference be eliminated by selective permeabilization **and** tissue selection (e.g. tissue low in interfering organelles)?
Constrained by analytical requirements
Permeabilized preparations, tissue selection driven by analytical requirements
Constrained by analytical requirements
No
Yes
Yes
No
Can non-mitochondrial interference be eliminated by isolation (centrifugation, density gradient, antibody pull down)?
Isolated mitochondria
Constrained by analytical requirements
Constrained by analytical requirements
No
Yes
Yes
No
Will inversion of mitochondrial inner membrane eliminate interference?
Proteoliposomes or other highly manipulated systems may be required
Inverted/coupled submitochondrial particles
Mitochondrial preparations may not be appropriate for question

Figure 4



1131

1132 Figure 5

1133

1134

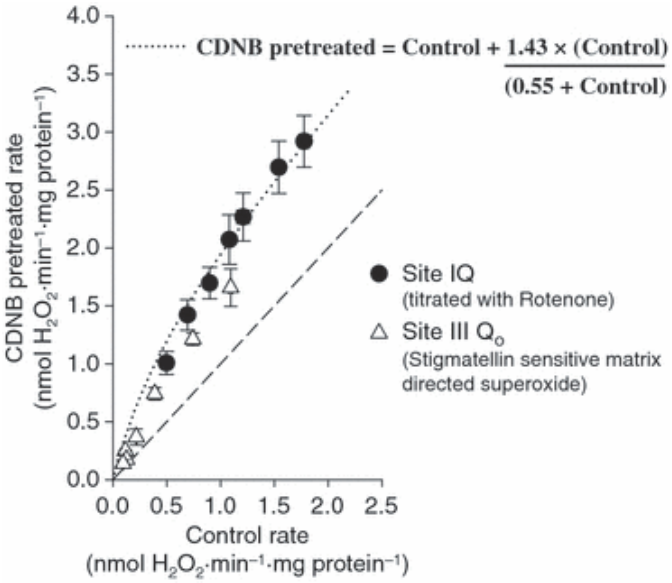
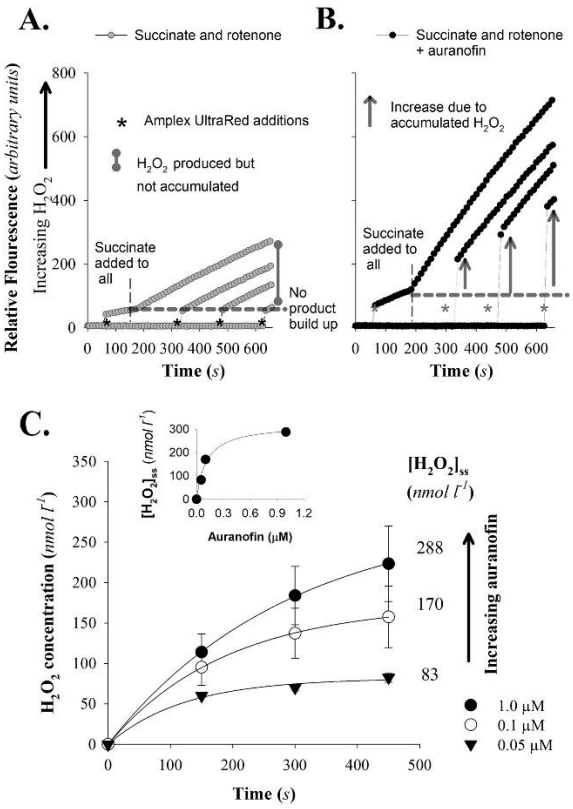

CDNB pretreated = Control + 1.43 × (Control) / (0.55 + Control)
CDNB pretreated rate (nmol H_2O_2·min⁻¹·mg protein⁻¹)
Control rate (nmol H_2O_2·min⁻¹·mg protein⁻¹)
Site IQ (titrated with Rotenone)
Site III Q_o (Stigmatellin sensitive matrix directed superoxide)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0

Figure 6

1140

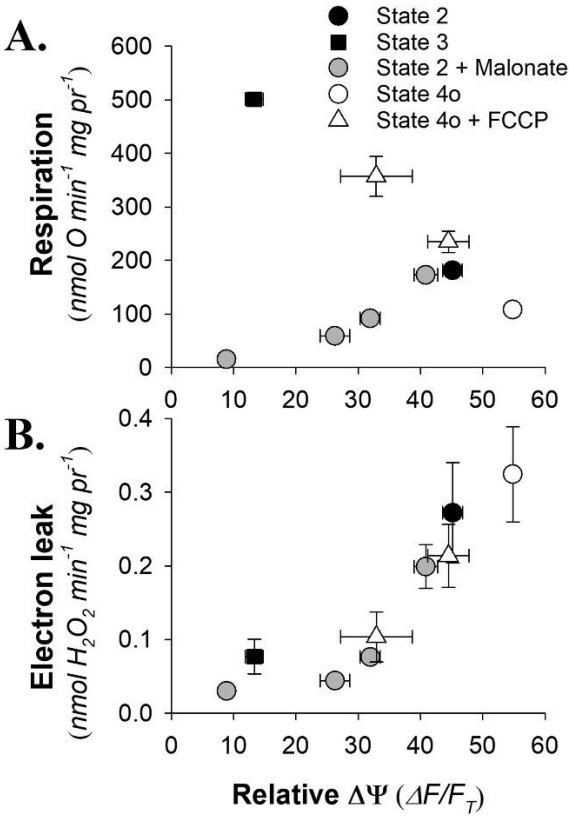


1141

1142

1143 Figure 7.

1144



1145

1146 Figure 8

1147