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SYMPOSIUM

Comparing Electron Leak in Vertebrate Muscle Mitochondria

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Synopsis Mitochondrial electron transfer for oxidative ATP regeneration is linked to reactive oxygen species (ROS) production in aerobic eukaryotic cells. Because they can contribute to signaling as well as oxidative damage in cells, these ROS have profound impact for the physiology and survival of the organism. Although mitochondria have been recognized as a potential source for ROS for about 50 years, the mechanistic understanding on molecular sites and processes has advanced recently. Most experimental approaches neglect thermal variability among species although temperature impacts mitochondrial processes significantly. Here we delineate the importance of temperature by comparing muscle mitochondrial ROS formation across species. Measuring the thermal sensitivity of respiration, electron leak rate (ROS formation), and the antioxidant capacity (measured as H_2O_2 consumption) in intact mitochondria of representative ectothermic and endothermic vertebrate species, our results suggest that using a common assay temperature is inappropriate for comparisons of organisms with differing body temperatures. Moreover, we propose that measuring electron leak relative to the mitochondrial antioxidant capacity (the oxidant ratio) may be superior to normalizing relative to respiration rates or mitochondrial protein for comparisons of mitochondrial metabolism of ROS across species of varying mitochondrial respiratory capacities.

Introduction

It has been known for approximately 50 years that the overall process of electron transfer in mitochondria is not completely efficient (Jensen 1966; Loschen et al. 1971; Boveris et al. 1972). As electrons move through the various supply pathways upstream of the electron transport system (ETS) and subsequently flow down the ETS, some of them prematurely exit, or leak out, before reaching the terminal acceptor (oxygen) at the level of complex IV. Part of the inefficiency pertains to the capacity of electronegative molecular species, such as dioxygen (O_2), to snatch electrons away from proteins in the ETS and the various electron supply routes (Krebs cycle, mGPDH, β-oxidation of lipids) (reviewed in Andreyev et al. 2005; Brand 2016). This electron leak forms the two primary reactive oxygen species (ROS) produced by mitochondria: superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2).

The role of mitochondria in cellular ROS metabolism or redox balance is at the core of many hypotheses relating to mitochondrial function (a very short list of reviews: Slimen et al. 2014; Barja 2007; Schieber and Chandel 2014). Skeletal muscle mitochondria, from the rat, have been extensively characterized for the mechanisms and sites of electron leak and the contribution of H_2O_2 to ROS metabolism (reviewed in Brand 2016; Munro and Treberg 2017). Skeletal

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muscle is a major component to metabolic demand (Rolfe and Brown 1997) and differences in muscle mitochondrial volume are a key correlate between interspecific differences in locomotory or aerobic performance, at least in mammals (Weibel et al. 2004). Altered mitochondrial function has also been linked to aging-related declines in performance (Barazzoni et al. 2000; Short et al. 2005). The detailed mechanistic knowledge combined with the ubiquitous role of skeletal muscle in animal physiology makes muscle an ideal system for comparative studies. However, exploring linkages between mitochondrial ROS and muscle function in a comparative context raises the important question of how to compare mitochondrial ROS metabolism.

The appropriate answer for comparative research requires review of (i) fundamental processes of electron leak in mitochondria to produce ROS and (ii) factors known to influence these processes. Here we examine the core aspects of mitochondrial ROS metabolism and explore strategies for comparing mitochondrial electron leak across species. In particular, we address the issue of varying temperature effects across different components of mitochondrial metabolism for comparing ectotherm and endotherm species.

Fundamental concept of electron leak

The simplest system to produce ROS via electron leak is a single enzyme with one redox center (Fig. 1A). For clarity we show the reaction catalyzed in two steps: first a substrate (S1) is oxidized by the enzyme, meaning at least one electron has been transferred to the redox center of the enzyme followed by release of the product (P1). The hypothetical enzyme now binds a second substrate (S2) and transfers the electron(s), reducing S2 to the second product (P2). Assuming that oxygen has access to the redox center in this enzyme then there is a possible interaction where the electrons are prematurely transferred to oxygen instead of to the normal electron acceptor. If a single electron is transferred to oxygen superoxide is formed whereas if two electrons are donated this leads to the direct formation of H_2O_2. Of note, not all oxygen accessible redox centers produce ROS. Many redox centers have midpoint potentials that do not lead to favorable electron transfer to oxygen. For example the electron carrier cytochrome c so favorably accepts electrons that no matter how reduced the pool of cytochrome c becomes, there is little risk of direct superoxide formation from the redox center of the cytochrome even though oxygen has access. Indeed, cytochrome c is an exquisitely good scavenger of superoxide, as evidenced by use of cytochrome c reduction in many superoxide dismutase (SOD) activity assays.

Based on this simple model we can now argue that for any given candidate "site" of electron leak, "X" in this case, the rate of ROS formation will be a function of the number of enzyme complexes present (protein expression level) and the relative reduction state ($[X_{\text{reduced}}]/[X_{\text{oxidized}}]$) of the population of enzyme complexes or specific redox centers (Fig. 1B). This model is overly simplistic because it ignores the importance of oxygen availability (Boveris and Chance 1973; Treberg et al. 2018), as well as how the nature of the electron leaking species (Quinlan et al. 2011) or substrate and product binding (Quinlan et al. 2012a; Siebels and Dröse 2013) can influence electron leak. Nevertheless, this conceptual model leads to a straightforward inference: the likelihood of electron leak increases with increasing electron availability within the systems linked to mitochondrial respiratory substrate oxidation.

Assaying rates of mitochondrial electron leak and the relevance of H_2O_2 metabolism

We and many other groups use H_2O_2 efflux from mitochondria to measure the rate of electron leak. This approach accepts that both superoxide and H_2O_2 are products of electron leak in mitochondria but matrix SOD activity combined with the relatively rapid spontaneous dismutation of superoxide leads to the production of the membrane permeant and more stable (longer half-life) H_2O_2. This assay further relies on the capacity of H_2O_2 to traverse biological membranes and escape the mitochondrion. In most cases extramitochondrial H_2O_2 is detected using an enzyme coupled assay that destroys H_2O_2 to produce a stable fluorescent end product that can be monitored kinetically (for example see Affourtit et al. 2012). In our assays we also add high levels of extramitochondrial SOD to both prevent auto-oxidation of Amplex Red (Starkov et al. 2002; Starkov and Fiskum 2003) and to convert any small amounts of extramitochondrially directed superoxide that escape the intermembrane space to H_2O_2. It is important to stress that this assay only detects the ROS that escapes the mitochondrion.

Underestimation of electron leak rate due to matrix H_2O_2 consumption

Although the measurement of H_2O_2 efflux provides a convenient kinetic assay of electron leak with intact mitochondria, there are compartmentalization issues. The mitochondrial matrix possesses several

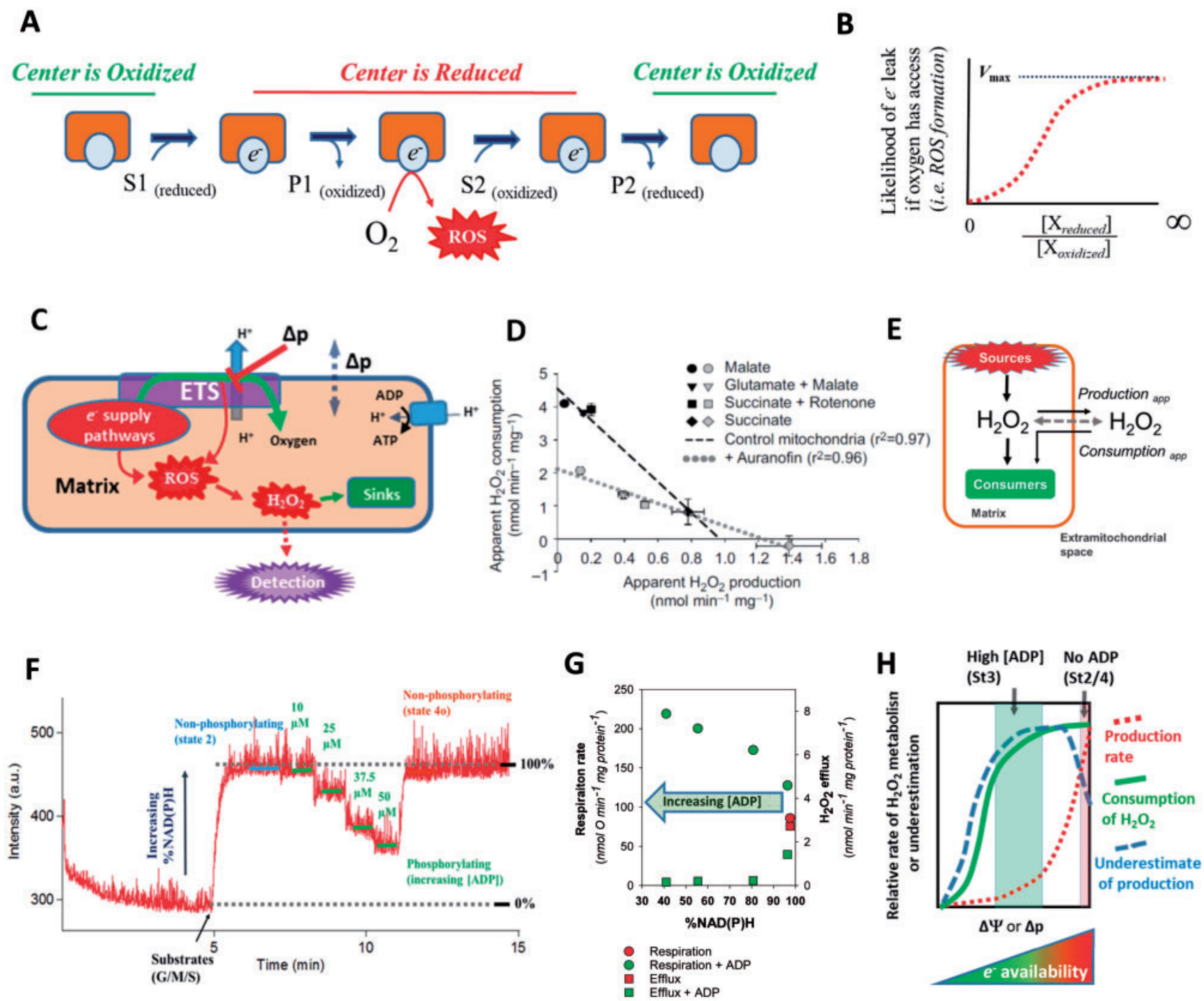

Fig. 1 Cartoon of hypothetical redox center capable of electron leak to oxygen (**A**) through one catalytic cycle and (**B**) the relationship between the redox center's reduction state and the likelihood of electron leak (see text for additional details). (**C**) Graphical summary of the major components involved with integrating electron flow, oxidative phosphorylation, electron leak, and antioxidant H_2O_2 consumers. Note that the protonmotive force (Δp) can impede electron flow through the system. (**D**) Illustration of the relationship between the apparent production of H_2O_2 (measured as efflux) and the H_2O_2 consumption capacity of isolated rat muscle mitochondria (from Munro and Treberg [2017] used with permission). Note the *y*-intercept should estimate the actual rate of consumption when there is no confounding effect of production. (**E**) Synthetic model illustrating underestimation of H_2O_2 metabolism in isolated mitochondria leading to apparent (app) rates of production and consumption (summarized and redrawn based on concepts in Treberg et al. [2015]). (**F**) Representative trace of %NAD(P)H autofluouresence with rat skeletal muscle mitochondria at 37°C assayed as described elsewhere (Munro et al. 2016) with the exception of having an ADP regenerating system comprised of 10 mM glucose and 0.25 units/mL of exogenous hexokinase added. Oligomycin will block proton return by ATP synthase. (**G**) Comparison of the %NAD(P)H reduction state, as a measure of electron availability and a proxy correlate of Δp (under these conditions as %NAD(P)H declines so does Δp whereas respiration rates increases) and H_2O_2 efflux rate (measured as described in Munro et al. 2016) under the same assay conditions. (**H**) Conceptual model incorporating concepts on mitochondrial H_2O_2 balance and underestimation of electron leak rates for a specific substrate mixture (see Starkov et al. 2014; Treberg et al. 2015; Munro et al. 2016; Munro and Treberg 2017) and electron availability in relation to respiratory states: non-phosphorylating conditions; also called LEAK conditions or State 2/4 (St 2/4) and phosphorylating states; also called State 3 (St. 3). The LEAK conditions will maximize electron leak rates and provide a stable capacity for H_2O_2 consumption therefore minimizing the degree of interference that occurs due to underestimation of electron leak rate measurements.

antioxidants routes capable of consuming H_2O_2. In particular it is now known that the capacity of mitochondria to consume H_2O_2 is typically much greater than the observed rates of electron leak under the same substrate/effectors conditions (Starkov et al. 2014; Munro and Treberg 2017).

Some inevitable underestimation of the actual electron leak rates is caused by intramitochondrial H_2O_2 consumers that have preferential access to the H_2O_2 produced (Fig. 1C) during assays where the detection system cannot reach the matrix. The capacity of mitochondria to consume H_2O_2 can be measured by adding exogenous H_2O_2 and monitoring the disappearance from the medium (Zoccarato et al. 2004; Drechsel and Patel 2010; Starkov et al. 2014; Treberg et al. 2015; Munro et al. 2016). These assays have shown a reciprocal relationship between the observed rates of H_2O_2 efflux and the capacity of mitochondria to consume exogenous H_2O_2 (Fig. 1D). We interpret this relationship to indicate that over a range of assay conditions there is competition for access to H_2O_2 consuming enzymes between the extramitochondrial H_2O_2 and the H_2O_2 produced by electron leak within the mitochondrion. Therefore, conditions of higher electron leak appear to have low rates of consumption. To uncover this competition we add auranofin, which inhibits the thioredoxin reductase-dependent consumption of matrix H_2O_2, and apparent rates of H_2O_2 efflux increase while apparent H_2O_2 consumption decreases (Fig. 1D). Therefore, we predict that, as the internal production of H_2O_2 increases, or internal consumption decreases, the disappearance of the exogenous H_2O_2 will decline and *vice-versa*. This model of H_2O_2 metabolism (Fig. 1E) thus asserts that true "actual" rates are occurring within the mitochondrion, but these true rates cannot be measured directly due current methodological limitations. Furthermore, the interaction between the intra- and extramitochondrial processes leads to some underestimation of these actual rates regardless if one is measuring electron leak as H_2O_2 efflux or using H_2O_2 consumption as a measure of mitochondrial antioxidant capacity (Fig. 1E).

Linking mitochondrial energy transformation to electron leak

A central consideration for our model is that the interaction between ROS production and energy transformation functions via feedback between these processes. Specifically, the electron flow through the energy transforming complexes I, III, and IV is coupled to proton translocation and thus generating the protonmotive force. The protonmotive force acts as a resistance to electron flow through these complexes as it will be harder to move protons against the proton gradient as the gradient increases. Thus, the electron availability should increase upstream of the proton translocating steps with increasing protonmotive force. In other words, under these conditions where the mitochondria are coupled the flow of electrons through the ETS can generate feedback against itself.

The linkage between electron availability and the protonmotive force can be readily demonstrated with isolated rodent mitochondria in the absence of respiratory inhibitors. Because Complex I catalyses a near-equilibrium reaction (Brown and Brand 1988), the relationship between protonmotive force and electron availability upstream of Complex I in the ETS can be monitored with isolated muscle mitochondria by measuring the relative reduction state of the NAD-pool. This can be performed in real-time by measuring the fluorescence of NADH, which has different fluorescent properties than the oxidized form (NAD^+). These measurements are well suited for muscle mitochondria because only a small part of the fluorescent signal derives from the NADP(H) pool relative to the NAD(H) pool (see Treberg et al. [2010] for a brief discussion of additional caveats of this assay and demonstration of application). Hence, this assay shows that the matrix NAD-pool becomes highly reduced when a respiratory substrate cocktail is added (Fig. 1F). The addition of ADP leads to protons flooding back to the matrix via ATP synthase, which results in a lower steady-state protonmotive force and an increase in the rate of respiration. Step-wise increases in ADP availability, across a physiologically relevant range (Dudley et al. 1987; Askenasy and Koretsky 2002), leads to a series of increasingly oxidized states for the NAD-pool (Fig. 1F), and an expected concomitant decline in the protonmotive force (Quinlan et al. 2012b).

An inverse relationship between electron flow and electron leak?

The respiratory substrates added (5 mM each of glutamate, malate, and succinate) in the %NAD(P)H experiment (Fig. 1F) were chosen to reach a very high level of electron availability and associated high capacity for electron leak. Parallel experiments were run to measure the rate of oxygen consumption and H_2O_2 efflux under these conditions (Fig. 1G). We find that increasing ADP concentrations increase the rate of respiration as expected. Simultaneously, the rate of H_2O_2 efflux deceases markedly as the %NAD(P)H (and protonmotive force) declines (Fig. 1G). This reciprocal relationship emphasizes how factors that increase electron flow through the ETS may also reduce ROS formation because of lower electron residency on potential sites of electron leak (lower $[X_{reduced}]/[X_{oxidized}]$ in our model).

A linkage between declining protonmotive force (or membrane potential) and lower rates of electron leak can be demonstrated with respiratory substrates that feed electrons into the NAD-pool or the Q-pool (Korshunov et al. 1997; Starkov and Fiskum 2003; Quinlan et al. 2012b; Treberg et al. 2017). Indeed, we find this pattern of declining electron leak with addition of ADP in muscle or heart mitochondria respiring on pyruvate/malate or other substrate mixes, including glutamate/malate, palmitoylcarnitine/malate, or succinate plus rotenone (L. Wiens and J. R. Treberg, unpublished data). This suggests that the pattern between the protonmotive force and electron leak may be generalizable independent of substrate; however, some exceptions to the pattern may occur (Kikusato and Toyomizu 2015).

How should mitochondrial processes be compared?

With the above context we return to the question, how should mitochondrial electron leak be compared across species? Answering this involves experimental decisions like (i) the respiratory state for mitochondrial assays and substrate choice(s), as well as, (ii) deciphering a means of normalizing, which may be highly influenced by physiological determinants like temperature.

Respiratory state

Given that measurements of electron leak with isolated mitochondria often rely on H_2O_2 efflux, which is confounded by the matrix consumption, it seems that minimizing the effects of these underestimates should be a priority. This can be best achieved for a given substrate choice by assaying under a "LEAK" respiration condition where the mitochondria are respiring but not phosphorylating (also called State 2, or State 4 depending on the source [for example see Estabrook 1967]). These respiratory conditions should maximize the actual rate of electron leak by maximizing protonmotive force, which leads to a lower underestimation given that the fractional underestimation of H_2O_2 production seems to decline as the absolute rate of production increases (Munro et al. 2016). Unfortunately, measuring rates in a LEAK respiratory state will also maximize the matrix H_2O_2 consumption capacity; however, if our model (Fig. 1H) is accurate then this is an acceptable compromise because the actual consumption capacity will at least be relatively consistent across assay conditions.

Conversely, it can be argued that mitochondria undergoing some degree of ADP-phosphorylation is a more physiologically relevant condition to test, as ATP turnover is always present in living cells. But, is a phosphorylating state the best way to compare mitochondrial electron leak? Addition of ADP introduces uncertainty to the assays of H_2O_2 metabolism because under most conditions this will decrease the absolute rate of electron leak, which should lead to a greater degree of underestimation (Treberg et al. 2015; Munro et al. 2016). If the goal is to have comparable assays across species and the number of analyses is limited then we recommend that ADP addition should be omitted. The degree of underestimation due to matrix consumption influences the results of comparative studies. High levels of ADP may cause a modest decline in H_2O_2 consumption capacity in the matrix or none at all (Munro and Treberg 2017). The effect of adding ADP could be quite variable between species depending on how much control phosphorylation of ADP has on the energetics of mitochondria from a given species (Fig. 1H). Therefore, if consistency in the comparisons is a benefit, then measuring electron leak under LEAK respiration conditions would seem to be the superior choice due to minimizing, or at least stabilizing, the confounding effects of underestimations (Fig. 1H).

Substrate choices

The major sites of electron leak depend heavily on the substrate choice (see review by Brand 2016). Since the relative ratios of the various mitochondrial ROS forming enzyme complexes may vary by tissue, species, or physiological condition it becomes challenging to determine which substrate(s) should be tested *a priori*. For comparative studies, we would recommend starting with saturating levels to maximize rates or at least minimize variation. Simplicity is key for initial comparisons of various systems and species.

Even if different respiratory substrates can lead to a range of apparent rates of electron leak, because the H_2O_2 consumption capacity is generally maximized across various conditions of respiratory substrate (Treberg et al. 2015; Munro and Treberg 2017), the relative rank-order of different substrates will be conserved for a species or experimental group despite underestimation of the actual rates. This allows for comparing across species which substrates, and by inference which enzyme complexes, may be most potent at leading to electron leak. But, caution is warranted in statements about absolute rates of electron leak if marked differences occur in H_2O_2 consumption capacity. For this reason, we now need to consider how comparisons can be made

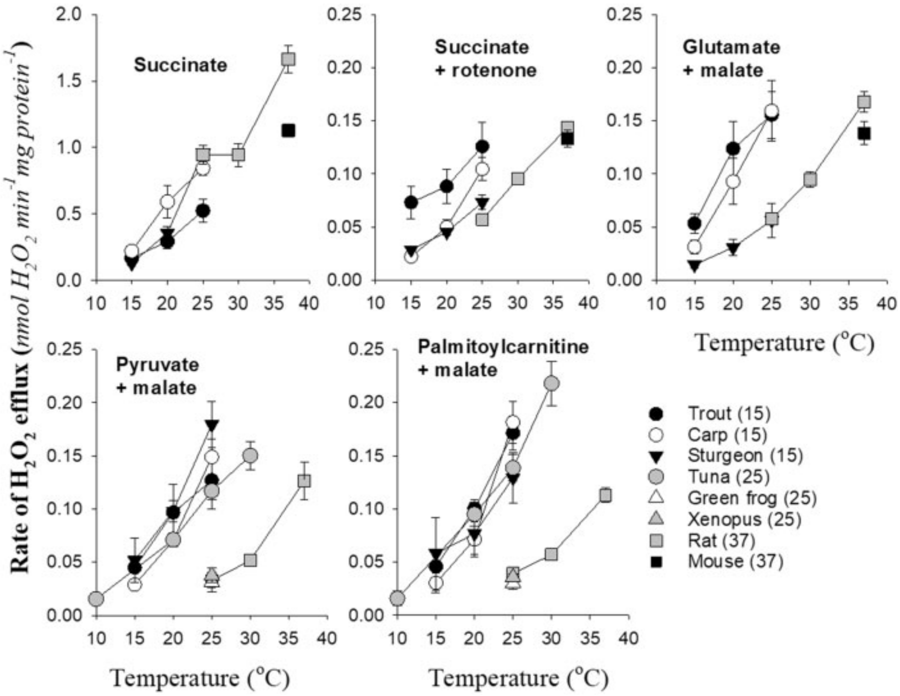

Fig. 2 The effect of assay temperature and substrate used on the rates of apparent electron leak, measured as H_2O_2 efflux in mitochondria isolated from the muscle of several vertebrates. The assay temperature is indicated on each panel and the physiological temperature for each species' muscle that was the source of the mitochondria is in parentheses in the symbol legend. The substrate cocktail added is indicated in each panel and in all cases additions were 5 mM except rotenone (4 μM) and palmitoylcarnitine (50 μM). Values are from Banh et al. (2016) or Wiens et al. (2017) with the exception of mouse (strain C57BL/6N), Green frog (*Lithobates pipiens*), and *Xenopus* (*X. laevis*), which are all previously unpublished and were isolated and assayed according to the same methods described in Wiens et al. (2017). Data are mean ± SEM (n = 3–6) with the exception of *Xenopus* which is mean ± range for n = 2. Where error bars are not visible they are obscured by the symbol.

across species by examining the data set we have developed on vertebrate muscle mitochondria from ectotherms and endotherms. This of course raises the important issue of temperature.

Temperature

It is well known that temperature influences the rate of biological processes and therefore needs to be considered when looking at species/systems with different physiological temperatures. The comparisons to be made can be considered at either physiological temperatures or a common test (assay) temperature; but which provides the better comparison? If the processes being compared show a common temperature sensitivity then it becomes straightforward to make all measurements at a common temperature. With isolated vertebrate muscle mitochondria assayed at different temperatures the electron leak shows marked temperature sensitivity with a wide range of respiratory substrates irrespective if the organism is an endotherm or an ectotherm (Fig. 2). In essence, as might be expected, the warmer the assay temperature, the higher the rate of H_2O_2 efflux but generalities between ectotherms and endotherms are elusive. The mitochondria from the endothermic muscle of Bluefin Tuna appear similar to mitochondria from ectothermic fish even though the physiologically relevant temperatures were 25°C and 15°C, respectively (Fig. 2). However, the rates for fish mitochondria at 25°C generally approach or exceed rates seen in rodent muscle mitochondria at 37°C. In all cases the fish mitochondria have higher rates than tetrapods at a common temperature of 25°C regardless if the mitochondria are from endothermic rat muscle or ectothermic amphibians acclimated to 25°C (Fig. 2). Of note, muscle fiber type could influence the nature of the mitochondria, and thus of ROS production. All the fish mitochondria were isolated from slow twitch muscle whereas the rodent muscle will be a mix of fast and slow fibers and the amphibians will be predominantly fast twitch fibers. However here, the fiber type falls short of explaining the higher rate of ROS formation in fish since mitochondria from fast twitch fibers (amphibian) reportedly have higher rates of electron leak than slow twitch fibers (Leary et al. 2003; Anderson and Neufer 2006).

Normalizing rates of mitochondrial electron leak

While the amount of mitochondrial protein is a very common means of normalizing rates with isolated mitochondria this assumes that the relative assemblage of proteins within the mitochondrial proteome is comparable across species or comparison groups. This contention has been challenged with respect to mitochondrial respiration, where the content of cytochrome A or adenine nucleotide translocase has been argued as potentially better means of comparing mitochondria from ectotherms and endotherms (Hulbert et al. 2006). Alternatively mitochondrial enzyme activities have been used to normalize, with the Krebs cycle enzyme citrate synthase and complex IV of the ETS (cytochrome c oxidase) being popular choices. But kinetic assays, like enzyme activities, may also be poor choices for normalizing in experiments on temperature if the enzymes do not possess identical sensitivity to temperature as the other processes being investigated.

However, as addressed above, mitochondrial electron leak is influenced by the overall mitochondrial system. Therefore, using an individual part of that system to represent a normalization of the whole seems less favorable than adopting other processes that reflect the mitochondrial system as an integrated whole. For this reason, we and others have used the respiratory capacity to compare rates of electron leak by expressing these processes as ratios. While a ratio of electron leak and respiration may be useful to address the efficiency of electron transport, this approach is compromised by the underestimations of true electron leak. However, the underestimation of electron leak should be a function of the H_2O_2 consumption capacity, leading to a new means of comparing across species and temperatures: the oxidant ratio, where the rate of H_2O_2 efflux is expressed relative to an estimate of H_2O_2 consumption capacity. A useful inference from Fig. 1D is that extrapolation of the regression lines to the *y*-axis intercept will predict the rate of consumption when there is no confounding influence of production. However, rates of H_2O_2 consumption with substrate mixes that have relatively low rates of H_2O_2 production provide a convenient approximation of this maximal value for H_2O_2 consumption capacity (Fig. 1D).

Comparing mitochondrial electron leak across vertebrate muscle

Rather than iteratively going through every possible permutation of the data in Fig. 2 we will focus on a simplified data set to illustrate and compare methods of contrasting mitochondria from different species. In the vertebrates used in Fig. 2 locomotion relies heavily on carbohydrates as a major fuel (Kieffer et al. 1998; McClelland 2004). For this reason we will focus on the data for pyruvate and malate (both at 5 mM), with the exception of mouse where data for glutamate and malate (both at 5 mM) will be considered as a similar assay condition relying on NADH generating substrates.

Using respiration as a denominator: comparing electron transfer efficiency

Comparative studies often use the fraction of electrons leaking to oxygen relative to overall electron flow to examine patterns across species. While often called the free radical leak, we prefer the term fractional electron leak (FEL) because some of the electron leak can produce non-radical H_2O_2 directly. We also considered the possibility that the State 3 respiration rate (presence of high ADP) may be a better indicator of the overall metabolic machinery involved in respiration and therefore a better normalizing trait. When assayed across a range of physiological temperatures or at a common temperature (25°C) a number of patterns emerge (Fig. 3). First, mitochondria from the fish and amphibians have similar respiration rates, albeit lower than those from rodents examined, even at a common assay temperature in the case of the rat (Fig. 3B, D). Regardless if respiration is under LEAK (Fig. 3B) or State 3 (Fig. 3D), changing the assay temperature influences the rate for mitochondria isolated from ectothermic and endothermic skeletal muscle, which is to be expected (Brooks et al. 1971; Johnston et al. 1998).

Maximal rates of respiration will be in part a function of how many enzyme complexes and electron carriers are present in the mitochondria. It is also expected that mitochondria with a relatively high density of enzyme complexes could have higher capacity for ROS production. Mitochondrial respiration rates in State 3 (high ADP availability) approach maximal respiration rates under many conditions. Therefore it could be argued that State 3 respiration should better reflect the overall total metabolic "machinery" of the mitochondria and be a better denominator for electron leak rates. Nevertheless, and important to past comparative studies, we find the same overall pattern regardless if using FEL or relying on State 3 respiration as the denominator, indicating that FEL is likely a reasonable simplification when considering the efficiency of electron transfer across species. However, even though the

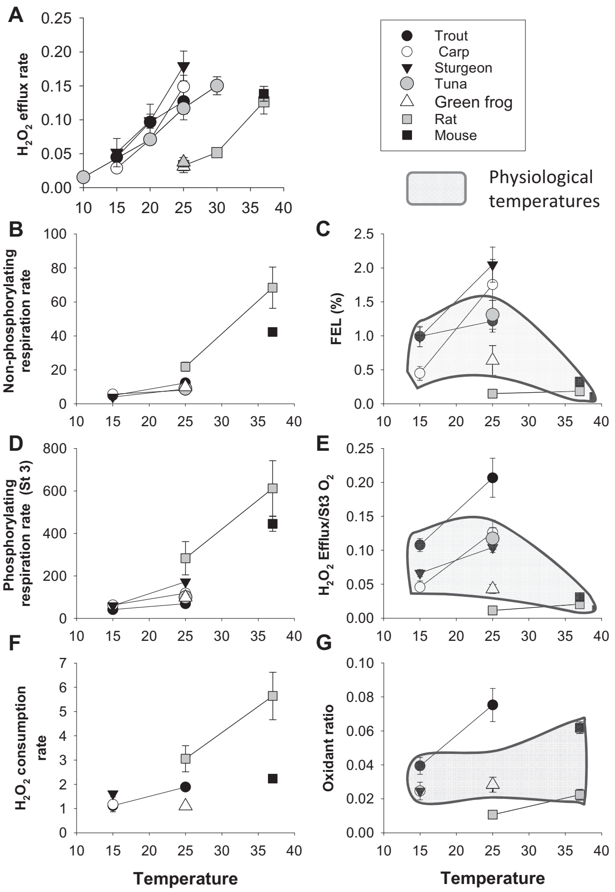

Fig. 3 Comparison of electron leak across different means of normalizing over different assay temperature. (**A**) Mitochondrial H_2O_2 efflux rates to estimate the electron leak. In all cases rates are from Fig. 2 and determined for isolated muscle mitochondria respiring on pyruvate and malate (5 mM of each) with the exception of mouse where the substrate was glutamate and malate (5 mM of each). Note that these two substrate cocktails give similar values for rat (Fig. 2). (**B**) The non-phosphorylating (LEAK) respiration was used to determine the fractional electron leak (FEL) in (**C**), see text for description. (**D**) The State 3 respiration rate (St3 O_2), where mitochondria are stimulated with high levels of ADP, was also tested as a denominator for efflux data (**E**). (**F**) The H_2O_2 consumption rate (measured as described in Munro et al. 2016), see text for details, was also determined and used to assess the Oxidant ratio (rate of electron leak: rate of H_2O_2 consumption) to evaluate comparisons across assay and physiological temperatures (**G**). Data are as described in Fig. 2 and are in nmol O min^{-1} mg $protein^{-1}$ or nmol H_2O_2 min^{-1} mg $protein^{-1}$.

rate of electron leak is also sensitive to temperature, the ratios of electron leak with LEAK respiration (Fig. 3C) or State 3 respiration (Fig. 3E) demonstrate that electron leak and respiration show markedly different sensitivities to temperature. Changes in respiration rates fail to keep pace with rates of electron leak leading to these ratios having a positive relationship with increasing assay temperature. This discrepancy between temperature sensitivities for the two processes indicates that normalizing by respiration and assaying at a common temperature are not necessarily a straightforward comparison.

Since using a common assay temperature appears to be a complicated and confounded means of comparing across these species, we consider comparisons made across the physiological temperatures (outlined by gray shading in Fig. 3C, E). At physiological assay temperatures a complex pattern between electron leak and respiration emerges with fish and amphibians appearing to have far greater potential for ROS production than the rodents assessed in this study. Although further species need to be studied, the potential implication of this pattern is that muscle mitochondria from ectotherms, and at least one endothermic fish, display disproportionately high electron leak compared with tetrapod endotherms.

The oxidant ratio: pro-oxidant electron leak relative to antioxidant H_2O_2 consumption

If some species have higher mitochondrial electron leak than others, it is possible they may also possess a concomitantly high antioxidant capacity. To test this hypothesis we use the oxidant ratio, which we define as a rate of electron leak under a defined condition of substrate addition relative to the estimate of H_2O_2 consumption. To maximize H_2O_2 consumption a respiratory substrate needs to be added (Treberg et al. 2015) and based on the rationale in Fig. 1D we assert that conditions of relatively low electron leak (malate alone or malate + glutamate) can give reasonable approximation of this maximal consumption capacity. This oxidant ratio will increase when electron leak predominates over the relative H_2O_2 consumption capacity. Thus, higher values for the oxidant ratio would be consistent with a more pro-oxidant poise for the mitochondria.

The H_2O_2 consumption capacity for mitochondria from all species examined is markedly higher (Fig. 3F) than the rates of electron leak (Fig. 3A). For an ectothermic fish (Rainbow Trout) and a rodent (rat) we find the H_2O_2 consumption capacity is sensitive to assay temperature and there is a positive relationship between temperature and the rate of H_2O_2 consumption (Fig. 3F). However, the oxidant ratio for the Rainbow Trout and rat mitochondria at physiological temperature, 15°C and 37°C respectively, is markedly different (Fig. 3G) than that for these species at a common assay temperature (25°C). This difference indicates that the processes of mitochondrial electron leak and H_2O_2 consumption also have different sensitivities to temperature, at least for muscle from these two species. Such differential temperature sensitivity between pro-oxidant and antioxidant processes may have physiological implications to tissues experiencing rapid changes in temperature, such as during hibernation (Brown et al. 2012) or acute environmental warming in ectotherms (Bagnyukova et al. 2007); however, as a means of comparing across species we see that the oxidant ratio may also be fraught with confounding temperature effects if a common assay temperature is used across species of differing physiological temperatures. For this reason we recommend using the physiological temperatures to assess muscle mitochondria from this group of animals.

Interestingly, when assayed at the physiologically relevant temperatures (highlighted by gray in Fig. 3G) the oxidant ratio shows a relatively narrow range across the vertebrates species examined. Indeed, despite the pattern seen with rodents having very low FEL (Fig. 3C) the oxidant ratio for rodents is not clearly different from ectotherms. Unfortunately, we do not have data for the endothermic Bluefin Tuna muscle for the oxidant ratio. Nevertheless, our data suggest that species with disproportionately inefficient electron transfer relative to respiration (high FEL values) may also display a compensatory increase in antioxidant capacity. Therefore, the oxidant ratio data suggest that it may be inappropriate to make inferences on the role of mitochondria in oxidative stress based solely on FEL or similar means of normalizing comparative data.

Summary

To begin constructing a comparative dataset on mitochondrial function, be it electron leak or other processes, we recommend starting with assay conditions that are stable and reproducible. Given the innate underestimations involved with measuring H_2O_2 metabolism our solution is to assay in a non-phosphorylating (LEAK) state of respiration under high concentrations of specific substrates. This should minimize the confounding effects of underestimations allowing for better estimates of maximal potential electron leak. The observation that mitochondrial processes like respiration, electron leak, and H_2O_2 consumption differ in their temperature sensitivity indicates that assaying processes at a common temperature (or any temperature significantly departing from physiological) is an inappropriate solution to study species having different physiological temperatures. This may have particular implications for comparative studies using temperature acclimation or contrasts between ectotherms and endotherm. For comparison across groups where mitochondrial performance may vary (e.g., respiratory capacities are different) the FEL approach may give insight into relative efficiency of electron transfer but is likely misleading with respect to understanding mitochondria in oxidative/redox balance. For the latter the oxidant ratio may give better insight.

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