A radical shift in perspective: mitochondria as regulators of reactive oxygen species

Daniel Munro\textsuperscript{1,2} and Jason R. Treberg\textsuperscript{1,2,3,*}

**ABSTRACT**

Mitochondria are widely recognized as a source of reactive oxygen species (ROS) in animal cells, where it is assumed that over-production of ROS leads to an overwhelmed antioxidant system and oxidative stress. In this Commentary, we describe a more nuanced model of mitochondrial ROS metabolism, where integration of ROS production with consumption by the mitochondrial antioxidant pathways may lead to the regulation of ROS levels. Superoxide and hydrogen peroxide ($\text{H}_2\text{O}_2$) are the main ROS formed by mitochondria. However, superoxide, a free radical, is converted to the non-radical, membrane-permeant $\text{H}_2\text{O}_2$; consequently, ROS may readily cross cellular compartments. By combining measurements of production and consumption of $\text{H}_2\text{O}_2$, it can be shown that isolated mitochondria can intrinsically approach a steady-state concentration of $\text{H}_2\text{O}_2$ in the medium. The central hypothesis here is that mitochondria regulate the concentration of $\text{H}_2\text{O}_2$ to a value set by the balance between production and consumption. In this context, the consumers of ROS are not simply a passive safeguard against oxidative stress; instead, they control the established steady-state concentration of $\text{H}_2\text{O}_2$. By considering the response of rat skeletal muscle mitochondria to high levels of ADP, we demonstrate that $\text{H}_2\text{O}_2$ production by mitochondria is far more sensitive to changes in mitochondrial energetics than is $\text{H}_2\text{O}_2$ consumption; this concept is further extended to evaluate how the muscle mitochondrial $\text{H}_2\text{O}_2$ balance should respond to changes in aerobic work load. We conclude by considering how differences in the ROS consumption pathways may lead to important distinctions amongst tissues, along with briefly examining implications for differing levels of activity, temperature change and metabolic depression.

**KEY WORDS:** Hydrogen peroxide, Energetics, Antioxidant, Skeletal muscle, Oxidative stress, Glutathione peroxidase, Thioredoxin, Peroxiredoxin

**Introduction**

Over 40 years ago (Jensen, 1966; Loschen et al., 1971; Boveris et al., 1972; Boveris and Chance, 1973) it was discovered that mitochondria can produce reactive oxygen species (ROS; see Glossary). Since then, the realization that mitochondria may be a source of potentially harmful ROS has profoundly influenced the perceived role(s) of mitochondria in cellular function. The proposed physiological effects of ROS include life-history and energetic trade-offs, cellular dysfunction and damage in response to intense or prolonged activity, and even senescence and ageing (Trushina and McMurray, 2007; Costantini, 2008; Monaghan et al., 2009; Dai et al., 2014; Day, 2014; Mason and Wadley, 2014). For example, during migration, a trade-off may occur between individual performance and survival or reproductive fitness, owing to oxidative damage accrued during intense exercise. Publications in these areas, and many others, often describe mitochondria as the ‘major source’ of ROS in animal cells, despite the high antioxidant capacity of mitochondria (Zoccorato et al., 2004; Dreschel and Patel, 2010; Banh and Treberg, 2013). However, the rationale behind the notion of mitochondria as the major source of ROS in animal cells has been questioned (Brown and Borutaite, 2012), if not directly challenged, based on the capacity of isolated mitochondria to consume substantial amounts of ROS (Zoccorato et al., 2004). It is worth noting that it can be challenging to test hypotheses on mitochondrial function using cells, whole tissue or organism systems because of the additional control exerted by the plasma membrane. For this reason, isolated mitochondria are a valuable tool in understanding how mitochondria may respond to changing cellular conditions (which can be simulated by manipulation of the assay medium). Findings can then inform the generation of hypotheses applicable to higher levels of biological organization.

Recently, a different and more nuanced perspective on the role of mitochondria in ROS balance has been emerging: the contention that the ROS-producing and antioxidant-mediated ROS-consuming pathways of the mitochondrion can be integrated into a regulatory system. This perspective views ROS as specific regulatory molecules, consistent with their role in signalling, rather than simply as inevitable toxic by-products of aerobic metabolism, although excess ROS will cause oxidative stress. To our knowledge, the formalization of these ideas is rooted in work by Andreyev and colleagues (reviewed in Andreyev et al., 2005), initially laid out as a hypothesis by Starkov (2008). Recent experimental evidence in redent brain and skeletal muscle mitochondria supports this hypothesis (Starkov et al., 2014; Treberg et al., 2015).

This Commentary will focus on the skeletal muscle system, and aims to illustrate how mitochondria can regulate ROS levels. After briefly addressing several concepts on the interplay between mitochondrial energetics (see Glossary) and the major pathways of mitochondrial ROS metabolism, we will illustrate how mitochondria can act as regulators of an important ROS, hydrogen peroxide ($\text{H}_2\text{O}_2$), \textit{in vitro}. Using the proposed model of mitochondria as regulators of $\text{H}_2\text{O}_2$, the impact of high ADP availability on mitochondrial $\text{H}_2\text{O}_2$ metabolism will be explored, followed by some consideration of how differing degrees of physical activity should affect the regulation of $\text{H}_2\text{O}_2$ by skeletal muscle mitochondria. Finally, we consider how temperature may influence mitochondrial $\text{H}_2\text{O}_2$ metabolism, the impact our model could have on metabolic depression during torpor and how the relevant processes may vary across tissues.

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Influence of H$_2$O$_2$ on glutathione (GSH) status

Recently, it has also been proposed that energy metabolism-linked regulators of mitophagy (Scherz-Shouval and Elazar, 2011). Mitochondrial ROS also act as biogenesis and modulation of the activity of Krebs cycle enzymes oxidative phosphorylation capacity through mitochondrial cells (reviewed in Sies, 2014). Mitochondrial ROS in particular impact insulin signalling and redox signalling (see Glossary) in tumour wound healing and cell-shape changes, and which also play a role in cascades controlling, for example, apoptosis, cell differentiation, wound healing and cell-shape changes, and which also play a role in mitochondrial energetics. Because electron flow through the mitochondrial electron transport system (ETS) and Krebs cycle to form ROS. The superoxide radical is the main ROS formed at most sites of production. This free radical (see Glossary) is rapidly dismutated to the non-radical ROS H$_2$O$_2$, which is membrane permeant and can thus diffuse between cellular compartments. The concentration of H$_2$O$_2$ in the mitochondrion (Cochemé et al., 2011) and in the cytosol (Arniaz et al., 1995; Palomero et al., 2008) appears to be maintained in the submicromolar range by antioxidants, which probably limits the formation of the membrane-permeant and highly reactive hydroxyl radical (Fig. 1B,C).

Recent studies have identified site-specific inhibitors of ROS production – at least for the outer ubiquinone-binding site in complex III – which have minimal effects on mitochondrial energetics (Orr et al., 2015). The potential for ROS production to cause damage to cells is nearly universal in aerobes – in light of the pharmacological effects discussed above, this raises the question of whether there might be evolutionary trade-offs between energy transformation in aerobic metabolism and ROS production. In other words, if ROS are universally damaging, then why has their production not been mitigated by natural selection to be effectively negligible?

The answer may lie in the now-recognized signalling role of ROS, which are often involved as second messengers in signalling cascades controlling, for example, apoptosis, cell differentiation, wound healing and cell-shape changes, and which also play a role in insulin signalling and redox signalling (see Glossary) in tumour cells (reviewed in Sies, 2014). Mitochondrial ROS in particular have been suggested to mediate feedback signalling to the nucleus and mitochondrial transcription machinery in order to adjust oxidative phosphorylation capacity through mitochondrial biogenesis and modulation of the activity of Krebs cycle enzymes (Moreno-Loshuertos et al., 2006). Mitochondrial ROS also act as regulators of mitophagy (Scherz-Shouval and Elazar, 2011). Recently, it has also been proposed that energy metabolism-linked mitochondrial redox signals may be indirectly mediated via the influence of H$_2$O$_2$ on glutathione (GSH) status – the ratio of GSH to its oxidized form, GSSG, is modulated in response to H$_2$O$_2$ concentration. This influence on the GSH pool alters the activity of proteins both inside and outside the mitochondrion through S-glutathionylation – a reversible post-translational modification compatible with a signalling role (Mailloux and Treberg, 2016). Therefore, ROS (or H$_2$O$_2$ at least) play roles in cell function that may require further processing by specific enzymes. Thus, H$_2$O$_2$ may be better viewed as a bona fide metabolite rather than simply an inevitable or unavoidable ‘by-product’ of aerobic metabolism.

Energetics and mitochondrial H$_2$O$_2$ metabolism

In this Commentary, our central thesis is that mitochondria can differentially regulate the concentration of H$_2$O$_2$ (through observed changes in its production and consumption) based on their current energetic state. Because electron flow through the mitochondrial ETS is coupled to proton translocation, the flux of electrons through to oxygen is impeded as the protonmotive force (PMF; see Glossary) increases. This impedance leads to a buildup of electrons in redox centres within the enzyme complexes that are

**List of symbols and abbreviations**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tr>
<td>GSH</td>
<td>Reduced form of glutathione</td>
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<tr>
<td>GSSG</td>
<td>Oxidized form of glutathione also known as glutathione disulphide</td>
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<tr>
<td>[H$_2$O$<em>2$]$</em>{ss}$</td>
<td>Steady-state concentration of H$_2$O$_2$</td>
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<tr>
<td>$k$</td>
<td>Rate constant</td>
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<tr>
<td>PMF</td>
<td>Protonmotive force</td>
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<tr>
<td>%NAD(P)H</td>
<td>Percent of combined NAD and NADP pools that are in the reduced form</td>
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<tr>
<td>$V_c$</td>
<td>Rate of H$_2$O$_2$ consumption</td>
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<tr>
<td>$V_{app}$</td>
<td>Apparent (measurable) rate of H$_2$O$_2$ consumption</td>
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<td>$V_p$</td>
<td>Rate of H$_2$O$_2$ production</td>
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<tr>
<td>$V_{p,app}$</td>
<td>Apparent (measurable) rate of H$_2$O$_2$ production</td>
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**Glossary**

**First-order kinetics**

Description of a reaction rate that is dependent on the concentration of one reactant and can be summarized by the following rate equation: rate of reaction=$k[A]$, where $A$ is the reactant that on which the rate is dependent and $k$ is the rate constant. First-order reactions follow an exponential decay curve for $A$ over time, which can be described by $A=A_0e^{-kt}$, where $A_0$ is the initial amount of $A$, $k$ is the same reaction constant already described and $t$ is time.

**Free radical**

An atom or molecule that has an unpaired electron, designated by the raised dot as seen with the superoxide anion (O$_2^{−}$), which is formed by the univalent (single electron) reduction of dioxygen (O$_2$).

**Mitochondrial energetics**

The interacting processes of electron and proton flux in the mitochondrion that link substrate oxidation to energy transformation. Major components include the membrane potential ($\Delta\Psi$) and the pH gradient ($\Delta\mathrm{pH}$), which make up the protonmotive force (PMF), as well as the rates of reactions and status of the involved metabolic intermediates.

**Mitochondrial uncoupling**

Process in which the protonmotive force established by the electron transport system is dissipated by proton leak across the mitochondrial inner membrane without being coupled to ATP synthesis.

**%NAD(P)H**

A measurement of the relative reduction status of the combined nicotinamide cofactor [NAD(H) and NADP(H)] pools. Generally measured by autofluorescence because the reduced form is strongly fluorescent while the oxidized form is not. For isolated mitochondria this is influenced by both cofactor pools, but is biased toward reflecting the NAD(H) pool.

**Protonmotive force (PMF)**

The potential energy established by the electrochemical disequilibrium created by proton translocation. It includes an electrical component, the membrane potential ($\Delta\Psi$) and a concentration gradient ($\Delta\mathrm{pH}$). The protonmotive force is used to couple oxidation of substrate to phosphorylation of ADP to ATP, which together are referred to as ‘oxidative phosphorylation’.

**Reactive oxygen species (ROS)**

Molecules that contain oxygen and are chemically reactive, meaning they readily react with other molecules without the need for a catalyst. Examples include free radicals [superoxide anion (O$_2^{−}$) and hydroxyl radicals (‘OH)] and nonradicals [hydrogen peroxide (H$_2$O$_2$) and other peroxides].

**Redox signal**

Cellular signalling mechanism based on redox chemistry. Generally, reactive oxygen or nitrogen species or other chemical compounds are major messengers and the relative reduced to oxidized ratio of one or more major cellular redox couple(s) can be a central component (e.g. the NADH/NAD$^+$) of the signal.
To summarize, oxidation of respiratory substrates contributes to the establishment of the PMF. The PMF will feed back on the flux of electrons through the ETS and the Krebs cycle that feeds into the ETS, leading to the accumulation of electrons in the enzymes and mitochondrial electron carriers, and thus influencing the reduction status of the NAD⁺ and NADP⁺ [i.e. NAD(P)] pool in the mitochondrial matrix. Thus, substrate oxidation will prime both the ROS-producing sites and the respiration-dependent pathways of H₂O₂ consumption. Given that different respiratory substrates are not oxidized at equal rates, nor do they produce equal amounts of ROS, there can be a wide range of ROS production rates and NAD (P)H availability, depending on the substrate supply.

Fig. 1D shows the rate of H₂O₂ production and H₂O₂ consumption for a range of substrates. From these data, it is clear that H₂O₂ consumption capacity largely surpasses H₂O₂ production across a range of substrate conditions in muscle mitochondria. The figure also suggests that high and constant H₂O₂ consumption capacities are maintained across various respiratory substrates, and that any apparent decrease in H₂O₂ consumption is thus the result of an increase in production.

**Regulation of metabolite concentration**

In the simplest terms, the concentration of a metabolite is set by the interaction between the pathways for its formation and removal. Within a given system, the ability for regulation of a metabolite requires that the consumption or clearance capacity must be greater than the rate of production. This is, in fact, the case for H₂O₂ production for many respiratory substrates in rodent skeletal muscle (Fig. 1D; Treberg et al., 2015; Munro et al., 2016) and brain mitochondria (Starkov et al., 2014). An additional condition promoting metabolic regulation is that consumption of the metabolite should be able to respond to changes in production. Starkov et al. (2014) proposed that the consumption of H₂O₂ by mitochondria follows first-order kinetics (see Glossary), where the rate of disappearance is proportional to the [H₂O₂] and is described by a single rate constant (k), a simple means of satisfying this second requirement. Eqn 1 describes the relationship proposed by Starkov and colleagues, wherein for any rate of H₂O₂ production (V_p), the steady-state concentration of the metabolite (H₂O₂) will be achieved can be related to the first-order rate constant for consumption (k) as follows:

$$[\text{H}_2\text{O}_2]_{ss} = \frac{V_p}{k}. \tag{1}$$

This model predicts that when the concentration of the metabolite of interest starts at zero, then initially the rate of appearance or production (solid black line in Fig. 2A) will approximate the increase in metabolite concentration over time (dotted line in Fig. 2A). However, as the concentration of the metabolite increases, the rate of consumption (solid grey line in Fig. 2A) will also increase. As the system proceeds, the rate of consumption approaches the rate of production, and the metabolite concentration will approach a stable value ([H₂O₂]_{ss}). The system will tend towards this set-point regardless of whether the concentration of the metabolite starts at zero.

Using this simple model, we can explore what happens when parts of the system change. For example, if production (V_p) increases, but the first-order constant (k) for consumption is maintained, the resulting [H₂O₂]_{ss} will increase (Fig. 2B, left). Conversely, if V_p is maintained constant, but k is increased, then the [H₂O₂]_{ss} will decrease (Fig. 2B, centre). An important aspect of this model is that the speed at which the system approaches equilibrium increases with increasing values of k.
Fig. 1. Mitochondrial metabolism of H$_2$O$_2$.
(A) Major sites of superoxide and H$_2$O$_2$ generation in enzyme complexes of the electron transport system (ETS) and the Krebs cycle (reviewed in Brand, 2010, 2016). CI–CIV, complexes I–IV; PDH, pyruvate dehydrogenase; αKGDH, alpha-ketoglutarate dehydrogenase; ETF-QOR, electron-transferring flavoprotein:ubiquinone-oxidoreductase; Q, ubiquinone; C, cytochrome c. Note: several additional sites and metabolic intermediates have been omitted for clarity. (B) Generalized process of electron leaks leading to reactive oxygen species (ROS) production. Superoxide is produced by a single electron reduction of dioxygen, and is detoxified into H$_2$O$_2$ by superoxide dismutase (SOD); H$_2$O$_2$ can form the hydroxyl radical (‘OH) and the hydroxyl anion (OH$^-$) via the non-enzymatic Fenton reaction (rxn) in the presence of certain transition metals. (C) Mitochondrial H$_2$O$_2$ consumption. Unlike catalase, the thioredoxin (Trx)- and glutathione (GSH)-dependent pathways for H$_2$O$_2$ consumption require reducing equivalents from NADPH. These respiration-dependent pathways rely on regeneration of Trx and GSH via conversion of an oxidized (S–S) to reduced (–SH) form thiol intermediate between the NADPH-dependent reductase and the H$_2$O$_2$-consuming peroxidase. (D) Rates of H$_2$O$_2$ metabolism by isolated rat skeletal muscle mitochondria (data are expressed per mg of mitochondrial protein; from Munro et al., 2016). Note that the pathways for the consumption of H$_2$O$_2$ have been pharmacologically compromised in the production assay to minimize underestimation of the production rate. For the consumption assay, mitochondria are incubated with 2.5 µmol l$^{-1}$ H$_2$O$_2$ and allowed to simultaneously consume and produce H$_2$O$_2$ for 10 min. Very low consumption of H$_2$O$_2$ is observed in the absence of respiratory substrate, which suggests limited involvement of catalase. Mal, malate; Glu, glutamate; Succ, succinate; Rot, rotenone. Results are means± s.e.m. (n=6).
This is illustrated in Fig. 2B (right panel) where both production and consumption have been doubled as compared with panel A. It can be seen that the $[\text{H}_2\text{O}_2]_{\text{ss}}$ is set by $V_p/k$, which is the same as in panel A, but is reached much faster with the higher value of $k$.

**First-order kinetics of respiration-dependent $\text{H}_2\text{O}_2$ consumption pathways?**

Our general model (Fig. 2A,B) uses an assumption of first-order kinetics to describe the relationship between $[\text{H}_2\text{O}_2]$ and the capacity of mitochondria to consume this ROS, because the rate of consumption ($V_c$) is a function of $[\text{H}_2\text{O}_2]$. We have previously shown that the decay of extramitochondrial $\text{H}_2\text{O}_2$ fits reasonably with first-order kinetics when considering energized rat skeletal muscle mitochondria (Fig. 2C). However, the assumption of a simple first-order reaction describing the combined consumers of ROS allows for consumption to be infinitely high at infinite $\text{H}_2\text{O}_2$ concentration. An infinite value of $V_c$ is, of course, unrealistic; the ROS consumers do appear to approach saturation (Banh...
and Treberg, 2013; Munro et al., 2016). To clarify, all of the measurements of \( \text{H}_2\text{O}_2 \) consumption rate \((V_c)\) used here so far (Figs 1D, 2D) have an initial, approximately linear phase at high \([\text{H}_2\text{O}_2]\) from which we could calculate the rate of \( \text{H}_2\text{O}_2 \) consumption in terms of nmol min\(^{-1}\). Calculating linear rates provides a convenient means of comparing rates of \( \text{H}_2\text{O}_2 \) consumption \((V_c)\) with rates of production \((V_p)\), but the first-order kinetic approximation remains a useful means of describing the response of the consumers under conditions when \([\text{H}_2\text{O}_2]\) is low and more likely to be similar to physiological levels.

**At the crossroads: producers and consumers interact with a common matrix pool of \( \text{H}_2\text{O}_2 \)**

Much of the experimental evidence for mitochondria as regulators of \([\text{H}_2\text{O}_2]\) comes from assays monitoring extramitochondrial \( \text{H}_2\text{O}_2 \); this is because components of the assay system that is used to quantify \( \text{H}_2\text{O}_2 \) cannot cross biological membranes and are confined to the medium. It is conventionally assumed that only minor amounts of \( \text{H}_2\text{O}_2 \) produced in the mitochondrial matrix are consumed before reaching the extramitochondrial detection system. However, using rodent skeletal muscle mitochondria, we recently demonstrated that this is not the case (Treberg et al., 2010, 2015; Munro et al., 2016). This means that mitochondrial ROS production rates are largely underestimated when based on extramitochondrial detection of \( \text{H}_2\text{O}_2 \). This is an important additional complexity to consider when evaluating the model leading to Eqn 1.

**Differentiating between apparent and actual rates of \( \text{H}_2\text{O}_2 \) production and consumption**

The interaction between endogenous mitochondrial \( \text{H}_2\text{O}_2 \) metabolism and observed rates of change in extra-mitochondrial \( \text{H}_2\text{O}_2 \) is a clear demonstration that \( \text{H}_2\text{O}_2 \) is exchanged between the mitochondrial matrix and extramitochondrial pools. This means that there is a distinction between ‘actual’ rates of production and consumption of \( \text{H}_2\text{O}_2 \) \((V_p, \text{ and } V_c\) respectively), and their ‘apparent’ rates (those which are measured), \( V_{p,\text{app}} \) and \( V_{c,\text{app}} \). Appreciating this distinction led us to derive a means of estimating the actual values of \( V_p \) and \( V_c \), by measuring their apparent values and extrapolating to where the rate of the competing intramitochondrial process would be zero. For example, Fig. 2D shows how \( V_{c,\text{app}} \) relates to \( V_{p,\text{app}} \). As \( V_{c,\text{app}} \) declines, the rate of \( \text{H}_2\text{O}_2 \) production increases, which we interpret as competition between exogenous and endogenous supply of \( \text{H}_2\text{O}_2 \) to the consumption pathways. However, by extending the relationship to the \( y \)-intercept, an estimate of the actual rate of consumption of \( \text{H}_2\text{O}_2 \) can be made where the influence of \( \text{H}_2\text{O}_2 \) production will be zero (Fig. 2D). If the matrix \( \text{H}_2\text{O}_2 \) consumption capacity is inhibited with auranofin, which impairs the thioredoxin-dependent pathway (Munro et al., 2016), the slope of the relationship between \( V_{p,\text{app}} \) and \( V_{c,\text{app}} \) decreases, and the predicted rate of \( \text{H}_2\text{O}_2 \) consumption, in the absence of competition from endogenous production, also decreases.

Importantly, the strong correlation between \( V_{p,\text{app}} \) and \( V_{c,\text{app}} \) leads to the interpretation that the consumption rate \((V_c)\) is maintained across different substrate conditions. In other words, the supply of NADPH to the reductases for consumption of \( \text{H}_2\text{O}_2 \) is not sufficiently low to limit the rate of \( \text{H}_2\text{O}_2 \) consumption by the peroxidases, at least for the set of substrate conditions that we tested. This satisfies the requirement of the model leading to Eqn 1 for greater overall capacity for \( \text{H}_2\text{O}_2 \) consumption than production across a large range of energetic conditions, with the added benefit that it is reasonable to assert that the capacity of the \( \text{H}_2\text{O}_2 \)-consuming pathways is consistent across these substrate conditions.

**Direct evidence of mitochondrial \( \text{H}_2\text{O}_2 \) regulation**

As we have shown above, skeletal muscle mitochondria have the requisite traits described in our general model of metabolite regulation that led to Eqn 1. For the interested reader, more detailed explanations can be found elsewhere (see Starkov et al., 2014; Treberg et al., 2015), but the requirements include \([\text{H}_2\text{O}_2]\)-dependent consumption that can act on both intramitochondrial and extramitochondrial \( \text{H}_2\text{O}_2 \) and outpace production. Combining these concepts, we will now discuss how isolated mitochondria can act as regulators of \([\text{H}_2\text{O}_2]\).

Our model predicts how the system can regulate towards \([\text{H}_2\text{O}_2]\)\(_{ss}\). This \([\text{H}_2\text{O}_2]\)\(_{ss}\) should be a function of \( V_p \), set by the substrates added, and \( V_c \) described by \( k \), which does not change across substrate conditions (Fig. 2D). In order to determine whether \( \text{H}_2\text{O}_2 \) is accumulating in the medium or being consumed by the mitochondria, we used a modified version of the horseradish peroxidase-linked fluorometric Amplex UltraRed assay. Normally, using this assay to measure \( \text{H}_2\text{O}_2 \) that has escaped from the mitochondrion consumes all extramitochondrial \( \text{H}_2\text{O}_2 \), thus maintaining an outward diffusion gradient (Fig. 3A). We modified the assay by adding respiratory substrate as normal but withholding the Amplex UltraRed for a period of time. This allows assessment of whether \( \text{H}_2\text{O}_2 \) has accumulated or been consumed – an accumulation of \( \text{H}_2\text{O}_2 \) is measured as a ‘jump’ in the fluorescence following addition of Amplex UltraRed (Fig. 3B). For example, while using glutamate and malate as the respiratory substrates, we see negligible accumulation of \( \text{H}_2\text{O}_2 \) in the presence of energized mitochondria, in accordance with low \( V_p \) but high and maintained \( V_c \) (Fig. 3C). In this example, it is clear that some \( \text{H}_2\text{O}_2 \) escapes the mitochondrion and can be detected when the detection system is completed by the addition of Amplex UltraRed at different time points for the four parallel reactions (Fig. 3). But there is no detectable accumulation of \( \text{H}_2\text{O}_2 \) in absence of a complete detection system, suggesting the consumers are maintaining \( \text{H}_2\text{O}_2 \) at very low concentrations. However, compromising \( V_c \) with the inhibitor auranofin, which decreases \( k \) (Treberg et al., 2015), and adding the same substrates leads to the appearance of \( \text{H}_2\text{O}_2 \) in the medium before the addition of Amplex UltraRed (Fig. 3D). If \( V_p \) is high enough for a given substrate, we would expect that some \( \text{H}_2\text{O}_2 \) should accumulate in the medium in absence of auranofin. Succinate leads to very high \( \text{H}_2\text{O}_2 \) production rates in well-coupled muscle mitochondria (Fig. 1D). When using succinate as a respiratory substrate, \( \text{H}_2\text{O}_2 \) accumulates (Fig. 3E) and even approaches a steady-state concentration over time (Fig. 3F), as predicted. These results are consistent with the general model in Fig. 2, and we conclude that for any condition where the consumption capacity does not change, any increase or decrease in the rate of \( \text{H}_2\text{O}_2 \) production should lead to a concomitant increase or decrease in the established \([\text{H}_2\text{O}_2]\)\(_{ss}\).

**How should \([\text{H}_2\text{O}_2]\)\(_{ss}\) respond to changes in mitochondrial energetics? ADP as a test case**

ADP activates the mitochondrial ATP synthase, thus allowing protons to return to the matrix side of the inner mitochondrial membrane, and partially collapsing the PMF (Box 1). This reduction in the PMF allows for increased flux through PMF-generating complexes in the ETS of the inner membrane, thus decreasing the concentration of the reduced form of NAD\(^+\) and NADP\(^+\) [NAD(P)H] in the matrix. Here, we illustrate these effects under two different substrate conditions: (1) malate (alone), which is a poorly oxidized respiratory substrate in muscle mitochondria, and (2) malate with glutamate, which allows for more rapid oxidation of...
Fig. 3. Demonstrating the accumulation of H$_2$O$_2$ over time with isolated muscle mitochondria. (A) (Upper) Cartoon demonstrating the principle of the fluorometric detection of extramitochondrial H$_2$O$_2$ via the H$_2$O$_2$-consuming horseradish peroxidase (HPx)-linked enzymatic reaction. By eliminating all extramitochondrial H$_2$O$_2$, the assay maintains a strong outward diffusion gradient for H$_2$O$_2$ that can be followed over time as a measure of net H$_2$O$_2$ escape from the mitochondrion (lower). (B) (Upper) Illustration of conditions when the H$_2$O$_2$ detection system is incomplete because of absence of the fluorometric substrate. (Lower) Schematic showing how to use the jump in fluorescence, relative to a cuvette containing complete detection system at t=0, to determine extramitochondrial H$_2$O$_2$ accumulation in the medium. (C–E) All assay components, except the molecular fluorescent probe Amplex UltraRed (Amplex), were added before beginning the assay. Additions of Amplex UltraRed are indicated by vertical grey arrows. (C) There is negligible accumulation of H$_2$O$_2$ in the medium of mitochondria respiring on 5 mmol l$^{-1}$ glutamate and malate. (D) Addition of 2 µmol l$^{-1}$ auranofin to mitochondria respiring on 5 mmol l$^{-1}$ glutamate and malate results in H$_2$O$_2$ accumulation. (E) Mitochondria respiring on 5 mmol l$^{-1}$ succinate accumulate H$_2$O$_2$ in the medium in a time-dependent manner. Note that where the traces reach 1000 arbitrary units, the detector has reached its maximum for the sensitivity settings used. (F) Accumulation of H$_2$O$_2$ in the medium from mitochondria respiring on 5 mmol l$^{-1}$ succinate approaches a stable steady-state concentration as determined by Eqn 1 and based on experiments as illustrated in E. Data are means±s.e.m. (n=3). k and V$_p$ values are expressed per g of mitochondrial protein. C–E and legend are modified from Treberg et al. (2015) (Redox Biol. 5, 216-224; copyright © 2015 the authors; published by Elsevier B.V.). For the interested reader, values for kinetic parameters and explanation of units can be found elsewhere (Treberg et al., 2015).
respiratory substrates and a shift towards a more reduced state in the nicotinamide cofactor pools – that is, an increase in NADH/NAD⁺ and NADPH/NADP⁺ – as indicated by higher %NAD(P)H (see Glossary).

As expected, respiration with glutamate and malate leads to a higher PMF than with malate alone, and under both substrate conditions the presence of ADP markedly decreases the PMF (Fig. 4A) (data from Quinlan et al., 2012). Similarly, glutamate and malate in combination allow for a higher %NAD(P)H as compared with malate alone, and ADP markedly decreases %NAD(P)H for both substrate conditions. Note that measurement of PMF under the same experimental conditions demonstrates a strong relationship between PMF and the %NAD(P)H (Fig. 4A). The strength of the relationship allows us to exploit the effects of ADP on the mitochondrial energetic state in order to investigate the relationship between PMF and the production and consumption of H₂O₂.

As the PMF decreases, in response to ADP, the capacity to consume H₂O₂ shows relatively little response compared with the rate of ROS production, which drops by a factor of three to four (Fig. 4B). Similarly, large decreases in %NAD(P)H in response to ADP addition largely affect H₂O₂ production rates while having relatively little impact on the capacity for consumption (Fig. 4C). This is typical, in our experience, of skeletal muscle mitochondria respiring under conditions that can readily reduce NAD⁺ to NADH either by matrix dehydrogenase reactions and buildup of Krebs cycle intermediates or by the reversal of complex I of the ETS (J.R.T., S. Banh, P. Zacharias, L. Wiens, D.M., unpublished observations).

**Preferential activation of the consumption pathways over the production pathways at low %NAD(P)H**

The respiration-dependent H₂O₂ consumption pathways characteristic of muscle mitochondria require a constant supply of NADPH to maintain flux and should theoretically fail under low % NAD(P)H (Fig. 1C). Above, we show that, under two different substrate conditions, and with or without ADP, the decrease in H₂O₂ production is much more pronounced than the decrease in H₂O₂ consumption when %NAD(P)H declines, indicating a more oxidized state of the matrix nicotinamide cofactor pool. In other words, the consumers appear to be primed and ready to quench H₂O₂ well in advance of reaching energetic states that are associated with high rates of ROS production. This has implications for the signalling role of H₂O₂ in transmitting information about the energetic status of the mitochondrion to its transcriptional machinery and to the nucleus (Moreno-Loshuertos et al., 2006). The resilience of the consumers in the face of declining %NAD(P)H, relative to the producers, leads to a system where – under most conditions – the [H₂O₂]ₘ will reflect the changes in H₂O₂ production (Eqn 1), which is largely a function of the mitochondrial energetic state.

How the mitochondrion interacts with other cellular elements in ROS handling is currently unknown. Some cytoplasmic components (homologs of mitochondrial H₂O₂ consumption and production pathways) also have the potential to regulate [H₂O₂]ₘ in a manner similar to what we propose for mitochondria. However, these cytosolic consumers and extramitochondrial sources of H₂O₂ could have different [H₂O₂]ₘ set-points compared with that of the mitochondrial matrix. Mitochondria may therefore represent a net sink or source of H₂O₂, if the current set-point for matrix [H₂O₂]ₘ is lower or higher, respectively, than that of the cytosol.

**Implications and future work**

Here, we briefly elaborate on potential physiological consequences of our hypothesized role for mitochondria in H₂O₂ regulation on
working skeletal muscle. We also consider the influence of body temperature, inter-tissue variation and the possible role of post-translational protein modification.

**Skeletal muscle mitochondria, oxidative stress and intense activity**

Sustained intense physical activity, such as long-distance migration, increases levels of oxidative stress markers. This has been ascribed to a putative increase in mitochondrial ROS production during periods of intense oxygen consumption. However, physical activity increases the turnover rate of ATP, thus increasing ADP availability to the mitochondria. Here, we have shown that while H$_2$O$_2$ consumption is not significantly affected, H$_2$O$_2$ production decreases profoundly in the presence of high levels of ADP. Interestingly, a recent study provides some benchmarks for the physiological levels of %NAD(P)H in rat muscle mitochondria by comparing mixes of respiratory substrates and effectors mimicking the in vivo environment for three levels of physical activity: ‘rest’, ‘mild aerobic exercise’ and ‘intense aerobic exercise’ (Goncalves et al., 2015). The %NAD(P)H corresponding to these conditions are indicated in Fig. 4C. Comparing rest (the highest rate of H$_2$O$_2$ production) with intense exercise (the lowest rate of production) indicates an 85% decline in V$\text{c,app}$ across this range of activity (Goncalves et al., 2015). In contrast, our results (Fig. 4C), would predict minimal effects on V$\text{c,app}$ for the same decrease in %NAD(P)H. If this prediction and our model are correct, then according to Eqn 1 – ‘intense’ aerobic physical activity should lead to a decrease in the [H$_2$O$_2$]$_{ss}$ as compared with ‘rest’ conditions. Additionally, we have previously argued that the acidification that occurs with intense muscle activity may shift mitochondria towards a more antioxidant state (Banh and Treberg, 2013).

In view of the combined effects of the expected decline in H$_2$O$_2$ production and maintained H$_2$O$_2$ consumption, which should reduce the [H$_2$O$_2$]$_{ss}$ set by muscle mitochondria during physical activity, it seems unlikely that mitochondrial function promotes oxidative stress during periods of high demand for ATP in the cell. Reconciling this with the observed increase in markers of oxidative stress after intense exercise (reviewed in Powers and Jackson, 2008) will require further investigation, but these arguments support a role for extramitochondrial sources of ROS, including NADPH oxidases, phospholipase A2 and lipoxygenases, as the cause of the observed oxidative stress.

**Rapid changes in temperature**

We recently demonstrated that H$_2$O$_2$ production by fish red muscle mitochondria is more sensitive to changes in assay temperature than are the reductases that supply NADPH to the respiration-dependent H$_2$O$_2$ consumers (Banh et al., 2016). We hypothesized that this ‘thermal mismatch’ could implicate mitochondria as a bona fide source of oxidative stress during acute heat stress in ectotherms. A mismatch in the temperature sensitivity of H$_2$O$_2$ production and H$_2$O$_2$ consumers may be particularly significant for animals that experience large and regular temperature fluctuations, such as intertidal species (Somero, 2002).

**Mammalian torpor and hibernation**

Many small endothermic hibernators go through repeated bouts of metabolic depression and torpor at low body temperature which are interrupted by acute and rapid rewarming by 20°C or more during the interbout euthermia phase (Geiser, 2004). Applying our thermal-mismatch observation (see above) to hibernating mammals predicts that decreasing body temperature could passively contribute to decreasing [H$_2$O$_2$]$_{ss}$ during torpor. A previous study using isolated liver and skeletal muscle mitochondria of hibernating 13-lined ground squirrels found that mitochondrial H$_2$O$_2$ efflux often decreases as assay temperature declines from 37°C to 10°C (Brown et al., 2012). Although the interaction of physiological state (interbout euthemic, torpid or summer-active animals), tissue, assay and respiratory substrates added leads to a complex pattern to interpret (Brown et al., 2012), one consistency arises: efflux of H$_2$O$_2$ is lower for mitochondria isolated from torpid individuals when measured at a physiologically relevant temperature corresponding to torpor (10°C) compared with mitochondria isolated from individuals during interbout euthemia and measured at 37°C (Brown et al., 2012).

Factors that affect mitochondrial [H$_2$O$_2$]$_{ss}$ during torpor may, however, be much more diverse than simple temperature effects. Mitochondrial substrate oxidation capacity is markedly decreased, especially for succinate but also with NADH-linked substrates, during the torpor phase of hibernation (Staples, 2014, 2016). As discussed above, a decrease in mitochondrial capacity for substrate oxidation may affect the production of H$_2$O$_2$ more than the pathways for its consumption, thereby decreasing [H$_2$O$_2$]$_{ss}$. However, there are indications that proteomic and allosteric regulation of mitochondrial substrate oxidation may form part of the adaptation to torpor (reviewed in Staples, 2014, 2016). Post-translational modifications such as glutathionylation may decrease enzymatic flux and, at the same time, either decrease or increase ROS production as seen with complex I and II, respectively (reviewed in Mailloix and Treberg, 2016). Therefore, the declining substrate oxidation capacity may not necessarily lead to declining H$_2$O$_2$ production.

Very little research has been conducted on regulation (proteomic and/or allosteric) of the pathways for the consumption of H$_2$O$_2$ during hibernation and torpor. Levels of peroxiredoxins, including the mitochondrial-specific peroxiredoxin-3, increase in brown adipose tissue and heart of 13-lined ground squirrels during hibernation (Morin and Storey, 2007), which presumably should elevate the mitochondrial capacity for H$_2$O$_2$ consumption, thereby leading to lower [H$_2$O$_2$]$_{ss}$; however, this remains to be empirically tested. In contrast with baseline torpor, the short period of rewarming from a torpor episode may lead to increased cell-level oxidative stress. Support for this stress includes increased antioxidant (ascorbate) uptake from plasma during rewarming (Toïnen, 2001), and the recruitment of hypoxia-inducible factor 1α (Ma et al., 2005) and elevated protein carbonyls and TBARS in late arousal phase (Orr et al., 2009). Oxidative stress during arousal from torpor has traditionally been attributed by some to the increased oxygen consumption by mitochondria. For instance, a previous attempt to estimate the elevated ROS formation was based on an assumed direct relationship between oxygen consumption and the fraction of oxygen diverted to ROS production by mitochondria (% free radical leak) during rewarming (Orr et al., 2009). However, as discussed above, our model shows that elevated oxygen consumption (either by demand for ATP or increased proton leak) and the associated decrease in PMF is expected to temporally lower mitochondrial [H$_2$O$_2$]$_{ss}$. Clearly, the interaction between oxidative stress and rewarming is potentially more complex than the simple effect of oxygen consumption by mitochondria.

It will be important to reconcile how during hibernation, especially during the acute phase of rewarming leading into interbout euthermia, there is shifting metabolic capacity for mitochondrial substrate oxidation combined with alterations in mitochondrial H$_2$O$_2$ metabolism (both production and...
consumption). This may be an important test of our hypothesized role for mitochondria as regulators of H$_2$O$_2$.

Other organs and tissues

The nature and capacity of mitochondrial H$_2$O$_2$ consumers have only been explored for a limited number of tissues, so it is difficult to generalize the nature of H$_2$O$_2$ regulation across multiple tissues. Brain mitochondria are likely to regulate H$_2$O$_2$ similarly to muscle mitochondria, as they also rely on respiration-dependent pathways for the consumption of H$_2$O$_2$, with minimal involvement of catalase. Brain and muscle mitochondria also have similar H$_2$O$_2$ consumption rates: ~11 and 6 nmol min$^{-1}$ mg$^{-1}$ protein, respectively (Dreschel and Patel, 2010; Munro et al., 2016).

It is difficult to estimate how qualitative and quantitative inter-tissue differences in mitochondrial H$_2$O$_2$ consumption translate into functional differences in regulation of matrix [H$_2$O$_2$]$_{ss}$. Although the effectiveness of catalase in maintaining very low levels of [H$_2$O$_2$] is questionable owing to its low affinity [the $K_m$ for H$_2$O$_2$ is typically found in the range of 80 to 100 mmol l$^{-1}$ in mammals (Switala and Loewen, 2002)], catalase has an exceptionally high catalytic turnover ($k_{cat}$) and we have observed that liver mitochondria (which have a high catalase content) can readily consume a bolus of 2.5 µmol l$^{-1}$ H$_2$O$_2$ to below detection capacity (i.e. below 0.1–0.2 µmol l$^{-1}$) in the absence of additional respiratory substrates (D.M. and J.R.T., unpublished observations). Hence, we cannot exclude the idea that catalase may well achieve respiration-independent regulation of H$_2$O$_2$ in tissues where it is highly expressed. In fact, liver mitochondria have rates of H$_2$O$_2$ consumption that are ~10 to 20 times higher than those of muscle and brain; their high catalase content may help to explain this (Dreschel et al., 2010; Lopert and Patel, 2014; D.M., unpublished observations). Thus, there may be some tissues where mitochondria regulate H$_2$O$_2$ predominately in response to the current mitochondrial energetic state (skeletal muscle and brain), whereas in other tissues, such as liver, respiration-independent H$_2$O$_2$ consumption could be the dominant mitochondrial contribution. This will be important to consider when extending the arguments provided here to new tissues or other species.

Post-translational regulation

Except for brief mention in regards to hibernation and torpor, we have not considered the regulation of H$_2$O$_2$ at the level of specific enzymes; however, it is probable that allosteric or post-translational regulation may add additional levels of complexity. For example, macromolecular supercomplexes have been implicated in altering the production of superoxide (Genova and Lenaz, 2014), which – alongside modifications such as glutathionylation – could influence mitochondrial H$_2$O$_2$ production (Mailhoux and Treberg, 2016). Likewise, post-translational modification could affect H$_2$O$_2$ consumption pathways, which would alter $k$ in our model and thereby alter the [H$_2$O$_2$]$_{ss}$. Thus, our model fits a scenario where there should be a wide range of physiologically adjustable [H$_2$O$_2$]$_{ss}$ set-points possible in order to reflect and communicate mitochondrial status via redox signalling.

Conclusions

In this Commentary, we have shown that mitochondria have the attributes necessary to act as regulators of H$_2$O$_2$ concentration. The model described herein relies on the existence of a balance between H$_2$O$_2$ production and consumption by mitochondria. The existence of such a balance would indicate that mitochondrial antioxidant systems are not simply minimizing oxidative damage but, instead, are an integral component of the ROS regulatory system. Moreover, the model discussed here would allow for mitochondria to alter the [H$_2$O$_2$]$_{ss}$ based on changes in the dynamics of ROS metabolism. Mitochondrial H$_2$O$_2$ production appears to be far more sensitive to changes in mitochondrial energetics than are the consumption pathways. Thus, mitochondrial H$_2$O$_2$ production could be an important link in redox signalling within the mitochondrion and between the mitochondrion and the nucleus. If mitochondria are regulators of cellular H$_2$O$_2$ concentration, this will change our understanding of the role of mitochondrial ROS metabolism in environmental adaptation, oxidative stress in response to life-history or metabolic trade-offs, metabolic dysfunction and signalling. However, future work should expand these lines of investigation to other types of mitochondria (from different tissues and species) in order to further test this model. It will also be important to extend these experiments from isolated mitochondria to cell- and tissue-level work in order to test whether mitochondria also may act as significant regulators of H$_2$O$_2$ in situ.

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Competing interests

The authors declare no competing or financial interests.

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