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COMMENTARY

Are mitochondria the main contributor of reactive oxygen species in cells?

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ABSTRACT

Physiologists often assume that mitochondria are the main producers of reactive oxygen species (ROS) in cells. Consequently, in biomedicine, mitochondria are considered as important targets for therapeutic treatments, and in evolutionary biology, they are considered as mediators of life-history tradeoffs. Surprisingly, data supporting such an assumption are lacking, at least partially due to the technical difficulties in accurately measuring the level of ROS produced by different subcellular compartments in intact cells. In this Commentary, we first review three potential reasons underlying the misassumption of mitochondrial dominance in the production of cellular ROS. We then introduce some other major sites/enzymes responsible for cellular ROS production. With the use of a recently developed cell-based assay, we further discuss the contribution of mitochondria to the total rate of ROS release in cell lines and primary cells of different species. In these cells, the contribution of mitochondria varies between cell types but mitochondria are never the main source of cellular ROS. This indicates that although mitochondria are one of the significant sources of cellular ROS, they are not necessarily the main contributor under normal conditions. Intriguingly, similar findings were also observed in cells under a variety of stressors, life-history strategies and pathological stages, in which the rates of cellular ROS production were significantly enhanced. Finally, we make recommendations for designing future studies. We hope this paper will encourage investigators to carefully consider non-mitochondrial sources of cellular ROS in their study systems or models.

KEY WORDS: Mitochondrial respiration, Oxidative damage, Oxidative stress, Life-history tradeoffs, Mitochondria-targeted antioxidants

Introduction

More than half a century ago, Harman (1955) proposed the free radical theory of aging (FRTA), which described how free radicals produced during aerobic respiration cause cumulative oxidative damage, resulting in aging and death. Upon the discovery of the antioxidant enzyme superoxide dismutase (SOD) in 1969 (McCord and Fridovich, 1969), this theory gained substantial credibility and quickly influenced the fields of aging and biomedical research (McCord and Fridovich, 1969; Beckman and Ames, 1998). The use of SOD activity to trace subcellular superoxide ($O_2^{\cdot-}$) generation led to the discovery that mitochondria are one of the major sources of endogenous oxidants (Chance et al., 1979), and revealed that the production of mitochondrial oxidants causes oxidative stress (see Glossary) and is associated with a variety of diseases (Balaban et al., 2005). In the field of evolutionary and ecological physiology, the FRTA has been adopted as the 'oxidative stress life-history theory', and is thought to explain the evolution of life-history strategies (Speakman and Selman, 2011; Selman et al., 2012; Speakman et al., 2015). As the center of life-history theory, life-history tradeoffs (see Glossary) are presumed to have a physiological basis (Zera and Harshman, 2001). Oxidative stress life-history theory provided this, and therefore became an attractive hypothesis (Monaghan et al., 2009). The theory proposes that reactive oxygen species (ROS) are produced in direct proportion to metabolic rate as inevitable byproducts of mitochondrial respiration (Speakman and Garratt, 2014). The central idea of these theories in both biomedical and evolutionary fields is the assumption that mitochondria are the hub governing the production and release of ROS in cells (Turrens, 2003; Andreyev et al., 2005; Balaban et al., 2005; Zhang and Hood, 2016; Hood et al., 2018). Even though this theory has been applied in a vast swathe of literature, the evidence base of this idea is obscure (Brown and Borutaite, 2012). Subsequently, this notion was critically assessed by two papers, and it was concluded that no convincing data were available to support the role of mitochondria as the main source of cellular ROS (Brown and Borutaite, 2012; Munro and Treberg, 2017).

The rates and the origins of cellular ROS production can vary significantly. These depend mainly on (a) the kind of ROS being generated, (b) the type of cells that produce ROS, (c) the species from which the cells are derived, and to a very large extent (d) the specific physiological or pathological conditions that cells are experiencing. Regarding (a), ROS are a collection of molecular species derived from oxygen that demonstrate high biological and chemical reactivity. Although ROS is a large family, in biological systems most of them are derivatives of $O_2^{\cdot-}$ and/or hydrogen peroxide (H_2O_2) (Murphy, 2009). This, together with the fact that ROS other than $O_2^{\cdot-}$ and H_2O_2 are short lived (as a consequence of high reactivity), is the reason that most literature mainly focuses on $O_2^{\cdot-}$ and H_2O_2, this Commentary included. Regarding (b) and (c), we have put together a dataset to facilitate our discussion of the role of mitochondria in cellular ROS production in different cell types from various species using a recently developed cell-based assay. In order to address (d), we here extend our discussion to cover the changes in profiles of cellular ROS production in response to different stressors or pathological conditions. Lastly, we end this Commentary by providing some recommendations for future studies in the fields of evolutionary and ecological physiology.

Why are mitochondria thought to be the major source of ROS production?

As we mentioned above, direct evidence supporting the dominant role of mitochondria in cellular ROS production is very limited. Thus, we should ask what indirect evidence has led researchers to assume mitochondria are the main producer of cellular ROS? To our knowledge, three main reasons underlie this assumption. Firstly,

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Glossary

Life-history tradeoffs
The negative associations between different life-history traits. Life-history traits are often indicated by different fitness components such as growth rate, foraging strategies, reproductive strategies or lifespan.

Oxidative stress
An imbalance between the production of reactive oxygen species (ROS) by cells and the capacity of antioxidant mechanisms to control the damaging effects of ROS.

Peroxisome
A membrane-bound organelle found in the cytoplasm of almost all eukaryotic cells. Peroxisomes play key roles in hydrogen peroxide and lipid metabolism.

Respiratory burst
The rapid release of ROS from different types of cells. Usually it denotes the release of ROS from immune cells.

State 3 respiration
The ADP-stimulated respiration of isolated mitochondria in the presence of sufficient substrates, ADP and phosphate at saturating oxygen levels.

State 4 respiration
Obtained in isolated mitochondria after state 3 respiration, when added ADP is phosphorylated maximally to ATP. This is often referred as the 'idling' respiratory state of mitochondria.

early classical biochemical studies using isolated mitochondrial enzymes, submitochondrial particles, isolated intact mitochondria or organelle-enriched cell fractions demonstrated that mitochondria have a high capacity to generate ROS under optimized experimental conditions (Boveris et al., 1972; Oshino et al., 1973; Sligar et al., 1974; Sies and Summer, 1975; Malinska et al., 2009; Tahara et al., 2009; Treberg et al., 2010). In these pioneering studies, isolated mitochondria and cell fractions were always provided with excessive/optimal amounts of substrates. Respiratory inhibitors were also included in the assays to inhibit unrelated reactions and, more importantly, to maintain very reduced pools of NADH and ubiquinone, therefore driving high rates of ROS production. The experiments were performed under air-saturated conditions where oxygen supply was not limited, as it would be in the cell. These optimized experimental conditions allowed the maximum rates of ROS production by mitochondria to be assessed. As a result, mitochondria were documented among the organelles with a high capacity to generate cellular ROS (Chance et al., 1979). It is very important to note here that even though these biochemical studies provided valuable information on the mechanisms of ROS production from mitochondria, results from these studies can only reflect the capacities rather than the physiologically relevant rates of ROS production by various organelles and pathways in cells. For example, studies testing the 'native rate' of mitochondrial ROS production (i.e. the rate measured in the absence of respiratory inhibitors, but still under ambient room oxygen conditions) indicated that using the previous 'optimal' experimental conditions would result in a significant overestimation of mitochondrial ROS production (Quinlan et al., 2012; Goncalves et al., 2020). In short, mitochondria do have a high capacity to produce ROS, but this is not a good indicator of the actual rate of ROS production by mitochondria in intact cells or under physiologically relevant conditions.

Secondly, the abundance of antioxidant enzymes and endogenous antioxidants present in mitochondria suggests that these organelles might be the major site of ROS production in cells. Roughly one-third of the cellular content of antioxidants such as glutathione peroxidase and catalase resides in mitochondria, and manganese-dependent SOD is exclusively located in the mitochondria, indicating the significance of mitochondria in ROS production (Chance et al., 1979). The role of mitochondria in cellular ROS production is further supported by the discovery of various mitochondria-targeted antioxidants. These compounds were found to be efficacious in treating a wide spectrum of oxidative stress-related diseases *in vitro* and *in vivo* (Smith and Murphy, 2010; Ni et al., 2016; Chavez et al., 2020), thus providing strong evidence for the contribution of mitochondrial ROS to cellular pathology. In contrast, the high levels of antioxidants present in the mitochondria could act as a defensive system to ensure mitochondria are protected against oxidative damage (Starkov et al., 2014). Consequently, ROS produced by mitochondria is more likely to be quenched before inducing oxidative stress compared with ROS produced by organelles with low antioxidant levels. Furthermore, the fact that there are protective effects afforded by mitochondria-targeted antioxidants does not exclude the possibility of contributions of other cellular pathways in the processes. Given their ability to diffuse inside a cell, ROS generated by other cellular compartments may also be quenched by mitochondrial antioxidants, ultimately leading to a global amelioration of oxidative stress in cells and tissues. This is supported by the fact that many mitochondrially targeted antioxidants are reported to produce partial protection in disease models *in vitro* and *in vivo* (Lowes et al., 2008; Hu et al., 2018).

Thirdly, a significant number of researchers in both biomedical and evolutionary biology incorrectly associate an increase in ROS production with an elevated rate of mitochondrial respiration or increased ATP demand/production, with ROS considered as byproducts of the process. This is largely based on early reports that almost 2% of mitochondrial oxygen consumption may undergo one electron reduction to form $O_2^{\cdot-}$ (Boveris and Chance, 1973). In fact, the authors clearly stated that these findings were obtained during mitochondrial state 4 respiration (see Glossary; i.e. the 'idling' state of mitochondria). On the contrary, during states with high ATP demand/production, such as state 3 respiration (see Glossary), the rate of mitochondrial ROS production is negligible (Chance and Oshino, 1971; Boveris and Chance, 1973). In fact, the rate of mitochondrial ROS production is governed by the redox potentials of both NAD and ubiquinone pools but not the rate of mitochondrial respiration (Brand, 2016; Wong et al., 2017). A more reduced NAD pool (a higher NADH/NAD ratio) or quinone pool (a higher ubiquinol or ubisemiquinone/ubiquinone ratio) leads to increased production of $O_2^{\cdot-}$ and H_2O_2 from various mitochondrial sites. This mostly happens when ATP demand is low (Wong et al., 2017). During periods of high ATP demand/production, the rate of mitochondrial respiration speeds up to match an increased energy demand in cells. This results in more oxidized NAD and ubiquinone pools and therefore a decrease in mitochondrial ROS production. With this logic, life-history traits that are energetically demanding would be unlikely to lead to a direct increase in ROS production, at least from mitochondria (Munro and Treberg, 2017).

The sources of ROS production in the cell

If mitochondria are not the only source of cellular ROS, then what are the other sites/organelles that contribute significantly to total cellular ROS production? Inside a cell, every site or organelle that is involved in redox reactions has the potential to produce ROS (Fig. 1). From the mid-20th century, the ability of different cellular compartments to produce ROS has been evaluated in different types of cells using a variety of approaches. For example, the production of ROS by peroxisomes (see Glossary) was first described by Nobel Laureate Christian de Duve in 1965 during his identification of H_2O_2-producing oxidase and catalase in peroxisomes (De Duve and

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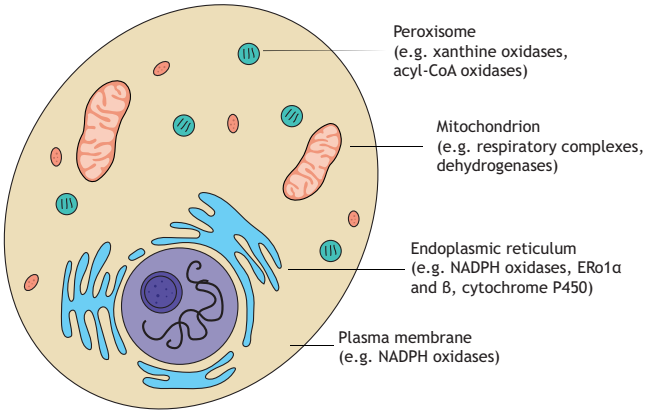

Fig. 1. Major sites of reactive oxygen species (ROS) production in cells. Enzymes responsible for ROS production at each of the cellular compartments are shown in parentheses.

Baudhuin, 1966). Following this, using tissue fractions, Boveris et al. (1972) identified that peroxisomes may be responsible for as much as 20% of a cell's oxygen consumption and could produce ROS at a high rate in rat livers. This study also demonstrated that microsomes (fractions of endoplasmic reticulum) account for roughly 45% of cellular ROS production. Iyer et al. (1961) documented that the respiratory burst (see Glossary) in phagocytes involved the generation of ROS, and this process was eventually identified to be mediated by NADPH oxidases (NOXs). A series of NOX family isoforms were then discovered; these transmembrane proteins are responsible for transporting electrons across biological membranes, leading to the reduction of oxygen to form ROS (Prieto-Bermejo and Hernández-Hernández, 2017). Most NOX isoforms reside in the plasma membrane, where they can produce extracellular ROS (Lambeth, 2007). NOXs can also be found in intracellular membranes, such as those of the mitochondria, endoplasmic reticulum, perinuclear areas and even the nuclear membrane, and can release ROS to the inside of cells (Bedard and Krause, 2007). Furthermore, other enzymes, including xanthine oxidase, the cytochrome P450 monooxygenases, myeloperoxidase and cyclo-oxygenase, found in different cellular organelles or intracellular spaces, are also involved in redox reactions and have the ability to produce ROS (Bae et al., 2011; Battelli et al., 2016; Di Meo et al., 2016).

ROS production from different cellular compartments under normal growing conditions

The existence of different ROS-producing sites in cells begs the question: which is the main contributor of cellular ROS? A lot of researchers have an impression of mitochondrial dominance in cellular ROS production and therefore consider mitochondria as important therapeutic targets and potential regulators of different life-history strategies. However, this assumption does not even match the maximal ROS-producing capacities of various subcellular fractions. Boveris et al. (1972) reported that mitochondria, microsomes, peroxisomes and other cytosolic enzymes have all been recognized as effective ROS generators; in rat liver, these contribute 15%, 45%, 35% and 5%, respectively, of cytosolic H_2O_2 production at a P_{O_2} of 158 mmHg when provided with excess substrates. This study concluded that mitochondria are a significant, but not the main, source of ROS production in rat liver. Surprisingly, to our best knowledge, this was the only study to quantitatively measure the relative ROS production by different cellular sources until recently. Using isolated mitochondria, recent efforts by a number of scientists identified that the rate of H_2O_2 production by mitochondria isolated from skeletal muscle, brain and heart varies between 0.2 and 2.5 nmol H_2O_2 min^{-1} mg^{-1} protein during state 4 (idling) respiration (Starkov, 2008; Malinska et al., 2009; Treberg et al., 2010). But in phosphorylating mitochondria (i.e. state 3), H_2O_2 production rate drops to 0.1 nmol min^{-1} mg^{-1} protein or less. Based on these findings, we postulate that the rate of mitochondrial ROS production under physiologically relevant conditions should fall between state 3 and 4 respiration and constitute around 0.1–0.2% of the total oxygen consumption rate (Staniek and Nohl, 2000; St-Pierre et al., 2002; Tahara et al., 2009). As a comparison, the rate of H_2O_2 production by intact liver peroxisomes has been estimated to be up to a thousand times greater than the mitochondrial rate above (Mueller et al., 2002). The phagocyte NADPH oxidase is also known to generate large quantities of $O_2^{\cdot-}$ and H_2O_2, which accounts for 10–90% of total oxygen consumption in activated neutrophils, macrophages, microglia and leukocytes (McBride and Brown, 1997; BalPrice et al., 2002; Souza et al., 2002). Using theoretical models, it has also been estimated that the endoplasmic reticulum accounts for 25% of H_2O_2 production in growing cells (Tu and Weissman, 2004; Gross et al., 2006). All of these studies indicate that isolated mitochondria from different tissues do not have the highest capacities of ROS production.

What about ROS production from these sites in intact cells under normal growing conditions? Cellular ROS levels are most commonly measured using intracellular fluorescent probes such as DCFH-DA and Mito-SOX (Woolley et al., 2013). These fluorescent probes have significant limitations such that they are only useful for qualification of ROS rather than quantification of the rates or amounts of ROS production in cells, let alone distinguishing ROS produced by different cellular compartments (Kalyanaraman et al., 2012). In order to address this question, here we employed a recently established cell-based assay to measure ROS release rates from different cellular compartments using a H_2O_2 detection probe, Amplex UltraRed (Wong et al., 2019). The peroxidase-coupled Amplex UltraRed oxidation system is a reliable method to measure extracellular H_2O_2 levels (Kalyanaraman et al., 2012). Exogenous SOD was also included in the reaction to capture the amount of $O_2^{\cdot-}$. This system is not permeable to cell membranes; although Amplex UltraRed itself displays some degree of cell permeability, the dependence of Amplex UltraRed oxidation on peroxidase, an enzyme to which the cell membrane is not permeable, limits the interference with intracellular processes. We adopted a pharmaceutical approach to inhibit or scavenge ROS produced from different subcellular sites (see Appendix and Fig. A1 for methods). We titrated a NOX inhibitor (GKT136901) to inhibit ROS produced by NOX enzymes, and Mito-Tempo was used to quench ROS ($O_2^{\cdot-}$ and H_2O_2) generated within the mitochondrial matrix (Fig. S1). Mito-Tempo is a mitochondrion-targeting antioxidant, composed of a piperidine nitroxide (Tempo, an antioxidant) linked to the lipophilic triphenylphosphonium (TPP). Tempo functions as a SOD mimetic (Trnka et al., 2008) and is also reported to exhibit some catalase-like activity (Samuni et al., 2016); however, a clear demonstration of its catalase-like mechanism has not been published. To justify the use of Mito-Tempo to sequester both $O_2^{\cdot-}$ and H_2O_2 within the mitochondrial matrix, we compared the effect of Mito-Tempo with $O_2^{\cdot-}$/H_2O_2 suppressors at mitochondrial site I_Q and site III_{Qo} (two main sources of mitochondrial ROS). Our findings showed that the degrees of suppression by $O_2^{\cdot-}$/H_2O_2 suppressors at mitochondrial site I_Q and site III_{Qo} and by Mito-Tempo are comparable; this suggests that Mito-Tempo can suppress the majority of $O_2^{\cdot-}$/H_2O_2 generated by mitochondria (Fig. S1H–J). Though it should be noted that the use of Mito-Tempo does not

provide a definitive measure of the contribution of mitochondria to total cellular H_2O_2 release, it should allow us to estimate this value. We also compared the effects of mixtures of Mito-Tempo and GKT136901 with the summed suppression by the individual compounds and found no significant difference between the summed individual effects and the extent of suppression by the mixture (Fig. S1G). These data indicate the full independence and additivity of $O_2^{\cdot-}/H_2O_2$ production from NOXs and mitochondria in cells. Because these compounds might interfere with mitochondrial membrane potential or mitochondrial electron transport at high dosages, we also examined their effects on cellular respiration (Fig. S1A–D). Only dosages that produced no effects on mitochondrial respiration were considered during data interpretation.

We surveyed a collection of cell types, including established cell lines and primary cells isolated from different tissues of various species (Fig. 2; Table S1). Overall, the significance of mitochondrial ROS production varied between cell types, with mitochondrial contribution to total cellular ROS of all tested cell types being less than 50% (Fig. 2; Table S2). Other cellular compartments/enzymes such as NOXs contributed a major portion of ROS production in some of the tested cell types under unstressed conditions (Fig. 2). In some cell types, a significant amount of the ROS signal was produced from unidentified sources (i.e. sites/enzymes other than mitochondria and NOXs). In the same species, ROS contribution from mitochondria varied significantly between cell types (for example, skin versus lung fibroblast in Siberian hamsters). Also, the ROS production profiles of the same cell types (such as lung fibroblast) differed between species. It is important to note that because of the limited research on the catalase-like action of Tempo, the mitochondrial contribution of H_2O_2 measured in this study may not be definitive. Consequently, these data provide speculative support that mitochondria do generate a significant portion of cellular ROS, but may not be the largest contributor in any cells tested in this study.

ROS production from different cellular compartments upon exposure to stressors

Most studies in both biomedical and evolutionary physiology fields focus on ROS production not only under normal growing conditions but also under pathological conditions or in response to different stressors. Under certain conditions when mitochondrial function is impaired – for instance, in some neurodegenerative diseases (McManus et al., 2011) and liver fibrosis (Rehman et al., 2016) – mitochondria can significantly increase their ROS production and therefore might become the main source of cellular ROS (Staniek and Nohl, 2000). However, not all diseases, toxins or life-history strategies would directly influence mitochondrial ROS production. Here, we measured changes in the ROS profile of cardiomyoblasts (H9c2 cells; a cell line derived from the laboratory rat *Rattus norvegicus*) upon hypoxia–reoxygenation. This is a well-defined model that is used to study the role of mitochondrial ROS-induced oxidative damage in heart tissue. During hypoxia–reoxygenation, succinate accumulates, and this drives extensive ROS generation by reverse electron transport at mitochondrial complex I (Chouchani et al., 2014). Here, we incubated H9c2 cells under hypoxic conditions (1% oxygen) for 30 min, and the profile of cellular ROS was subsequently immediately measured in normoxia using the approach described above. Hypoxia–reoxygenation did increase mitochondrial ROS production in H9c2 cells, but the mitochondria were never the largest contributor of cellular ROS throughout the course of experiment (Fig. 3). ROS production by the NOX system was also increased upon hypoxia–reoxygenation, and it remained as the major contributor of ROS throughout the experiment (Fig. 3). These data indicate that oxidative stress induced by hypoxia–reoxygenation in H9c2 cardiomyoblasts does not originate mainly

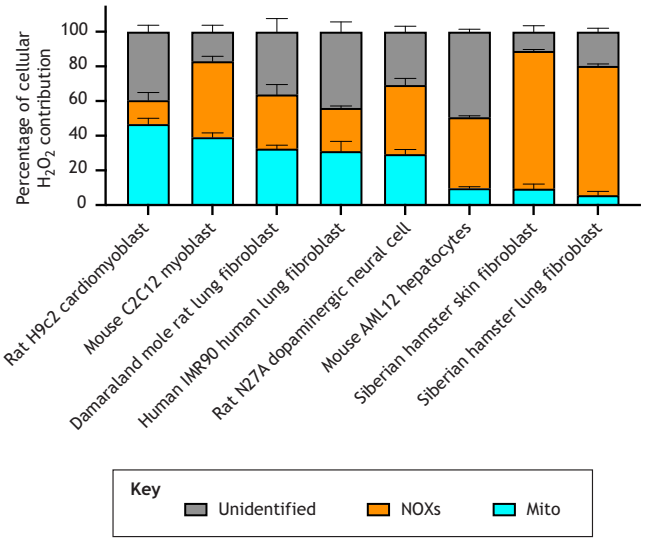

Fig. 2. Percentage of H_2O_2 contribution from different cellular compartments in a variety of cell types. Mito, mitochondria; NOXs, NADPH oxidases; Unidentified, cellular sites except for mitochondria and NOXs. Data are shown as means±s.e.m., with three biological replicates.

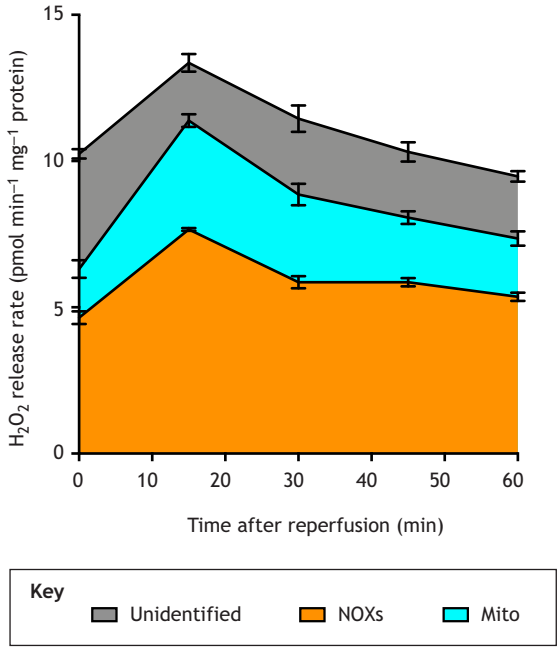

Fig. 3. H_2O_2 release rate from different cellular compartments at different times after reperfusion in H9c2 cardiomyoblasts. A Billups–Rothenberg modular incubator chamber was used to produce an *in vitro* hypoxia challenge. In essence, cells were incubated with pre-warmed KRB-BSA were placed in the sealed chamber, and the chamber was flushed with nitrogen for 15 min at a flow rate of 20 ml min^{-1}. After closing all sealable connectors, the chamber was transferred to an air incubator and the cells in the chamber were subjected to a 2 h period of hypoxia at 37°C. Reoxygenation was initiated by opening the chamber and then replacing with fresh KRB-BSA. The rates of H_2O_2 release were then determined at 0, 15, 30, 45 and 60 min after the start of reoxygenation. Mito, mitochondria; NOXs, NADPH oxidases; Unidentified, cellular sites except for mitochondria and NOXs. Data are shown as means± s.e.m., with three biological replicates. The graph represents the cumulative H_2O_2 release rate from different sites.

from mitochondria. Consistent with our observation, studies focused on the NOX family have also shown that these enzymes appear to be a main source of ROS production during reoxygenation (Abramov et al., 2007; Bedard and Krause, 2007).

Intense physical activity, such as long-distance migration or foraging, is another widely used example of mitochondrial ROS production under stress. This is supported by increased levels of free radicals and markers of oxidative damage during physical activity in fish, mammals and birds (Costantini et al., 2007; Powers and Jackson, 2008; Birnie-Gauvin et al., 2017; Yap et al., 2017; Costantini et al., 2019). Because of high ATP demand during intense physical activity, the increased ROS production is thought to be caused by elevated mitochondrial respiration. However, physical activity increases ADP availability to the mitochondria, which is associated with a lower rate of ROS production (Munro and Treberg, 2017). During intense physical activity, the rate of mitochondrial respiration goes up to match an increased energy demand in muscle fibers. This results in more oxidized NAD and ubiquinone pools and therefore a decrease in mitochondrial ROS production, as discussed above. In line with this concept, recent findings identified large non-mitochondrial sources, in particular NOX enzymes, as the dominant source of cellular ROS during muscle contraction (Sakellariou et al., 2013; Henríquez-Olguin et al., 2019). The discrepancy between the expected and actual role of mitochondria in the production of ROS during muscle contraction may be explained by the misconception of mitochondrial ROS as a byproduct of mitochondrial ATP production (Kanter, 1994; Urso and Clarkson, 2003; Powers and Jackson, 2008).

Recent advances in the use of mitochondrially targeted antioxidants (such as MitoQ, SkQ and Mito-Tempo) in different disease models could also provide good tools to study mitochondrial contributions to cellular ROS production. The mitochondrially targeted antioxidants have been used in models of diverse diseases such as neurodegenerative diseases, diet-induced metabolic syndrome, diabetic kidney disease, ischemia–reperfusion injuries and drug-induced liver toxicity (Smith and Murphy, 2010; Isaev et al., 2016; Oliver and Reddy, 2019). These bioavailable compounds would quickly accumulate in the mitochondrial matrix once they enter the cells. They can either stop lipid peroxidation (MitoQ and SkQ) or quench all H_2O_2 (Mito-Tempo) inside the mitochondrial matrix (Murphy and Smith, 2007). These compounds have been intensively tested in a large number of disease models and human clinical trials (Smith and Murphy, 2010). As shown in the literature, these mitochondrially targeted antioxidants often offer only partial protection against the disease phenotypes. *N*-Acetyl cysteine, which acts as a glutathione precursor, is often used as a positive control. This indicates that ROS produced from other cellular sites might also contribute to the diseases investigated (Lowes et al., 2008; Hu et al., 2018). Moreover, ROS production from different cellular sites might also vary between disease stages. In evolutionary studies, mitochondrially targeted antioxidants have been employed as a tool to study the role of mitochondrial ROS in evolutionary processes. For example, MitoQ has been used to study the growth trajectory of yellow-legged gulls (*Larus michahellis*), where fast growth during early development might be costly (Velando et al., 2019). It was thought that fast growth might increase cellular ROS production, which would induce damage and accelerate the aging process. In the study, mitochondrial DNA damage was negatively correlated with chick mass, but this relationship was not affected by MitoQ treatment, suggesting that MitoQ does not prevent oxidative damage in this system (Velando et al., 2019). Alonso-Alvarez and colleagues (Cantarero and Alonso-Alvarez, 2017; Cantarero et al., 2020) used both MitoQ and Mito-Tempo on red crossbills (*Loxia curvirostra*) and zebra finches (*Taeniopygia guttata*) to study carotenoid-based coloration. It has been hypothesized that birds display a trade off in the use of carotenoids – they can be used for body maintenance (particularly as antioxidants to directly mitigate ROS and prevent oxidative damage) or for ornamentation (Hill et al., 2019). However, these studies found different effects of MitoQ and Mito-Tempo treatments (Cantarero and Alonso-Alvarez, 2017; Cantarero et al., 2020). High doses of MitoQ decreased carotenoid levels in both bird species, whereas Mito-Tempo increased ketocarotenoid levels in the circulation. However, the results from these studies are hard to interpret, because antioxidant capacity and the level of oxidative damage were not measured. Consequently, we cannot conclude whether these antioxidants successfully decreased oxidative stress. Future comprehensive studies are warranted to reveal whether oxidative stress induced by mitochondrial ROS is the mechanism underlying these life-history tradeoffs.

Conclusion and future directions

In this Commentary, we have reviewed three potential reasons behind the misassumption that mitochondria are the main source of cellular ROS production. We have also introduced some other major cellular ROS-producing sites/enzymes that could contribute significantly to total cellular ROS production. Using a recently developed cell-based assay, we measured the contributions of different cellular compartments to ROS production in a collection of cell lines and primary cells. Based on our findings, mitochondrial ROS contribution varies between cell types but is never the main source of cellular ROS. This indicates that although mitochondria are significant sources of cellular ROS, they may not be the largest contributors of cellular H_2O_2 release under normal growing conditions. Furthermore, we have discussed the mitochondrial contribution of cellular ROS under a variety of stressors, life-history strategies and pathological conditions. Under these conditions, mitochondria also may not be the main source of ROS. Together with other authors, we urge scientists in both the biomedical and evolutionary physiology fields to also consider other sources of ROS in their study systems or models (Brown and Borutaite, 2012; Munro and Treberg, 2017).

It is important to note that our ROS measurements in cells are based on a number of assumptions. For the sake of simple interpretation, we assume a homogeneous distribution of cellular organelles, cells that are spherical in shape and unidirectional diffusion of H_2O_2 from the inside to the outside of cells. These are oversimplifications of the complex cellular system. For example, several substantial diffusion gradients of H_2O_2 have been documented, from the extracellular to intercellular space, between subcellular compartments and even within subcellular organelles (Sies, 2017). The complexities of the cellular system and the technical difficulties inherent in measuring ROS make it extremely hard to quantify the exact ROS production from different cellular compartments. More importantly, our measurements were done *in vitro*, where cells were cultured in artificial environments with higher P_{O_2} and substrate levels compared with the *in vivo* situation. Therefore, the contributions of cellular ROS from the different cellular compartments will be different to those *in vivo*. Lower P_{O_2} and substrate levels *in vivo* would be likely to lead to lower rates of ROS production by mitochondria, and thus would result in an even lower mitochondrial contribution of cellular ROS. Unfortunately, because of technical difficulties, measurements of ROS production levels *in vivo* are performed using boronated probes (such as MitoB) with mass-spectrometric techniques or

using genetically modified organisms that express redox-sensitive fluorescent proteins (Hanson et al., 2004; Cochemé et al., 2011). These methods can provide snapshots of ROS production, but cannot reveal kinetic measurements of flux as is the case *in vitro*. Technical difficulties limit our research, especially in evolutionary studies where genetically modified organisms are often not available for the species of interest (Salin et al., 2017). Because of this, we propose that researchers should adopt an integrative approach, studying redox status at the organelle, cellular and organismal levels concurrently. Methods to prepare primary cell cultures from a variety of tissues (such as muscle satellite cells, hepatocytes and macrophages) are fully established and could be used by evolutionary physiologists on their species of interest with only minor optimization. Using *in vitro* models to study the question would allow researchers to better delineate possible pathways and identify markers involved *in vivo*. This integrative approach may be effort intensive but it would offer substantial benefits when interpreting data to draw solid conclusions.

ROS and oxidative stress have been connected to aging and pathologies such as diabetes, neurodegeneration, cancer and cardiovascular disease. Much effort has been spent on developing pharmaceutical interventions against mitochondrial ROS production and oxidative stress. We encourage researchers to use these pharmaceutical compounds (almost all of which are commercially available) as tools to address their research questions. These compounds could be considered as good tools to bypass the lack of robust measurements of ROS production *in vivo* as we have discussed above. However, these compounds must be adequately characterized and used correctly at physiologically relevant dosages to avoid potential side-effects. Pilot studies to titrate dosages of these compounds in the study system with confirmational measurements of targeted changes in antioxidant and/or oxidative damage level, as well as positive and negative controls are preferred. We are confident that by using appropriate approaches and study design, and by understanding that ROS could be produced by many different cellular compartments, researchers will make significant progress on redox biology in both fields.

Last but not least, even though mitochondria might not be the largest site of ROS production in cells, they remain a major controller of cellular redox homeostasis. A number of researchers have hypothesized that mitochondria may serve as a ROS sink, rather than a source of ROS production under some circumstances. This idea is supported by the high abundance of antioxidant enzymes and endogenous antioxidants found in mitochondria (Starkov, 2008). As part of being a sink, mitochondria can also rapidly consume extra-mitochondrial $O_2^{\cdot-}$, and can even respire significantly on $O_2^{\cdot-}$ via cytochrome *c* and cytochrome oxidase (Mailer, 1990). This hypothesis can be tested by titrating the effect of Mito-Tempo on cellular ROS. If it is true, Mito-Tempo should be able to quench all cellular (including non-mitochondrial) ROS. However, in our experiments, excessive Mito-Tempo failed to remove all the $O_2^{\cdot-}$ and H_2O_2 produced by all tested cells (Fig. S2), suggesting that the hypothesis of mitochondria as a sink for ROS production might only be partially correct. Munro and Treberg (2017) have recently considered this topic and its application to life-history studies in another Commentary published in this journal.

In conclusion, the significance of different cellular compartments in the production of cellular ROS under various cellular conditions remains to be explored. However, current data indicate that mitochondria are a significant source of ROS production, but may not be the largest contributor. More studies are warranted to investigate the regulation of mitochondrial ROS production and metabolism in the context of different life-history traits and pathologies.

Appendix

Protocols for cellular hydrogen peroxide (H_2O_2) release measurements and cellular respiration measurement

Details of the cell culture conditions are listed in Table S1. Rates of H_2O_2 release were assessed based on the HRP-mediated oxidation of Amplex UltraRed (non-fluorescent) to Amplex UltroxRed (fluorescent) by H_2O_2 (see Wong et al., 2019; Fig. A1). Any extracellular release of $O_2^{\cdot-}$ was also captured by the addition of excess exogenous SOD1 to convert $O_2^{\cdot-}$ to H_2O_2. We assumed that intracellular $O_2^{\cdot-}/H_2O_2$ production is irreversible, so lowering extracellular H_2O_2 levels by adding HRP will not alter production rates. We also assumed a homogeneous distribution of organelles and spherical cell morphology. To assess net rates of H_2O_2 release,

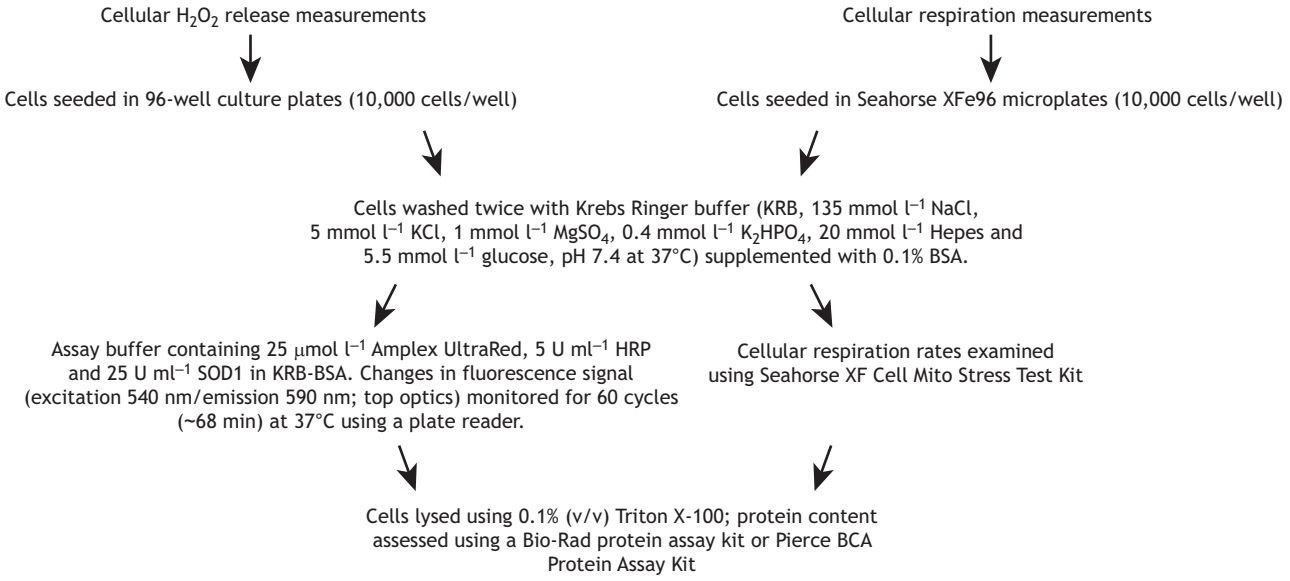

Fig. A1. Schematic diagram outlining the methods used to measure H_2O_2 release and cellular respiration.

cells were seeded to reach complete confluency in a 96-well microtiter plate (black, flat bottom). On the day of the experiment, cells were incubated with pre-warmed KRB-BSA at 37°C for 30 min. The measurement of H_2O_2 release was then initiated by changing KRB-BSA to a pre-warmed assay buffer containing 25 µmol l^{-1} Amplex UltraRed, 5 U ml^{-1} HRP and 25 U ml^{-1} SOD1 in KRB-BSA. Changes in fluorescence signal (excitation 540 nm/emission 590 nm; top optics) were monitored for 1 h at 37°C. Measured fluorescence in the operational range was linearly dependent on H_2O_2 added during signal calibration; fluorescence values were converted to pmol H_2O_2 using plate-matched calibration curves. The rate of H_2O_2 release from cells was calculated as the slope of a plot of H_2O_2 content against time after subtraction of the small rate of H_2O_2 release in the absence of cells in parallel wells for each experiment. The rate of H_2O_2 production was then normalized to the cell protein content of each well and expressed as pmol min^{-1} mg^{-1} protein. The contributions of NOXs and mitochondria to total H_2O_2 release were assessed by supplementing the assay buffer with NOX inhibitor (GKT136901) or Mito-Tempo. GKT136901 decreased the response of the HRP-Amplex UltraRed assay to added H_2O_2; this effect was corrected for by conducting all H_2O_2 calibrations in the presence of the appropriate concentration of each inhibitor. The rates of cellular respiration were assessed using a Seahorse XFe96 Analyzer (Fig. A1). Cells were seeded to reach complete confluency in Seahorse XFe96 cell culture microplates. On the day of the experiment, cells were washed twice with pre-warmed KRB-BSA followed by a 30 min incubation with KRB-BSA in an air incubator at 37°C. The rate of cellular respiration was then determined using Seahorse XF Cell Mito Stress Test Kit (cat. no. 103015-100, Agilent) in the presence of vehicle or test compounds according to the corresponding manufacturer protocols. The maximum capacity of mitochondrial respiration was defined as the maximum rate induced by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 1 µmol l^{-1}).

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Competing interests
The authors declare no competing or financial interests.

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Figure S1. Effects of Mito-Tempo (MT) and NOXs inhibitors (GKT136901,GKT) on hydrogen peroxide release rate and respiration in cells. These figures are representative graphs as these titrations were done in all cell types. A-B, Seahorse traces of different dosages of MT and GKT on respiration rates in AML12 hepatocytes. C-D, Titration of MT and GKT on basal, uncoupled, oligomycin induced, and non-mitochondrial respiration in AML12 hepatocytes. E-F, Titration of MT and GKT on hydrogen peroxide release in AML12 hepatocytes. Vertical dotted lines indicate the ranges of inhibitor concentrations that gave maximum inhibition; horizontal dotted lines indicate the rates of H_2O_2 release under un-inhibited and maximally-inhibited conditions. G, Rates of hydrogen peroxide release in AML12 hepatocytes. The contributions of mitochondrial matrix and NOXs were defined by the use of GKT (1 μM) and MT (1 μM) (left). The additivity of these reagents was further confirmed by incubating cells with a mix of GKT and MT (right). H-J, Rates of hydrogen peroxide release by H9c2 rat cardiomyoblasts (H), N27A dopaminergic neural cell (I) and IMR90 human lung fibroblast (J). The contributions of mitochondrial Complexes I and III, mitochondrial matrix and NOXs were defined by the use of S1QEL (1 μM), S3QEL (3 μM), MT (10 μM) and GKT (1 μM). The rate of H2O2 release from mitochondrial Complexes I and III was compared to the rate of H2O2 release from mitochondrial matrix and yield no significance. Values are means ± SEM (N = 3 independent experiments). Keys: Anti A – Antimycin A; FCCP - carbonyl cyanide p-trifluoromethoxyphenylhydrazone; Rot - rotenone

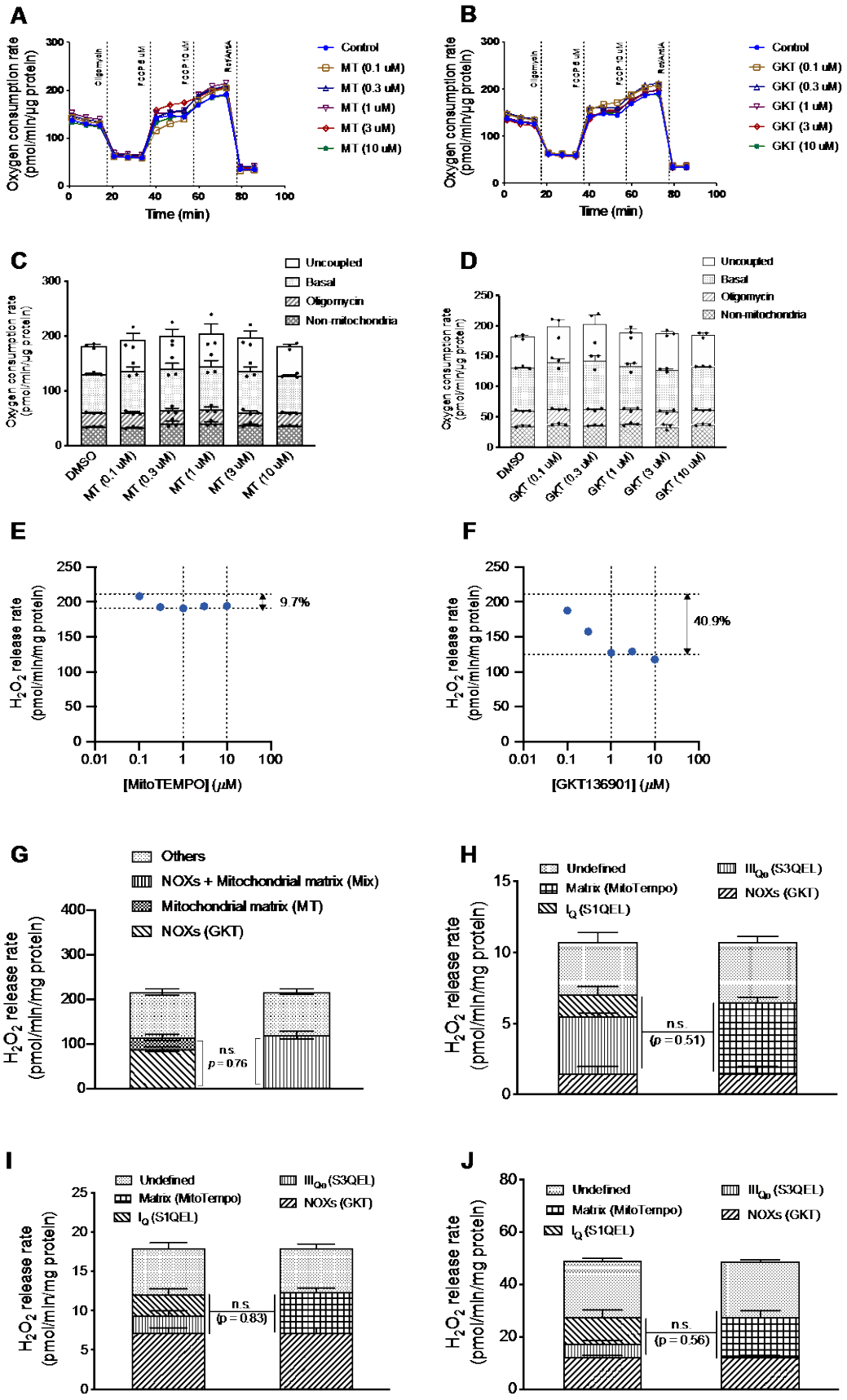
A
Oxygen consumption rate (pmol/min/µg protein)
Time (min)
Oligomycin
FCCP 1 uM
FCCP 10 uM
Rot/AntiA
Control
MT (0.1 uM)
MT (0.3 uM)
MT (1 uM)
MT (3 uM)
MT (10 uM)
B
Oxygen consumption rate (pmol/min/µg protein)
Time (min)
Control
GKT (0.1 uM)
GKT (0.3 uM)
GKT (1 uM)
GKT (3 uM)
GKT (10 uM)
C
Oxygen consumption rate (pmol/min/µg protein)
Uncoupled
Basal
Oligomycin
Non-mitochondria
DMSO
MT (0.1 uM)
MT (0.3 uM)
MT (1 uM)
MT (3 uM)
MT (10 uM)
D
Oxygen consumption rate (pmol/min/µg protein)
Uncoupled
Basal
Oligomycin
Non-mitochondria
DMSO
GKT (0.1 uM)
GKT (0.3 uM)
GKT (1 uM)
GKT (3 uM)
GKT (10 uM)
E
H2O2 release rate (pmol/min/mg protein)
9.7%
[MitoTEMPO] (µM)
F
H2O2 release rate (pmol/min/mg protein)
40.9%
[GKT136901] (µM)
G
H2O2 release rate (pmol/min/mg protein)
Others
NOXs + Mitochondrial matrix (Mix)
Mitochondrial matrix (MT)
NOXs (GKT)
n.s.
p = 0.76
H
H2O2 release rate (pmol/min/mg protein)
Undefined
Matrix (MitoTempo)
IQ (S1QEL)
IIIQo (S3QEL)
NOXs (GKT)
n.s.
(p = 0.51)
I
H2O2 release rate (pmol/min/mg protein)
Undefined
Matrix (MitoTempo)
IQ (S1QEL)
IIIQo (S3QEL)
NOXs (GKT)
n.s.
(p = 0.83)
J
H2O2 release rate (pmol/min/mg protein)
Undefined
Matrix (MitoTempo)
IQ (S1QEL)
IIIQo (S3QEL)
NOXs (GKT)
n.s.
(p = 0.56)

Table S1. Culture conditions for each cell type. H9c2, IMR90, N27A, AML12 cells were purchased from ATCC (Manassa, VA). Darmaraland mole rat lung fibroblast, Siberian hamster skin and lung fibroblast were isolated and cultured according to Seluanov et al. (2010).

Cell type	Culture condition
H9c2 rat cardiomyoblast	Dulbecco's Modified Eagle Medium (DMEM, 25 mM glucose), 10% v/v fetal bovine serum (FBS), penicillin (100 IU/ml)-Streptomycin (100 µg/ml). Cultured in tissue culture incubator at 37°C, 5% CO_2, ambient O_2.
Damaraland mole rat lung fibroblast	Eagle's Minimum Essential Medium (EMEM. 5.6 mM glucose), 10% v/v FBS, penicillin (100 IU/ml)-Streptomycin (100 µg/ml). Cultured in tissue culture incubator at 37°C, 5% CO_2, 3% O_2.
IMR90 human lung fibroblast	DMEM (25 mM glucose, without sodium pyruvate), 10% v/v FBS, penicillin (100 IU/ml)-Streptomycin (100 µg/ml). Cultured in tissue culture incubator at 37°C, 10% CO_2, 3% O_2.
N27A dopaminergic neural cell	RPMI-1640 Medium (25 mM glucose), 10% v/v FBS, penicillin (100 IU/ml)-Streptomycin (100 µg/ml). Cultured in tissue culture incubator at 37°C, 5% CO_2, ambient O_2.
AML12 mouse hepatocytes	DMEM:F12 Medium (17.5 mM glucose), 10% v/v FBS, penicillin (100 IU/ml)-Streptomycin (100 µg/ml), insulin (10 µg/ml)-transferrin (5.5 µg/ml)-selenium (5 ng/ml), dexamethasone (40 ng/ml). Cultured in tissue culture incubator at 37°C, 5% CO_2, ambient O_2.
Siberian hamster dermal fibroblast	EMEM supplemented with 5.6 mM glucose, 10% v/v FBS, penicillin (100 IU/ml)-Streptomycin (100 µg/ml). Cultured in tissue culture incubator at 37°C, 5% CO_2, 3% O_2.
Siberian hamster lung fibroblast	

Table S2. Hydrogen peroxide release rate (pmol/min/mg protein) from different cellular compartments in each cell types. Values are means ± STD (N = 3 independent experiments); values in brackets are percentages of total H_2O_2 release rate of respective cells.

Cell types	Total	Mitochondria	NOXs	Unidentified
H9c2 rat cardiomyoblast	10.7 ± 1.16	5.02 ± 0.08 (46.7%)	1.48 ± 10.7 (13.8%)	4.23 ± 0.90 (39.4%)
Damaraland mole rat lung fibroblast	28.1 ± 4.85	9.04 ± 0.70 (32.6%)	8.47 ± 1.31 (31.2%)	10.59 ± 5.14 (36.2%)
IMR90 human lung fibroblast	49.0 ± 0.76	15.2 ± 4.52 (31.0%)	12.2 ± 1.04 (24.9%)	21.57 ± 5.05 (44.1%)
N27A dopaminergic neural cell	17.9 ± 2.11	5.26 ± 0.84 (29.3%)	7.15 ± 1.19 (39.9%)	5.52 ± 0.97 (30.8%)
AML12 mouse hepatocytes	211 ± 12.6	20.5 ± 3.24 (9.70%)	86.6 ± 3.1 (41.0%)	104.1 ± 5.32 (49.3%)
Siberian hamster dermal fibroblast	80.0 ± 11.1	7.55 ± 2.98 (9.44%)	63.7 ± 9.40 (79.6%)	8.76 ± 3.30 (11.0%)
Siberian hamster lung fibroblast	109.1 ± 2.92	6.28 ± 2.52 (5.76%)	81.4 ± 62.0 (74.6%)	21.5 ± 13.7 (19.7%)

Reference

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