Methodological considerations for the determination of mitochondrial ADP sensitivity in skeletal muscle

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Summary

In skeletal muscle, mitochondria adapt to physiological (i.e. exercise, aging) and pathological scenarios (i.e. insulin resistance, muscle atrophy). Due to the kinetic regulation by adenylates within the oxidative phosphorylation system, a small increase in free ADP ($_f$[ADP]) within the cell results in a rapid utilization of oxygen and production of ATP, conferring to mitochondria the unique ability to detect, and respond, to small changes in the energetic status of the cells. The advent of high-resolution oxygraphy potentiated the studies on mitochondrial bioenergetics where it is now possible to record mitochondrial O_2 consumption in real-time with high sensitivity in various tissues and substrate protocols. While most of the studies rely on saturating concentrations of substrates and ADP to test the maximal respiratory capacity of mitochondria, such approaches may not fully recapitulate physiological conditions by which these organelles are exposed within skeletal muscle cells. Over the years, we and others have employed a mitochondrial ADP sensitivity assay, where we determine mitochondrial bioenergetic responses to a wide range of ADP concentrations, scaling from the physiological levels found in resting skeletal muscle cells (μM) to saturating values (mM). Here, we reviewed this methodology by offering practical guidance and insights from experiments performed in our laboratory, as well as examples of the applicability of such a protocol.

1. Introduction

Metabolism and mitochondrial bioenergetic are centrally involved in several physiological and pathological processes. Nowadays, diseases such as diabetes[1], cardiovascular diseases[2], Alzheimer's[3], and non-alcoholic fatty liver disease (NAFLD)[4], are somehow related to alterations in mitochondrial physiology. The alterations of mitochondrial bioenergetics lead to cellular changes that may result in dysfunctional cells, and eventually, the development of disease. Therefore, the comprehensive characterization of mitochondrial alterations in such conditions holds the promise to serve as a diagnosis as well as treatment of a wide range of conditions[5].

The advance of technology allowed the rapid growth of research in mitochondrial biology and metabolism. High-resolution respirometry equipment offers a sensitive and versatile approach to test mitochondrial bioenergetics[6–8], enabling researchers to address different questions with very little biological material[2,7,9,10]. Mitochondrial oxidative phosphorylation (OXPHOS), based on seminal work led by Chance[11], is classically assessed in extreme, non-physiological, states, ranging from the absence of ADP to saturating concentrations (mM range)[8,12,13]. Although such methods are useful for increasing our understanding of mitochondrial bioenergetics, they are quite artificial given mitochondria very rarely face such abrupt low/high levels of free ADP ($_f$[ADP]) within the cells[14]. For instance, ATP consumption induced by muscle contraction elevates ADP concentration within muscle cells by several fold[15–18], however, still below the mM range used in experiments with permeabilized fibers or isolated mitochondria. Such small fluctuations were, indeed, modeled more than 20 years ago when mitochondrial ADP sensitivity was assessed in skeletal muscle[19]. However, these studies reported mitochondrial apparent K_m to be too low (~100 μM ADP) to be biologically relevant[19]. It is now understood that these artificially low apparent K_m values are caused by spontaneous contraction of permeabilized muscle fibers, which can be prevented with specific myosin ATPase inhibitors[20]. As a result, after more than two decades following the original estimates of submaximal ADP responses in skeletal muscle[19], we now have the ability to determine more physiologically relevant mitochondrial bioenergetic responses in unique metabolic situations. The application of this approach is expected to unravel novel connections between mitochondrial bioenergetics and cellular homeostasis in diverse situations/pathologies.

Here, we have attempted to provide a resource for individuals interested in incorporating this methodology into their laboratory. Practical aspects as well as the pathophysiological implications of its use in skeletal muscle bioenergetics and metabolism are revised. Of note, routine methodology of respirometry and design of experiments were already revised by colleagues elsewhere and will not be critically analyzed here[6,8,21,22].

2. Methods

2.1 Ethical approval

All experiments were performed under institutional guidelines and approved by the Animal Care Committee at the University of Guelph (4241).

2.2   Mice

Ten 20-week-old female C57/Bl6N mice were used in this study. Mice were housed in collective cages (~22 °C) on a 12-hour light-dark cycle with food and water *ad libitum*.

2.3   High-resolution respirometry

Mitochondrial respiration was determined in saponin-permeabilized red gastrocnemius skeletal muscle fibers in an Oxygraph high-resolution respirometer chamber with 2 mL MiR05 at 37 °C as previously described with minor modifications[23,24]. Briefly, red gastrocnemius was excised, immediately placed in ice-cold BIOPS (50 mM MES, 7.23 mM K_2EGTA, 2.77 mM CaK_2EGTA, 20 mM imidazole, 0.5 mM dithiothreitol, 20 mM taurine, 5.77 mM ATP, 15 mM PCr and 6.56 mM $MgCl_2 \cdot H_2O$, pH 7.1) and fibers bundles were separated using fine-tipped forceps. Fibers were then transferred to 1.5 mL of BIOPS buffer and permeabilized with saponin (40 µg/mL) for 30 min at 4 °C with constant mixing. After permeabilization protocol, fibers were transferred to a 1.5 mL MiR05 buffer (0.5 mM EGTA, 3 mM $MgCl_2 \cdot 6H_2O$, 60 mM K-lactobionate, 10 mM KH_2PO_4, 20 mM HEPES, 20 mM taurine, 110 mM sucrose, 1 g/L FFA-free BSA, pH 7.1) and washed twice for 15 min. Thereafter, fibers were inserted into the chamber to determine rates of oxygen consumption by high-resolution respirometry (Oroboros Oxygraph-2k, Innsbruck, Austria). Mitochondrial respiration was assessed following the addition of 5 µM blebbistatin (or absence – Figure 4), 5 mM pyruvate (or glutamate 10 mM) and 2 mM malate, ADP was titrated in various concentrations (see Table 1), and 10 µM cytochrome *c* (to assess mitochondrial membrane integrity) were sequentially added. Experiments with cytochrome *c* responses greater than 10 % were excluded from the analysis. Two measurements were excluded due to cytochrome *c* response greater than 10 % (One in Figure 3A-C and one in Figure 4A-C). Full ADP titration experiments were performed in a range of 160-200 µM O_2 by opening and closing the chambers after every single injection. Experiments to address the variance of O_2 concentration in mitochondrial respiration were performed in the presence of 1 mM ADP with or without the injection of pure O_2. Michaelis-Menten kinetic analysis was used to estimate mitochondrial ADP sensitivity (apparent K_m) whereby O_2 consumption in the absence of ADP (only pyruvate/glutamate + malate) was defined as V_0 and analysis was constrained to maximal ADP-supported respiration (100 % maximal mitochondrial respiration). Importantly, the addition of oligomycin (1 µM) and antimycin A (1 µM) at the end of the ADP titration protocol fully blunts ADP-driven respiration, confirming the coupling state of skeletal muscle fibers.

2.4   Statistical analysis

Raw data was extracted from DatLab 8.0 and analyzed using Prism 8.0 (GraphPad Software, La Jolla, CA, USA). Correlation analysis was done in R version 4.3.2. All raw data as well as script is available upon reasonable request.

# 3.   Practical aspects

3.1   Experimental design

To test mitochondrial ADP sensitivity in permeabilized muscle fibers (Fig. 1A), the first step is to provide substrates to guarantee mitochondria are properly energized. In

general, we use a mix of pyruvate/malate to provide NADH to Complex I and generate the necessary protonmotive force to allow ATP synthesis by the mitochondrial F_1F_0-ATPase (or ATP synthase). Once substrates are provided and mitochondrial respiration is stable in state 2, an ADP titration can be initiated. Table 1 provides an example of an ADP titration protocol that can be applied in skeletal muscle fibers. The titration starts with submaximal (or physiological) ADP injections, mimicking the concentrations found in resting muscle. Next, a progressive increase in ADP concentration is performed until the maximal capacity of OXPHOS is reached.

Table 1 – Example of ADP concentrations used in mitochondrial ADP sensitivity assay.

ADP injection	Stock concentration	Volume injected	Final concentration
1	50 mM	4 µL	100 µM
2	50 mM	3 µL	175 µM
3	50 mM	3 µL	250 µM
4	50 mM	10 µL	500 µM
5	500 mM	2 µL	1000 µM
6	500 mM	4 µL	2000 µM
7	500 mM	8 µL	4000 µM
8	500 mM	8 µL	6000 µM
9	500 mM	8 µL	8000 µM
10	500 mM	8 µL	10000 µM

It is important to highlight that the incorporation of an ADP sensitivity experiment into the SUIT protocol does not affect maximal responses. In fact, state 2, maximal state 3, and respiratory control ratios (i.e. state 3/state 2) can be calculated regardless of the ADP sensitivity protocol in this methodology. Figure 1B shows a schematic representation where an ADP titration is inserted into the classical SUIT protocol instead of a single bolus of maximal ADP (Table 1). Of note, rotenone and uncouplers can be added after cytochrome c if necessary. Figure 1C shows the quantification (normalized by dry weight) of such an experiment. As can be observed, state 2, state 3, and *RCR* are assessed despite the ADP sensitivity protocol being incorporated (Fig. 1D). Once respiration values are obtained, to examine the dynamic response to ADP, one can plot the JO_2 values as a % of the maximal respiration obtained with ADP, setting the ordinate as the JO_2 in the absence of ADP (i.e pyruvate/malate; Fig. 1E). Importantly, given ADP titration protocol takes much longer than a single bolus of saturating ADP concentrations, integrity of mitochondrial preparation may be subject of concern. To test this, we performed an experiment where oligomycin and antimycin A were added separately at the end of the titration protocol (Fig. 1F). We observed, however, no impairment in coupling respiration caused by ADP titration protocol, where mitochondria were fully coupled with respiration returning to baseline values following oligomycin addition (Fig. 1 G).
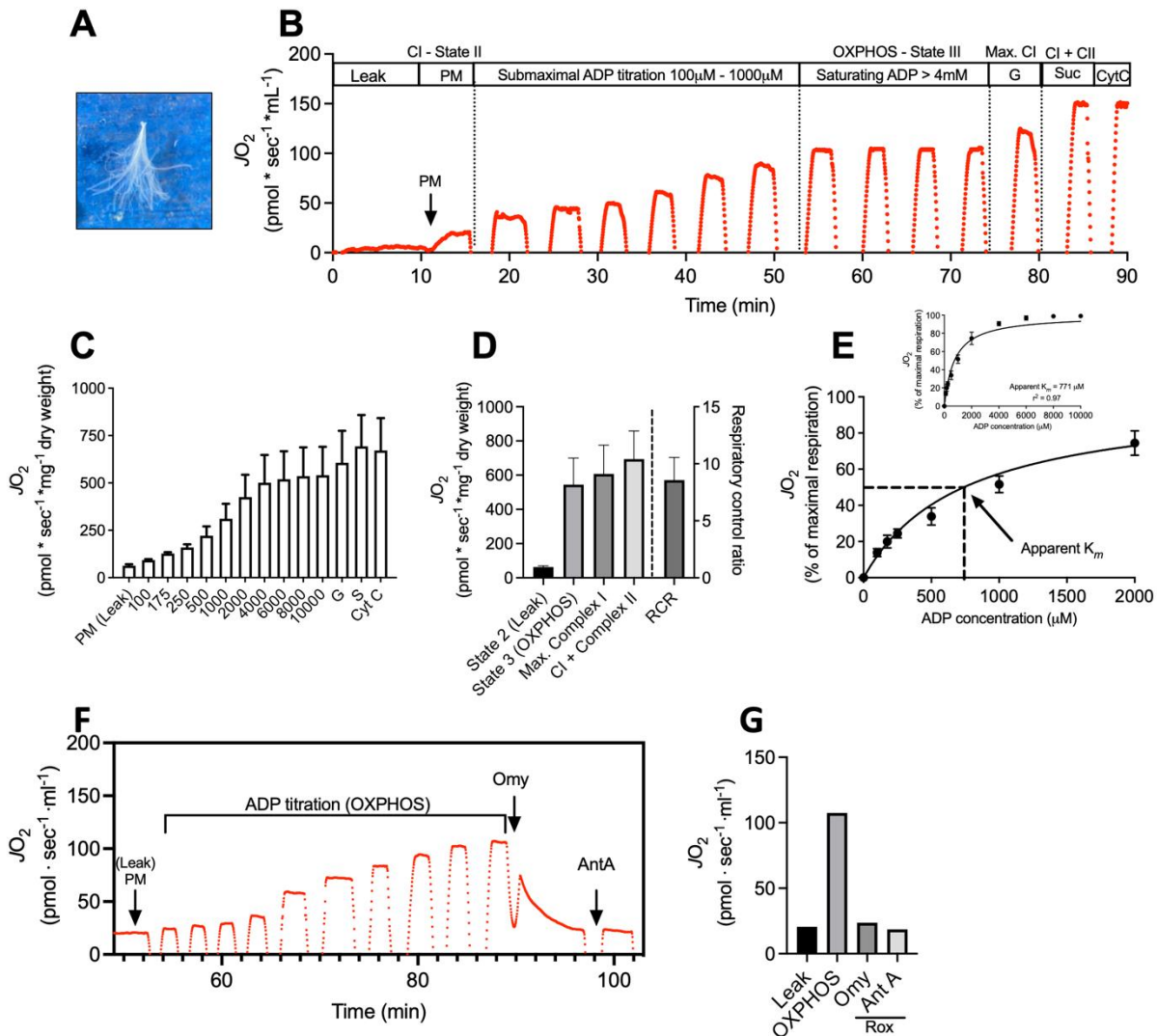
Figure 1. Example of incorporation of mitochondrial ADP sensitivity method into the SUIT protocol. (A) Skeletal muscle (red gastrocnemius) fibers after mechanical separation. **(B)** Schematic example of the ADP sensitivity protocol inserted into the classical SUIT protocol. **(C)** Absolute mitochondrial respiratory values after all injections of the protocol; **(D)** State 2, state 3, maximal Complex I (pyruvate/malate/glutamate), Complex II-linked respiration, and respiratory control ratio (state 3/state 2); **(E)** ADP-driven respiration as % of maximal ADP-supported respiration (inset) and zoomed ADP-driven respiration to highlight the ADP concentration necessary to reach 50 % of maximal mitochondrial respiration. **(F)** Representative respiration trace with the addition of oligomycin and antimycin A at the end of the protocol and **(G)** quantification of mitochondrial respiration in the presence and absence of oligomycin. AntA – Antimycin A; ADP – adenosine diphosphate; P – pyruvate; M – malate; S – succinate; Cyt *c* – cytochrome *c*; G = glutamate; Omy – Oligomycin; *RCR* – respiratory control ratio; OXPHOS – oxidative phosphorylation. C-D sample size = 4, B, F, G sample size =1.

As observed in Figure 1, the Michaelis-Menten equation (Equation 1) is applied to the generated data to calculate the apparent mitochondrial ADP K_m (i.e. ADP sensitivity).

$$\text{Equation 1. } y = \frac{V_{\max} \times x}{(K_m + x)}$$

It is important to highlight that the Michaelis-Menten equation was first designed for enzymatic activity where a single enzyme is tested against one substrate. In this condition, the Michaelis-Menten equation provides the K_m values, which is the substrate concentration necessary to achieve 50 % of the maximal enzymatic activity, and the V_{max}, which is the maximal activity of the enzyme when the substrate is not limiting. Given the estimation of mitochondrial ADP sensitivity by oxygen consumption measurements relies on several proteins and complexes (i.e. ATP synthase, ANT, electron transfer system, mitochondrial content), it is not appropriate to use the Michaelis-Menten constant term directly. Therefore, the term apparent K_m is more appropriate, given it better recapitulates the information provided by this methodology.

3.2   Effect of oxygen tension on oxidative phosphorylation and ADP sensitivity

Mitochondrial respiration may be affected by oxygen tension inside the chamber. While low oxygen tension is not a major concern when working with isolated mitochondria, this is particularly important when permeabilized muscle fibers are being analyzed. Figure 2 highlights the effects of low oxygen tension on mitochondrial respiration in state 3 in permeabilized muscle fibers (Fig. 2A). In general, we notice O_2 tension/diffusion to be a limitation around 130-140 nmol/mL (or µM) (Fig. 2A), and a dramatic 50 % reduction in state 3 respiration when oxygen tension is below 100 µM. While the threshold for O_2 diffusion depends on several aspects, particularly the quality of mechanical separation of the fibers, maintaining an $[O_2] > 150$ µM ensures O_2 is not limiting. Moreover, once O_2 diffusion limitation has occurred, reoxygenation of the chamber with pure O_2 can rescue mitochondrial respiration (Fig. 2A). However, artificial (i.e. pure O_2 injection) changes in O_2 tension increase JO_2 values above those observed before O_2 limitation is developed (Fig. 2A). This ischemic-reperfusion artefact creates a challenge for establishing a full ADP kinetic range to assess ADP sensitivity. While one solution is to start with hyperoxia to prevent the need for reoxygenation, room air possesses ~4-fold greater O_2 content than skeletal muscle interstitial fluid in a resting situation, and therefore room air is already non-physiological. Alternatively, we find that reoxygenating when O_2 tension above is 150 µM and not limiting does not affect JO_2 (Fig. 2B). In this respect, opening/closing the chamber to allow repeated room-air equilibrium does not alter mitochondrial respiration in the presence of ADP (Fig. 2B-C). As a result, our experimental approach is to keep oxygen tension during the experiment in a range that does not affect oxidative phosphorylation, essentially 'clamping' O_2 between room air and 150 µM. We achieve this by opening and closing the chamber after every single injection. Figure 2D shows a representative run of a mitochondrial ADP sensitivity protocol where O_2 concentration was kept between room air (~190 µM) and 150 µM. While this approach negates a possible artifact to rapid fluctuations in O_2, another benefit is that all JO_2 determinations occur at a relatively consistent O_2 tension. As recently considered by Walsh et al. (2023), oxygen tension is an overlooked confounding factor when assessing mitochondrial bioenergetics that should be considered, and reported, in the methods section [8].
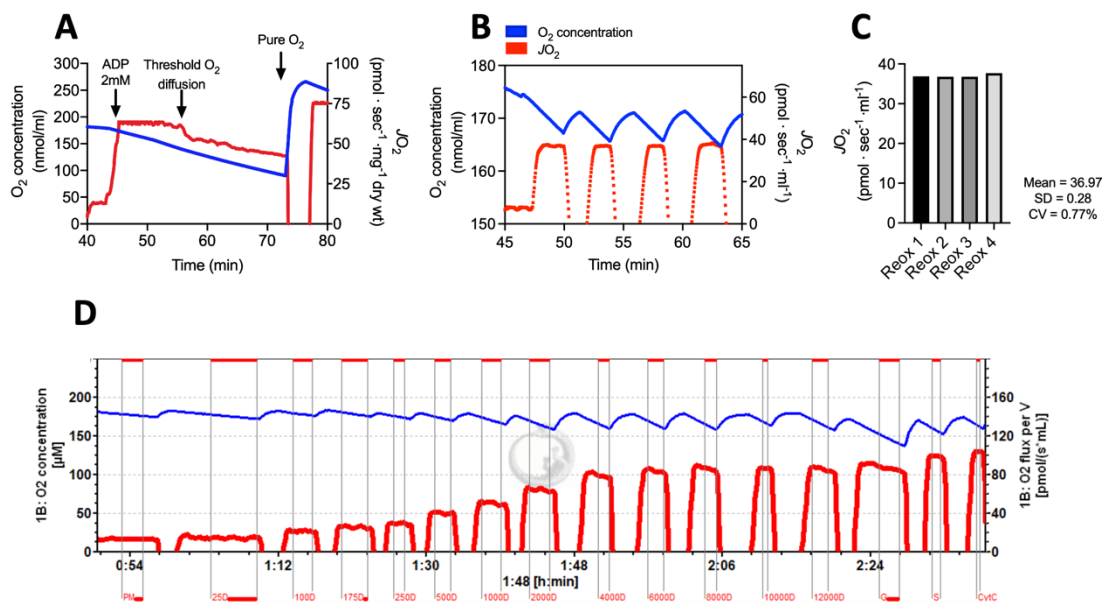
Figure 2. Oxygen tension is critical for mitochondrial ADP sensitivity assay. (A) Low oxygen tension limits state 3 mitochondrial respiration in muscle-permeabilized fibers. **(B)** Representative experiment showing reoxygenation of the chamber and mitochondrial state 3 (ADP 2 mM) respiration. **(C)** Quantification of state 3 after reoxygenation of the chamber when performed in non-limiting O_2 diffusion. **(D)** A representative experiment with a chamber being opened after every ADP injection Sample size = 1.

3.3   Substrate-dependency of mitochondrial ADP sensitivity

Several substrates may be considered for assessing mitochondrial ADP sensitivity, but a combination of pyruvate and malate is the most common. Pyruvate/malate (5 mM and 2 mM, respectively) provides NADH to Complex I, allowing the membrane potential gradient formation necessary to test mitochondrial ADP responsiveness. However, in combination with malate, glutamate can also be used to supply NADH to Complex I whereas succinate alone can support electron entry/reduction of a flavoprotein within Complex II. Figure 3A-B shows a typical ADP kinetic curve in the presence of PM and GM in the presence of blebbistatin. It can be observed that mitochondrial respiration kinetics are quite different depending on which substrate is used to energize mitochondria. As a result, the Michaelis-Menten constant delineates an almost one-fold lower mitochondrial ADP sensitivity (i.e. higher apparent K_m) when glutamate is used instead of pyruvate (Fig. 3C). This difference may be explained by the necessity of high mitochondrial membrane potential for glutamate transport into mitochondria and possible fluctuations in glutamate transport throughout the ADP titrations. Regardless of the possible mechanism-of-action, the intrinsic differences in substrates supporting mitochondrial membrane potential during ADP titration should be kept in mind when designing experiments addressing submaximal mitochondrial bioenergetics in different physio-pathological conditions.
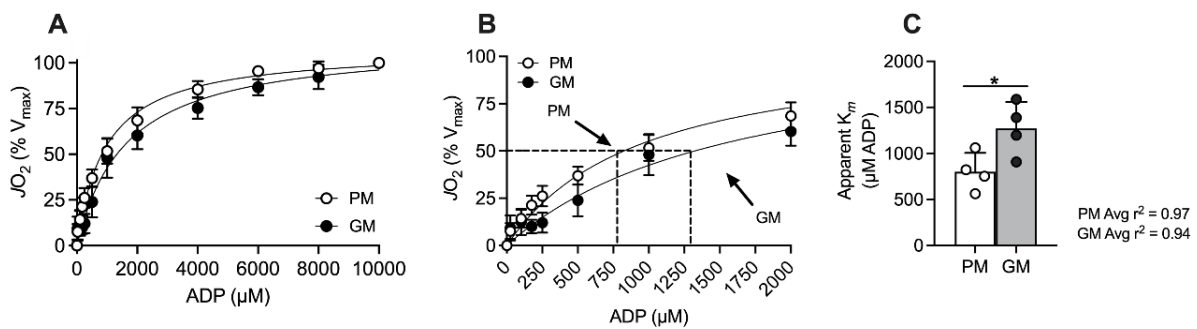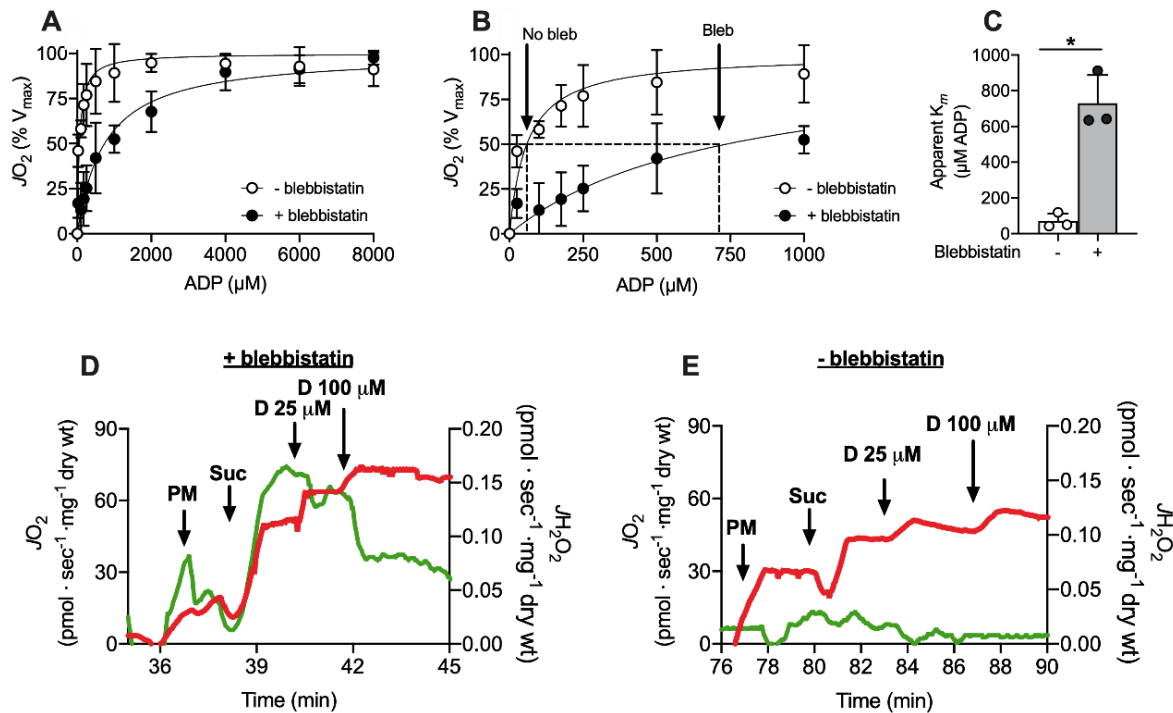
Figure 3. Mitochondrial ADP sensitivity is influenced by substrate used. (A) ADP kinetics curve in the presence of pyruvate or glutamate as substrates. **(B)** Zoomed ADP kinetic curve to highlight the apparent ADP K_m achieved by using glutamate or pyruvate. **(C)** Apparent ADP K_m in resting skeletal muscle permeabilized fibers. All experiments were performed in the presence of blebbistatin. P – pyruvate; M – malate; G – glutamate. Statistical test: one-tail Student's t-test. Sample size = 4.

3.4   Effects of muscle contraction on mitochondrial ADP sensitivity

In experiments with permeabilized muscle fibers, muscle contraction is a confounding factor when investigating mitochondrial ADP sensitivity[25]. The effect of muscle contraction can be appreciated by comparing mitochondrial ADP responsiveness in fibers in the presence or absence of blebbistatin, an inhibitor of myosin II (Fig. 4A-B). The presence of blebbistatin increases the apparent mitochondrial ADP K_m ~8-10-fold to~700-1000 μM (Fig. 4C), a value above $_f$[ADP] within muscle (50-200 μM), which appears to be more physiologically meaningful when compared to previous estimates[19]. In addition to supporting JO$_2$ assessments, blebbistatin affects hydrogen peroxide (H$_2$O$_2$) measurement in permeabilized fibers. Importantly, the impact of muscle contraction on the assessment of mitochondrial ROS production has been previously shown by Anderson et al. (2009)[26] and future studies should carefully consider this variable in their measurements as well as interpretation of the findings. Modern Oroboros apparatus allows for the simultaneous measurement of oxygen consumption and hydrogen peroxide (H$_2$O$_2$) emission, enabling unparalleled interrogations into mitochondrial ADP responses, which stimulate JO$_2$ while attenuating H$_2$O$_2$ emission. To highlight this point, we tested mtROS in the presence and absence of blebbistatin (Fig. 4D-E). In the presence of blebbistatin, succinate stimulated mitochondrial ROS emission, while the addition of two different concentrations of ADP (25 μM and 100 μM) gradually reduced mtROS emission and, as expected, increased O$_2$ consumption (Fig. 4D). In contrast, in the absence of blebbistatin, the rates of O$_2$ consumption were not dramatically different than those in the presence of blebbistatin, however the absolute rate of mtROS was ~6-fold lower (Fig. 4E). In summary, addition of blebbistatin provides a better resolution to measurements of mitochondrial O$_2$ consumption and H$_2$O$_2$ emission upon submaximal ADP levels.

Figure 4. Effect of muscle contraction on mitochondrial ADP sensitivity. (A) ADP kinetics curve in the presence or absence of blebbistatin. **(B)** Zoomed ADP kinetic curve to highlight the apparent ADP K_m achieved by the presence or absence of blebbistatin. **(C)** Apparent ADP K_m of contracted or relaxed permeabilized muscle fibers. Simultaneous measurement of mitochondrial oxygen consumption and hydrogen peroxide production in the presence **(D)** and absence **(E)** of blebbistatin. Bleb – blebbistatin; PM – pyruvate/malate; Suc – succinate; D – ADP; $J\mathrm{O}_2$ – oxygen flux (red line); $J\mathrm{H}_2\mathrm{O}_2$ – hydrogen peroxide flux (green line). Statistical test: two-tailed Student's t-test. Sample size = 3. * $p<0.05$.

3.5 Variability inter-experiment

Given the relative nature of the apparent ADP sensitivity protocol (i.e. % of the maximal respiration achieved upon saturating concentrations of ADP), the variability of each experiment must be considered. For that, we assessed mitochondrial ADP sensitivity in red gastrocnemius from one single animal across four different Oroboros equipment where the average of technical replicates is shown in Figure 5. Individual oxygen consumption kinetic curves show great overlap among experiments (Fig. 5A and 5B). In addition, we plotted the apparent ADP K_m (Fig. 5C) and maximal oxygen consumption (Fig. 5D) individually. The coefficient of variation across equipment was <15 % regardless of the measurement (i.e. apparent ADP K_m or maximal respiratory capacity). Of note, the source of such variability, although small, could likely be explained by intrinsic differences in mitochondrial content depending on the portion of the muscle extracted (i.e. fiber type differences), mechanical separation of fibers, and/or recovery of fibers from the chamber to weigh.
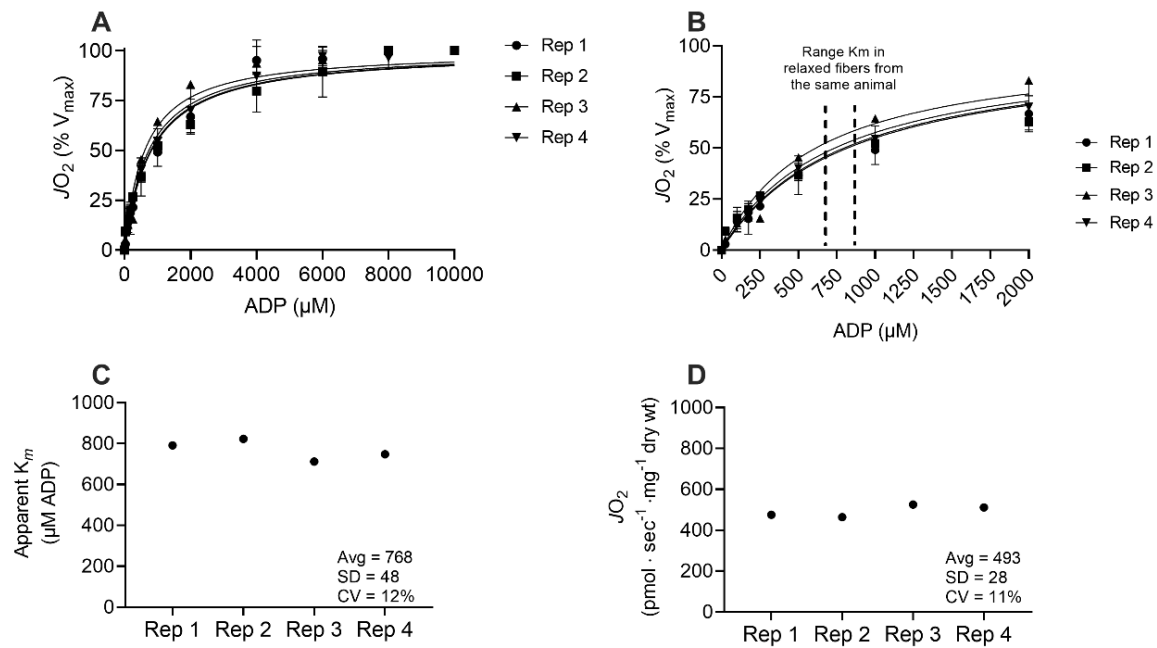
Figure 5. Assessment of mitochondrial ADP sensitivity presents low variability across oxygraphy equipment. (A) Individual whole ADP kinetics and **(B)** zoomed ADP kinetic curve to highlight the apparent ADP K_m. **(C)** Individual apparent ADP K_m and **(D)** maximal oxygen consumption in the presence of saturating ADP concentrations in the buffer. Avg - average; SD – standard deviation; CV – coefficient of variation.

4.   Physiological relevance of mitochondrial ADP sensitivity

By applying mitochondrial ADP sensitivity methodology in skeletal muscle fibers, we have observed the plasticity of mitochondria in different contexts to be much more dynamic than previously hypothesized. For instance, acute interventions such as insulin bolus or a single bout of exercise [23,27,28], can modulate ADP sensitivity without changing the maximal respiratory capacity of mitochondria (Figure 6). Although the mechanisms are still unclear, these findings suggest metabolic scenarios imposed by these two conditions are somehow matched by mitochondrial bioenergetics modulation without changes in mitochondrial content.
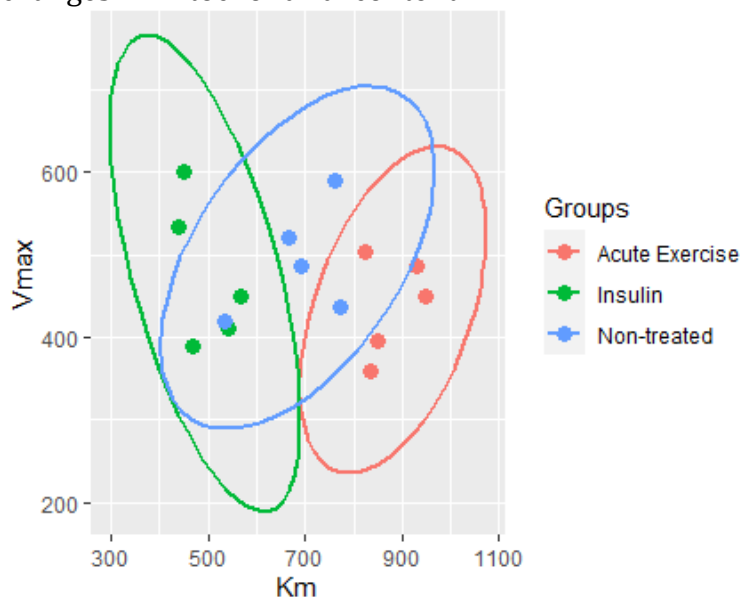
Figure 6. Rapid modulation of mitochondrial ADP sensitivity without changes in maximal respiratory capacity skeletal muscle fibers from mice. Correlation between apparent ADP K_m with maximal respiratory capacity in pmol·s^{-1}·mg^{-1} of dry weight. Data retrieved from Miotto & Holloway, 2016[29] and Brunetta et al., 2021[23].

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In addition, chronic interventions such as exercise training or high-fat diet (HFD) feeding are also able to modulate mitochondrial ADP sensitivity[27,29,30]. Of note, interpretations from these experiments must be made carefully, whereas, on one side, beneficial metabolic effects caused by exercise training are well-known, the reduction in ADP sensitivity, similar to what is seen after HFD-feeding, cannot be interpreted as maladaptation given the marked increase in mitochondrial content within skeletal muscle following training. Therefore, mitochondrial ADP sensitivity, as much as any other measurement of oxygen consumption, is confounded by mitochondrial content in the biological sample[31]. The effects of mitochondrial content on ADP sensitivity are further supported by the differences between glycolytic *vs.* oxidative fibers, where ADP sensitivity is found to be 2-4-fold greater in glycolytic fibers (i.e. lower apparent K_m) compared to oxidative muscle [32–34]. Conversely, we found that 8 weeks of high-fat diet-feeding reduced mitochondrial ADP sensitivity without changes in the maximal respiratory capacity despite the increase in markers of mitochondrial content[24,29]. Similarly, we observed that ADP responsiveness in skeletal muscle is modulated by aging[35], exercise[28,36], lipids[37], hormones[23], and ADP recycling (i.e. creatine cycle)[38]. Mechanistically, we have shown the attenuation in ADP responsiveness may be linked to post-translational modifications of adenine-nucleotide transporter (ANT)[29], but this remains an important area of research. In the future, deeper characterization of mitochondrial adaptations under these conditions must be performed to identify the mechanistic regulation of mitochondrial ADP sensitivity which may also involve post-translational modifications to ATP synthase.

Variability during respirometry experiments is a concern among several laboratories around the world[8]. We have been able to reduce such a confounding factor by recovering the fibers after every experiment, drying them in a cold vacuum, and weighing them. Therefore, our data is normalized by dry weight and not wet weight. This approach avoids errors in the weighing process that may occur when any reminiscent buffer drops remain within the fibers. Although such drops are small, they significantly contribute to the final fiber weight given the small fiber size (i.e. ~1 mg wet weight or ~0.2-0.3 mg dry weight). We have also noticed that the drying process on paper necessary to accurately weigh fibers before putting them into the Oroboros chamber results in fiber contraction that cannot be recovered with blebbistatin. As a result, although maximal coupled respiration is not affected by this procedure, the apparent ADP K_m is artificially reduced (i.e. higher ADP sensitivity) by drying for wet weight normalization, creating a large source of variability for submaximal ADP experiments. As a result, we recover the fibers after the experiment to normalize raw data to dry weight, which offers several advantages when assessing ADP kinetics, including the prevention of contraction-mediated reduction in the apparent ADP K_m. In addition, by recovering fibers after the experiment, we can control for any experimental problems that may happen during the protocol. For instance, fibers can get stuck to the stopper of the Oroboros chamber or rip apart during the experiment, which is more common in contracted fibers (i.e. absence of blebbistatin). Recovering the fibers provides a visual inspection of the integrity of fiber bundles, and when fiber bundles rip apart it is our standard procedure to remove that experiment from the final analysis given the inaccurate tissue weight for normalization. Of note, a cloudy buffer is observed when the fibers rip apart, providing insight into the integrity of the preparation and full recovery of the fibers. Additionally, recovered fibers can also be utilized for Western blotting[39], providing an opportunity to directly compare OXPHOS protein abundance and fiber type to bioenergetics.

It is important to highlight some limitations when it comes to *in vitro* modeling energetic perturbations and substrate flux. Although our approach gradually exposes mitochondria to low/high concentrations of ADP, biologically, saturating substrates (pyruvate) do not exist in muscle in the absence of ADP. While this approach ensures an ADP limitation on respiration, it would be interesting to test ADP responsiveness upon a range of energetic conditions (i.e. low-to-high pyruvate levels). Also, our approach assesses mitochondrial bioenergetics in conditions that are far from physiological, including O_2 tension, substrate concentration, the absence of contraction (i.e. presence of blebbistatin) and varying content of adenosine. As mentioned in the manuscript, these variables affect ADP responsiveness and must be taken into account when designing and analyzing experimental data. Finally, mitochondria handle several different substrates at the same time, and the interaction between those substrates is generally overlooked. Recently, we addressed this question by using a mix of several substrates simultaneously titrated to interrogate mitochondrial respiration upon a variety of pHs [37]. Although this approach may offer a different angle to analyze mitochondrial bioenergetics, it is difficult to pinpoint a mechanism given the complex nature of using a combination of several substrates. Therefore, we encourage the continued use of ongoing classical approaches for comparison/validation against historical data, and the incorporation of non-canonical substrate combinations and experimental approaches to further interrogate mitochondrial biology, which may lead to new findings and interpretations regarding the role of mitochondrial bioenergetics in a variety of conditions.

5.   Concluding remarks

Measuring mitochondrial metabolic fluxes via oxygen consumption rate has been essential to the modern understanding of mitochondrial physiology. Although landmark studies were performed in extreme conditions by using non-ADP in the media (state 2) or a bolus of saturating ADP (mM range) to achieve state 3, such approaches may not fully recapitulate the physiological fluctuations mitochondria experience within the cells. By determining mitochondrial oxygen consumption under a wide range of ADP concentrations, over the last decade, we have been able to identify subtle changes in mitochondrial bioenergetics that could not be visualized by measuring maximal OXPHOS capacity using saturating ADP concentrations. In addition, Fisher-Wellman et al. (2018)[38] have developed a similar approach where mitochondria are clamped in different energetic pressures to allow the interrogation of mitochondrial bioenergetics in a much more comprehensive angle than by using single-injection of a bolus of ADP. In summary, modeling physiological scenarios of transient energetic status is an interesting strategy for interrogating mitochondrial bioenergetics[39]. Such approaches have the potential to reveal nuances in mitochondrial adaptations previously overlooked when applying methods to determine the maximal capacity of these organelles. By combining classical approaches with creative new experimental designs, this exciting field can move forward toward a better understanding of mitochondrial bioenergetics and biology.

Abbreviations

f[ADP]	free ADP		JO$_2$	O$_2$ flux
GM	glutamate and malate		PM	pyruvate and malate

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