Published in final edited form as:

Curr Opin Clin Nutr Metab Care. 2010 September ; 13(5): 511–517. doi:10.1097/MCO.0b013e32833cc93d.

Mitochondrial Metabolic Function Assessed *In Vivo* and *In Vitro*

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Abstract

Purpose of review—Mitochondrial content and function vary across species, tissue types, and lifespan. Alterations in skeletal muscle mitochondrial function have been reported to occur in aging and in many other pathological conditions. This review focuses on the state of the art *in vivo* and *in vitro* methodologies for assessment of muscle mitochondrial function.

Recent findings—Classic studies of isolated mitochondria have measured function from maximal respiratory capacity. These fundamental methods have recently been substantially improved and novel approaches to assess mitochondrial functions *in vitro* have been emerged. Non-invasive methods based on magnetic resonance spectroscopy (MRS) and near-infrared spectroscopy (NIRS) permit *in vivo* assessment of mitochondrial function and are rapidly becoming more accessible to many investigators. Moreover, it is now possible to gather information on regulation of mitochondrial content by measuring the *in vivo* synthesis rate of individual mitochondrial proteins.

Summary—High-resolution respirometry has emerged as a powerful tool for *in vitro* measurements of mitochondrial function in isolated mitochondria and permeabilized fibers. Direct measurements of ATP production are possible by bioluminescence. Mechanistic data provided by these methods is further complimented by *in vivo* assessment using MRS and NIRS and the translational rate of gene transcripts.

Keywords

mitochondria; skeletal muscle; respiration; magnetic resonance spectroscopy; mass spectrometry

1. INTRODUCTION

Mitochondria have attracted the attention of scientists for decades, but widespread interest in these organelles has grown tremendously in recent years. We now know that the role of mitochondria in cell biology goes beyond their function in generating ATP. Mitochondria are also critical in cell signaling [1], reactive oxygen species (ROS) production [2], regulation of apoptosis and cellular aging [3]. The content and function of these organelles increases with physical training [4], decreases with aging and physical inactivity [5,6], and are altered with many diseases such as type 2 diabetes [7], obesity [8], heart failure [9], stroke [10], and Alzheimer Disease [11]. Furthermore, mitochondrial biogenesis appears to be regulated, in part, by insulin [12]. Sensitive and reliable assays for measuring mitochondrial function is critical to further understand the role of this organelle in normal physiology and as the basis of many pathological states. In this short review we will discuss several methodologies for assessing mitochondrial function, including *in vivo* techniques as well as *in vitro* methods using freshly prepared, non-frozen tissue samples. We will also

highlight an emerging methodology for measuring *in vivo* synthesis rates of individual mitochondrial proteins that, in combination with transcript levels, offer new insight in to the translational regulation of both nuclear and mitochondrial genes encoding mitochondrial proteins.

2. MAXIMAL ATP SYNTHESIS

The main function of mitochondria is to generate ATP by oxidizing nutrients (glucose, fatty acids, and some amino acids). In the tricarboxylic acid cycle (TCA), energy is released from acetyl groups as reduced coenzymes (NADH, FADH₂). Subsequently, the energy generated by electron transport is conserved by phosphorylation of ADP to ATP. The capacity for ATP synthesis is a property of tissue or mitochondria that is frequently used to define its functionality.

2.a. In vitro approaches

Firefly luciferase catalyzes the following reaction[13].

Using a luminometer to measure light emission, it becomes possible to quantify the maximal (i.e., state 3) rates of ATP production in a biological sample [14,15]. Mitochondria must be isolated from fresh tissue because freezing disrupts the membrane structure. With exogenous glutamate and malate, electron flow is provided exclusively through complex I (NADH dehydrogenase). The combination of succinate and rotenone selectively assesses complex II (succinate dehydrogenase). The substrate combination of palmitoyl carnitine and malate allows measurement of ATP synthesis from fatty acid β -oxidation. ADP is added to the reaction to stimulate state 3 ATP synthesis. The applications for this methodology are vast. For example, in the past 18 months, our group has used this approach to demonstrate that endurance exercise can largely prevent the age-related decline in oxidative capacity in humans [6], muscle mitochondrial function is dissociated from insulin sensitivity and obesity [16], and muscle mitochondrial capacity is increased by branched chain amino acids in young but not elderly people [17].

2.b. In vivo approaches

Measurements in isolated mitochondria provide mechanistic insight, but there is critical need for valid, reliable *in vivo* measurements of mitochondrial ATP production capacity. Magnetic resonance has been used for many years to noninvasively measure oxidative capacity in human skeletal muscle. Technological advances and increasing availability of higher field strength MR systems have made these types of measurements possible for many clinicians and researchers. Phosphorous magnetic resonance spectroscopy (³¹P-MRS) allows non-invasive measurement of phosphorous-containing metabolites, including high-energy phosphates ATP and phosphocreatine (PCr). Under non-steady-state conditions, maximal ATP production capacity can be assessed by measuring the kinetics of PCr recovery following depletion by a brief bout of muscle activity. The recovery rate constant is proportional to muscle mitochondrial oxidative capacity, which can be calculated as the product of k_{PCr} and the concentration of PCr in the resting skeletal muscle [1820]. This approach has been found to be reproducible [21,22] and well-correlated with citrate synthase activity measured *in vitro* [23]. An exciting new adaptation of the classic dynamic ³¹P-MRS method has been recently published [24,25]. Spatially resolved dynamic ³¹P spectra were acquired by chemical shift imaging (CSI) and used to estimate oxidative capacity from PCr recovery kinetics. This was accomplished by having subjects perform very brief, submaximal muscle contractions repeatedly with sufficient recovery to prevent fatigue or

acidosis. The liberal duty cycle and low intensity permitted multiple gated acquisitions following each contraction [24,25]. This approach is a major advancement because it overcomes many limitations of the classic dynamic spectroscopy protocols, specifically sacrificing signal-to-noise for time resolution, acidosis, fatigue, glycolytic contribution during exercise, and heterogeneity of metabolic events within a muscle group. Using this approach, Forbes and colleagues compared oxidative capacities among several muscle groups with strikingly different phenotypes in humans (lateral gastrocnemius, medial gastrocnemius, soleus, and tibialis anterior) [25]. Paradoxically, the tibialis anterior muscle, which is primarily composed of oxidative type I muscle fibers, exhibited a lower oxidative capacity than the more glycolytic gastrocnemius muscles. It appears that the relationship between fiber-type composition and oxidative capacity may not be as straightforward as once believed. Similar observations were recently made by Larsen et al., who found that PCr recovery kinetics were faster in vastus lateralis (mixed fiber phenotype) than in the tibialis anterior muscle [26]. These two recent MRS-based studies support previous work *in vitro* that showed dissociation between fiber type composition and mitochondrial enzyme activities [27]. A strong association between PCr recovery kinetics and moderate to vigorous physical activity in the vastus lateralis but not the tibialis anterior supports a notion that the oxidative capacity of a muscle is dictated by their patterns of use than by their fiber composition [26].

The widespread use of ^{31}P -MRS to investigate muscle energetics has prompted some to claim that it is now the “gold standard” method to non-invasively assess intramuscular energy metabolism [21]. In light of such claims it is important to carefully examine the precision and accuracy of the methodology. Surprisingly few investigators have directly assessed the reliability of PCr recovery kinetics [28,29,21,22]. Recent publications demonstrated excellent reproducibility of PCr recovery kinetics [21,22]. It is reassuring to know that several investigators are conducting carefully controlled, reproducible experiments, but it is critical that more research groups demonstrate the reliability and accuracy of their experiments, particularly in human studies in which it is difficult to control diet and physical activity before studies.

3. MAXIMAL OXYGEN CONSUMPTION

Oxygen is the final electron acceptor in the respiratory chain where it is reduced to water at complex IV (cytochrome c oxidase). Because the reduction of oxygen is a necessary precursor event to ATP synthesis, mitochondrial capacity is often assessed from the rates of oxygen consumption.

3.a. In vitro approaches

Several instruments are commercially available which allow measurements of oxygen consumption in biological samples. Recent advances in the field of high resolution respirometry permit measurements of oxidative capacity in very small amounts of isolated mitochondria (0.01 mg) or permeabilized muscle fibers (2mg) [30] from needle biopsy samples. At this point, the state-of-the-art in this field comes in the form of a twin-chamber instrument manufactured by Oroboros Instruments (Innsbruck, Austria). The distinguishing features that make this instrument so well-suited for measurements in small amounts of tissue are high sensitivity, low instrumental background, precise temperature and stir control, all of which allow experiments to be performed at high dilutions (2ml). Using an appropriate respiration buffer at high sample dilutions, it is possible to perform sequential measurements involving the titration of substrates, inhibitors, and uncouplers [31]. These types of experiments would be impossible with microchamber instruments where the oxygen concentration would quickly be exhausted. Key advantages of this approach include 1) the ability to assess the respiratory capacity at multiple levels of the respiratory chain, 2) serial

measurements in the same tissue sample, and 3) measurements from very small amounts of isolated mitochondria or permeabilized muscle fibers [32,31]. The utility of this methodology was nicely demonstrated in a recent paper by Anderson et al. in permeabilized muscle fibers from lean and obese men. Obese men exhibited lower state 3 respiration with glutamate + malate and increased H_2O_2 production rates [32]. Interestingly, a single high-fat meal acutely increased mitochondrial H_2O_2 production rates in lean men [32]. The above study provides valuable insight into the potential etiology of insulin resistance with a link between mitochondrial bioenergetics, oxidant production, and insulin action.

3.b. In vivo approaches

At the whole-body level, the maximal rate at which an individual consumes oxygen (VO_{2max}) can be approximated by indirect calorimetry during near-maximal exercise (VO_{2peak}). Although VO_{2max} is determined in part by the amount of oxygen extracted from the blood by respiring mitochondria, it is also a function of the oxygen-carrying capacity of the blood, tissue perfusion, and cardiac output. Thus, although VO_{2max} correlates with mitochondrial enzyme activities [33], the impact of central hemodynamics complicates its role as an *in vivo* equivalent to tissue respiration measurements performed *in vitro*. Furthermore, it is impossible to glean any tissue-specific information using whole-body indirect calorimetry

Near-infrared spectroscopy (NIRS) is another *in vivo* approach to measure tissue oxygenation by measuring optical absorption changes in oxy- and deoxy-heme groups (i.e., hemoglobin, myoglobin, and cytochrome c). During exercise, it is possible to continuously monitor oxygenation in the muscle, but the NIRS signal represents the balance between oxygen delivery and consumption. By interrupting oxygen supply by local ischemia, the rate of oxygen consumption can be determined by the change in the NIR signal [34]. Although NIRS systems are relatively inexpensive and portable, the major challenge in studying muscle metabolism relates to differentiating between multiple heme-containing molecules [35]. A recent paper addressed this challenge using a mathematical model of oxygen transport and metabolism during exercise [36]. This approach was able to predict the relative contribution of hemoglobin and myoglobin to the NIRS signal during exercise, and model predictions were compared with experimental data [36]. This approach, as well as other emerging methodologies such as contrast-enhanced ultrasound and arterial spin labeling allow tissue perfusion to be measured.

Myoglobin oxygen saturation can be measured with 1H -MRS, making it possible to assess intracellular oxygenation *in vivo*, without contributing signals from hemoglobin [37,39]. When myoglobin is fully oxygenated, its signal is virtually impossible to observe because its resonance overlaps with the resonance of tissue water [38]. When the protein becomes deoxygenated, its resonance shifts approximately 80 ppm downfield and can be observed in a proton spectrum [38]. Intracellular PO_2 can be calculated from the relative saturation of myoglobin based on the relationship between intracellular PO_2 and myoglobin saturation. While this approach holds promise for measuring tissue oxygen consumption rates, much like NIRS, the challenge remains in how to differentiate the reciprocal contributions of oxygen supply and demand to the overall signal. Tevald et al. recently applied this approach to further investigate the phenomenon whereby skeletal muscle becomes deoxygenated during ischemic muscle contractions, but to a lesser extent than deoxygenation during resting ischemia [40]. Using 1H -MRS to measure myoglobin deoxygenation, these investigators suggest that low intramyocellular PO_2 limits mitochondrial oxygen consumption during ischemic contractions, thus preventing complete desaturation of the muscle [40]. Figure 1 provides a summary schematic of the advantages and drawbacks of several *in vivo* and *in vitro* approaches for probing mitochondrial function.

4. MITOCHONDRIAL COUPLING

Electron transport and ATP synthesis are tightly coupled, but some of the energy generated by electron transport is uncoupled from ATP synthesis. The efficiency of oxidative phosphorylation can be defined by the ratio of the number of moles of ATP generated for each atom of oxygen consumed (P/O ratio). Some of the redox energy generated by electron transport is dissipated by movement of protons back into the matrix in a way that is uncoupled from the phosphorylation of ADP and accounts for anywhere from 15 to 50% of mitochondrial respiration, depending on the species and mitochondrial state [41,43]. The physiological impact of mitochondrial uncoupling continues to be a topic of debate. Uncoupling may attenuate reactive oxygen species (ROS) production by dissipating the mitochondrial membrane potential, as has been demonstrated in long-lived nematodes [44] and bats [45]. Furthermore, the “thrifty phenotype” or “thrifty genotype” hypotheses have been put forth to explain why some populations are susceptible to obesity and insulin resistance [46]. Nevertheless, mitochondrial coupling or efficiency is an important component of mitochondrial function that goes beyond the capacity of the organelle for oxygen consumption of ATP synthesis.

4.a. In vitro approaches

The efficiency of oxidative phosphorylation can be measured in isolated mitochondria by high-resolution respirometry [30]. The general concept underlying this method is to measure the amount of oxygen consumed for a given amount of ATP synthesized by the mitochondria. As a proxy for this, it is possible to determine ADP/O flux ratios. When isolated mitochondria are added to a physiological buffer, the vast majority of endogenous substrates and ADP have been washed away. Then state 3 is transiently stimulated by adding substrates and ADP at a concentration that is below the k_m of oxidative phosphorylation for ADP. This pulse injection of ADP increases oxygen consumption until the ADP has all been phosphorylated to ATP. By quantifying the amount of oxygen consumed in response to a known amount of ADP, it becomes possible to measure the efficiency of oxidative phosphorylation, assuming that all ADP is phosphorylated. This approach is carefully described in the paper by Gnaiger and colleagues, including the critical corrections for instrumental background, and the volume and oxygen injected during the ADP pulse [30]. Furthermore, these investigators developed a novel approach for measuring ADP/O flux by measuring the rate of oxygen consumption during steady-state ADP injection [30].

An important determinant of mitochondrial efficiency is the passage of protons back into the mitochondrial matrix, uncoupled from ATP synthesis. It is possible to measure the extent of this proton leak during state 4 respiration when ATP synthase is inhibited by oligomycin. The residual oxygen consumption in the presence of oligomycin represents respiration that is needed to maintain the mitochondrial membrane potential in the presence of uncoupling. The respiratory control ratio, or the ratio of state 3 to state 4 is a common indicator of the tightness of coupling between oxygen consumption and phosphorylation.

4.b. In vivo approaches

In vivo measurements of mitochondrial coupling are technically challenging, but several research groups have demonstrated that this parameter can be successfully assessed *in vivo* using unique non-invasive approaches. One approach was demonstrated by Conley and colleagues who combined information from ^{31}P -MRS and NIRS to assess ATP production and oxygen consumption, respectively [47,48]. The contributions of hemoglobin and myoglobin in the optical spectra were separated using partial least squares analysis and calibration sets from which spectra of oxy and deoxy hemoglobin and myoglobin, and oxidized and reduced cytochrome c are recorded and used to generate coefficients to predict

myoglobin and hemoglobin saturation *in vivo* [47]. The next challenge in this approach is to quantify Hb and Mb concentrations. This is accomplished using a 2-point calibration (breathing 100% oxygen vs. limb ischemia) and ^1H -MRS to measure [Mb] *in vivo* [48]. These investigators recently used this approach to demonstrate that the human tibialis anterior muscle is mildly uncoupled at a young age and is less susceptible to age-related declines in function. In contrast, the first dorsal interosseus is well-coupled at a young age, but exhibits substantial uncoupling and evidence of mitochondrial dysfunction with aging [48]. The authors posit that some low-level degree of uncoupling may protect against some of the detriments of aging, possibly by attenuating damage by ROS.

Another *in vivo* approach to measure mitochondrial coupling involves two independent magnetic resonance methods. ^{13}C -MRS is used to measure citric acid cycle flux while ^{31}P -MRS is used to measure ATP synthesis [49,50]. By combining the independent measurements of mitochondrial oxidative function (TCA flux) with ATP synthesis, it becomes possible to estimate the coupling between fuel oxidation and phosphorylation. When labeled acetate [$2\text{-}^{13}\text{C}$] is infused intravenously, it rapidly reaches an isotopic steady-state and is oxidized in the TCA cycle. ^{13}C -MRS is used to measure the time course of ^{13}C incorporation into carbons 2 and 4 of glutamate. Determination of TCA cycle flux was accomplished by computer software to simultaneously solve several differential equations to model the metabolic fate of the labeled acetate [49]. In a separate experiment, the unidirectional rate of ATP synthesis is measured by a saturation-transfer approach. Briefly, the gamma ATP peak is selectively irradiated with a radiofrequency pulse, which causes a decrease in the signal of the Pi peak. This decrease is proportional to the rate constant of the phosphate exchange between the two species and can be used to calculate the phosphorylation rate during steady-state conditions [49]. This approach was most recently used by Befroy and colleagues to demonstrate that endurance trained humans exhibit increased mitochondrial uncoupling [49]. Both of the aforementioned *in vivo* approaches represent the cutting edge in non-invasive assessment in mitochondrial coupling. The drawbacks of these methods are 1) they require separate experiments to measure oxidation and phosphorylation, 2) they require a large amount of technical expertise, and 3) many standard clinical imaging systems may not be equipped for many of the MRS-based measurements.

5. PROTEIN SYNTHESIS RATES *IN VIVO*

Proper mitochondrial function is dictated to a large extent by the expression of mitochondrial proteins, but also by the integrity and functionality of individual mitochondrial proteins or protein complexes. Proteins undergo numerous posttranslational modifications that can interfere with their intended function such that increased expression of a particular protein may not necessarily reflect the abundance of functional proteins. Maintenance of a functional proteome is accomplished by the constant turnover of the protein pool as a consequence of degradation of old proteins and synthesis of new proteins to take their place. It is now possible to measure the synthesis rates of individual skeletal muscle mitochondrial proteins *in vivo* [51]. Briefly, muscle proteins are labeled *in vivo* by intravenous infusion of L-[$\text{ring-}^{13}\text{C}_6$]phenylalanine, followed by extraction and rapid freezing of tissues. Individual muscle proteins are purified by 2-dimensional gel electrophoresis, and the fractional synthesis rates of these proteins are calculated from the isotopic enrichment (measured by tandem mass spectrometry) of gel spots. This calculation is performed using the tissue fluid free phenylalanine enrichment as the precursor pool. This new methodology has wide-reaching applications since it can be performed in animals and in humans in combination with transcript levels of the specific proteins thus offering an opportunity to determine whether a condition or an intervention are accompanied by changes at the transcriptional or translational levels. Because the systemic infusion of

isotope simultaneously labels all proteins being synthesized, it is possible to adapt this methodology to measure the synthesis rates of many proteins from nearly any organ tissue or biological fluid.

6. CONCLUSION

There are numerous approaches to probe mitochondrial function. Recent advances in high-resolution respirometry are quickly leading to widespread use of this methodology as a gold standard for *in vitro* assessment in isolated mitochondria or intact tissue samples. *In vivo* methodologies using near infrared and magnetic resonance spectroscopies are advancing rapidly and allow non-invasive assessment of both mitochondrial capacity and efficiency of fuel oxidation. Finally, the combination of novel protein purification techniques and mass spectrometry enable the *in vivo* synthesis rates of individual proteins to be measured in many types of tissue and body fluids.

Acknowledgments

This work was supported by National Institutes of Health (NIH) Grants R01-AG-09531, R01-DK-41973, and UL1-RR-024150-01 from the National Center for Research Resources, a component of the NIH, and NIH Roadmap for Medical Research. K.S. Nair is also supported by a David Murdock Dole Endowed Professorship. I.R. Lanza is supported by NIH KL2 RR024151.

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Figure 1.
In vivo and *in vitro* methods for assessing muscle mitochondrial function