

Methods for Assessing Mitochondrial Function in Diabetes

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A growing body of research is investigating the potential contribution of mitochondrial function to the etiology of type 2 diabetes. Numerous *in vitro*, *in situ*, and *in vivo* methodologies are available to examine various aspects of mitochondrial function, each requiring an understanding of their principles, advantages, and limitations. This review provides investigators with a critical overview of the strengths, limitations and critical experimental parameters to consider when selecting and conducting studies on mitochondrial function. *In vitro* (isolated mitochondria) and *in situ* (permeabilized cells/tissue) approaches provide direct access to the mitochondria, allowing for study of mitochondrial bioenergetics and redox function under defined substrate conditions. Several experimental parameters must be tightly controlled, including assay media, temperature, oxygen concentration, and in the case of permeabilized skeletal muscle, the contractile state of the fibers. Recently developed technology now offers the opportunity to measure oxygen consumption in intact cultured cells. Magnetic resonance spectroscopy provides the most direct way of assessing mitochondrial function *in vivo* with interpretations based on specific modeling approaches. The continuing rapid evolution of these technologies offers new and exciting opportunities for deciphering the potential role of mitochondrial function in the etiology and treatment of diabetes. *Diabetes* 62:1041–1053, 2013

There has been a dramatic expansion in research investigating the role of mitochondria in the etiology of type 2 diabetes. This trend has been fueled, in part, by recent technological advances for assessing mitochondrial function. The advent of new tools and methodologies requires an understanding of the potential advantages and limitations of each. Unfortunately, relatively few consolidated resources are available to introduce methodologies for assessing mitochondrial bioenergetics—a field that focuses on direct and indirect measures of ATP synthesis as well as redox and ion homeostasis. While fundamentals of bioenergetics are covered elsewhere (1–3), the purpose of this review is to provide an introduction to the principles, advantages, and limitations to key methodologies used to study mitochondrial function *in vitro*, *in situ*, and *in vivo*. Our intent is

to assist in the selection of appropriate methodologies by highlighting relevant characteristics of major approaches, and to discuss recent issues in optimizing established methodologies.

DEFINING AND MEASURING “MITOCHONDRIAL FUNCTION”

The synthesis of ATP via oxidative phosphorylation is the most common function ascribed to mitochondria. This process is typically determined indirectly through measurement of mitochondrial oxygen (O₂) consumption, or respiration. Hence, altered respiratory kinetics in response to specific substrates are often interpreted as reflecting changes in oxidative phosphorylation and the regulation of cellular energy homeostasis *in vivo*. However, in addition to ATP synthesis, mitochondria play a central role in establishing and regulating cellular redox homeostasis. The present review provides an overview of the leading methods used to examine both of these functions.

Respirometry. In 1956, Chance and Williams (4) published the definitions of different respiratory steady-states based on the typical order of substrate additions made during an experiment on isolated mitochondria. These definitions have since been modified by Nicholls and Ferguson (2), reflecting an alternative sequence of substrate additions used during many mitochondrial experiments (Table 1). Under this system of nomenclature, state 1 refers to the O₂ consumption rate of mitochondria alone prior to addition of exogenous substrates or ADP. State 1 O₂ consumption rate is minimal. Addition of substrate(s) provides the fuel necessary to support the mitochondria, generating an increase in O₂ consumption (state 2) that largely reflects the basal rate of proton conductance (i.e., leak) back into the mitochondrial matrix. State 3 refers to the rate of respiration generated upon addition of ADP, and therefore exceeds state 2. State 4 refers to the rate of respiration remaining after all ADP has been converted to ATP. Although state 2 and state 4 respiration are empirically equivalent, state 4 is conventionally used when referring to respiration under basal, nonphosphorylating conditions. The respiratory control ratio, defined as the quotient of maximal state 3 to state 4 respiration, is often used as an index of mitochondrial coupling of oxidation (O₂ consumption) to phosphorylation of ADP to ATP, and thus the functional integrity of mitochondrial preparations. Familiarization with the development of these and other conventions are strongly recommended by referring to several key resources (1,2,4–9).

Measuring mitochondrial respiration. Measuring the rate of mitochondrial respirometric O₂ flux is accomplished by a number of methods, including O₂-dependent quenching of porphyrin-based phosphors (10) and amperometric O₂ sensors (7). Although phosphorescent probes are growing in use for *in vitro/in situ* respiratory measurements (e.g., the Seahorse Bioscience XF Extracellular

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See accompanying articles, pp. 1032 and 1036.

TABLE 1
A comparison of the respiratory steady-state conventions

Author(s)	Respiration terminology	Relative O ₂ flux	Characteristics
Chance and Williams (4)	State 1	Negligible	Mitochondria alone
	State 2	Negligible or low	ADP added, low/no substrate*
	State 3	High	ADP present, substrate added
	State 4	Low	Low ADP (converted to ATP), substrate present
	State 5	Negligible	Oxygen depleted from experimental chamber
Gnaiger (130)	LEAK	Low	Maximal substrate, no ADP
	OXPPOS	High	Maximal ADP and substrate
	ETS	Very high	Maximal substrate, uncoupler present

*State 2 defined as “substrate present, ADP low” by Nicholls and Ferguson (2). ETS, electron transport system; LEAK, respiration due to basal proton conductance across the inner mitochondrial membrane; OXPPOS, oxidative phosphorylation.

Flux Analyzer or Luxcel MitoXpress) and are particularly useful for in vivo estimates of O₂ consumption in cultured cells (11), the amperometric approach has historically been the most common for in vitro investigations of mitochondrial respiration. The Clark electrode contains a gold or platinum cathode and an Ag/AgCl anode separated by a KCl solution. A voltage is applied to the two half-cells, which are separated from the experimental assay media by a membrane of O₂-permeant material (e.g., polyvinylidene difluoride). O₂ diffuses from the assay media through the membrane and is reduced by electrons at the cathode, yielding hydrogen peroxide (H₂O₂). The H₂O₂ then oxidizes the Ag of the Ag/AgCl anode, which generates an electrical current proportional to the partial pressure, and in turn concentration, of O₂ in the experimental solution (7). Changes in [O₂] in the assay media (typically 1–2 mL) therefore correspond to the inverse of the respiratory rate of a biological sample (e.g., mitochondria) and allow for quantification of O₂ consumption (7).

The introduction of the XF Extracellular Flux Analyzer (Seahorse Bioscience) in 2006 represented a technical breakthrough for the field of bioenergetics by providing the first instrument with the capacity to measure in vivo O₂ consumption in intact adherent cells in culture. The system uses a 24- or 96-well cell culture microplate format that is mated from above with a sensor cartridge containing an equivalent number of individual probes, each containing fluorophores sensitive to O₂ or H⁺ embedded within the polymer that comprises the probe. This piston-like sensor cartridge is introduced periodically into the wells of the microplate, forming transient microchambers just above (~200 microns) the cell monolayer. Fiber optic bundles inserted simultaneously by the machine into the probes of the sensor cartridge provide the excitation and collect the emission light for each fluorophore. The change in O₂ and H⁺ concentration in the media (7–10 μL) is measured over several minutes, reflecting the rate of cellular O₂ consumption and H⁺ production. Raising the sensor cartridge then allows the media above the cells to mix/reequilibrate. The XF Extracellular Flux Analyzer can also measure extracellular acidification rates and carbon dioxide evolution rates as indices of glycolysis and tricarboxylic acid cycle (TCA) kinetics.

Technical considerations and limitations. Accurate amperometric measurement of O₂ consumption is best achieved through high-resolution respirometric systems incorporating materials and structural designs that provide minimal O₂ leak and maximal sensitivity to O₂. To this end, manufacturers of respirometric systems (e.g., OROBOROS

Instruments, Strathkelvin Instruments, Hansatech Instruments, etc.) incorporate 1) closed, air-tight reaction chambers; 2) glass and/or low O₂-permeable polymers comprising all components (chamber seals, stir bars, etc), which minimizes O₂ back-diffusion and over-estimations of respiration; and 3) sensors capable of detecting changes in [O₂] with minimal noise. High resolution designs (i.e., O2k, OROBOROS Instruments) maximize respirometric sensitivity and precision (minimal O₂ leak and highly sensitive electrodes), reducing the biological sample size required. Software advances in flux derivations of changes in chamber PO₂ also permit real-time reporting of respiratory kinetics (Datlab, OROBOROS Instruments), which improves data analyses over other systems requiring visual assessments of steady-state kinetics. Moreover, the unavoidable background O₂ consumption by the electrode itself, combined with varying degrees of O₂ back-diffusion into the oxygenic chamber, constitute potential sources of systematic error. In high-resolution systems, global instrumental background O₂ consumption (i.e., nonbiological O₂ flux), which increases positively as a function of PO₂, must be corrected for accurate determinations of absolute O₂ consumption.

All amperometric-based approaches are suitable for studying isolated mitochondria, permeabilized cells/tissue and/or intact cells in suspension. Most provide injection ports and/or infusion systems for introducing substrates, reagents, and/or inhibitors without exposing the chamber solution to ambient O₂. Units with peltier control of temperature permit comparisons of functional kinetics at precise temperatures, a critical parameter (see “Controlling critical experimental parameters: temperature”), but this is not available in all systems. In addition, the capability of the O2k system (OROBOROS Instruments) has recently expanded beyond respiration to permit simultaneous fluorescence-based measurements, potentiometric measurements of H⁺ (i.e., pH), triphenylphosphonium (membrane potential probe), and Ca²⁺, as well as amperometric measurement of nitric oxide. High stirring capacities (i.e., several hundred rpm) in all closed systems prevent gradients from forming between the biological source in solution (i.e., isolated mitochondria, permeabilized tissue/cells, etc.) and the detector(s). Positioning the O₂ electrode at a 45° angle relative to the bottom of the chamber (e.g., O2k) also insures stirring about the electrode is optimized (7). Sample stirring also ensures maximal exposure of tissue surface area to the surrounding assay media, which is critical for optimizing diffusion of substrates and O₂ to sample cores. However,

as most cell culture-based studies are conducted on adherent cells, the utility of solution-based systems is obviously limited when the experimental objective is to assess cellular bioenergetics in intact cultured cells. The effect(s), per se, of rapid stirring on the function of either isolated mitochondria or permeabilized fibers and cells has not, to our knowledge, been systematically tested. However, it has been noted that with the gentle stirring involved in the preparation of saponin-permeabilized human skeletal muscle fibers, less than 4% of total citrate synthase is liberated in both the permeabilization and wash steps (12).

As described above, the XF Extracellular Flux Analyzer (Seahorse Bioscience) minimizes the potential limitation for adherent cells by transiently limiting the volume of solution in which O_2 and H^+ are measured to just above the monolayer of cultured cells. However, this temporary microenvironment is not completely isolated from atmospheric O_2 because of the potential for exchange with the surrounding media (i.e., the piston-like sensor probes do not form a seal when lowered into the well) and the plastic comprising the microplate wells (i.e., polystyrene is permeable to O_2 [13,14]). Consequently, as $[O_2]$ declines in the chamber volume above the cells during longer measurement periods, a gradient is created favoring diffusion of O_2 from the media surrounding the sensor probe and the walls of the well into the measurement volume. This counteracts declines in assay $[O_2]$ due to respiration leading to an underestimation of cellular respiration rate. Likewise, in protocols in which anoxia is reached and the media is then reoxygenated by mixing, diffusion of O_2 back into the depleted chamber from the chamber walls can lead to overestimation of low rates during the subsequent measurement period. This limitation has been addressed through the development of a compartment-model-based algorithm to account for both sources of O_2 diffusion (15).

Aside from the obvious biological relevance of assessing bioenergetic function in intact cells, the XF Extracellular Flux Analyzer offers a much higher throughput platform than the traditional Clark electrode-based systems. This is advantageous for screening-based applications; e.g., screening drug candidates for potential mitochondrial toxicity. Since its introduction, the XF Extracellular Flux Analyzer has been validated for a variety of different cell types (16–20), including single muscle fibers (21). However, many substrates and compounds used to target specific mitochondrial pathways do not cross the cell membrane. Therefore, targeted probing of potential changes in mitochondrial function often requires gaining direct access to mitochondria, either via permeabilization or by isolating mitochondria. Because of their versatility and expanding capabilities, amperometric-based closed systems have historically been the method of choice for isolated mitochondria or permeabilized cells/tissue. However, if high throughput is needed, the XF Extracellular Flux Analyzer may be used by adhering mitochondria to the bottom of the microplate with a brief centrifugation step (19). No specific coating of the microplate is required, and the mitochondria remain attached through repeated mixing/measuring cycles. Care must be taken not to overload the wells, which can cause O_2 to quickly become depleted under state 3 conditions. Substrates (e.g., ADP, etc.) may also become depleted from the small microchamber volume (7 μ L) and thus be rate limiting. Similar to the situation with O_2 , although the chamber is semiopen, rates of substrate utilization by the plated mitochondria may exceed the diffusion capacity from the media surrounding the microchamber, necessitating the testing and use of higher

initial substrate concentrations than typically required in closed systems that are not limited by volume or diffusion (19). Use of permeabilized muscle fiber bundles has not been validated in the XF Extracellular Flux Analyzer.

Oxidant emission. Another key function of mitochondria is the regulation of cellular redox homeostasis through the establishment of the redox circuitry (e.g., via the NADPH/NADP couple) and emission of reactive oxidants. The redox circuitry extends throughout the proteome and represents a major mechanism of controlling multiple cell functions (22–24). This has particular relevance to the study of metabolic health given that oxidant emission (oxidant production minus oxidant scavenging) and redox signaling have been implicated in the etiology of insulin resistance in multiple tissues, including skeletal muscle (reviewed in 3,25–27).

Reactive O_2 species (ROS) is an umbrella term used to describe molecules that are chemically reactive due to the incomplete reduction of O_2 within the molecule. These include free radicals possessing an unpaired electron such as the superoxide anion ($O_2^{\bullet -}$), hydroxyl radical (HO^{\bullet}), peroxy and nitroxyl radicals, as well as nonradical oxidants such as H_2O_2 (22). However, the term oxidants generally encompasses ROS, reactive nitrogen species, and reactive lipid species (26,28,29). Because of their relative instability, oxidants are generally quite difficult to study, a fact highlighted by the historical lack of widely available tools to measure oxidants (30). Electron spin resonance/electron paramagnetic resonance represents the most direct way to detect free radicals, an approach that can be used in vivo under physiological conditions (31,32). A variation on the technique, known as spin trapping, is also frequently used; it incorporates a detecting molecule that reacts with, but is considerably more stable than, the initial free radical. The resulting free radical product often has spectral properties that allow the original radical to be identified indirectly. Although clinical applications continue to develop (33), in general electron spin resonance/electron paramagnetic resonance spectroscopy is limited in both its specificity and sensitivity to quantitatively detect free radicals typically associated with biological systems (31,32). Furthermore, this method does not detect nonradical oxidants.

Several fluorescent, chemiluminescent, and, most recently, electrochemical/nanoparticle-based (34) approaches are available to detect oxidants. Fluorescent probes are arguably the most common method used to detect a variety of oxidants (35). For example, oxidation of the widely used 2',7'-dichlorodihydrofluorescein can give a sense of ROS burst. However, dichlorodihydrofluorescein is notorious for its relative lack of specificity and is subject to auto-oxidation and photo-oxidation (36). Mitochondrial $O_2^{\bullet -}$, the parent molecule of all ROS, can be detected fluorescently by the mitochondrial-targeted triphenylphosphonium hydroethidine-based probe MitoSOX Red (Invitrogen) (37). However, MitoSOX Red also reacts with other oxidant species to generate products with overlapping emission spectra, although this limitation can be overcome through use of an alternative detection system based on capillary electrophoresis (38,39). In biological systems however, mitochondrial $O_2^{\bullet -}$ is rapidly and efficiently converted by manganese superoxide dismutase to H_2O_2 , a membrane-permeable nonradical oxidant recognized for its role as a biological second messenger (22,30,40–42).

First described by Mohanty et al. (43), the redox-sensitive fluorogenic AmplexRed (*N*-acetyl-3,7-dihydroxyphenoxazine)

has become the most commonly used reagent for detecting H_2O_2 in the past ten years. Unlike its predecessor scopoletin, AmplexRed (and the latest AmplexUltraRed) is a colorless and nonfluorescent derivative of dihydroresorufin, which is oxidized by H_2O_2 via horseradish peroxidase (1:1 reaction stoichiometry) to the highly fluorescent resorufin (44). A number of ratiometric- as well as genetic-based fluorescent probes with high specificity for H_2O_2 have recently been developed for use in living cells, including sensors that are targeted to various cellular locations (45–48). These tools provide unprecedented ability to visualize by confocal microscopy in real time the spatiotemporal nature of cellular H_2O_2 production and its impact on local redox environments.

Measuring mitochondrial oxidant emission potential.

Measuring mitochondrial oxidant production has been accomplished using isolated mitochondria or permeabilized cells/tissue. The method is based on the general principle that the mitochondrial respiratory chain is a redox system reflecting the balance between the reducing potential of redox couples feeding into and through the system and the rate of proton conductance across the mitochondrial inner membrane into the matrix (2,3). Reducing potential within the electron transport system can be raised by increasing the supply and/or blocking the flow of electrons (e.g., with specific inhibitors), creating conditions that lead to electron leak and $O_2 \cdot^-$ generation, and in turn H_2O_2 . H_2O_2 is uncharged and therefore freely diffuses out of the mitochondrial matrix, possibly facilitated by aquaporin channels (49), where it can be measured by one of the detection systems mentioned above. Various substrates in combination with inhibitors to specific sites within the respiratory complexes have been used to identify the sites and topology of $O_2 \cdot^-$ production (50–52). It is important to recognize that both glutathione and thioredoxin provide significant H_2O_2 scavenging capacity in the mitochondrial matrix (21,27); thus, the emission rate of H_2O_2 from the mitochondria reflects the balance between H_2O_2 production and scavenging rates and therefore underestimates the rate of $O_2 \cdot^-$ production (44,45). Mitochondrial glutathione can be depleted by pretreatment with oxidizing agents such as 1-chloro-2,4-dinitrobenzene to obtain a more accurate measure of $O_2 \cdot^-$ production (approximately twofold higher rate of H_2O_2 emission by mitochondria) (53). It is important to note that measures of H_2O_2 emission in permeabilized cells or isolated preparations are performed under substrate and respiratory conditions that are defined experimentally but do not and cannot perfectly replicate conditions in vivo. Thus, these represent estimates of the mitochondrial oxidant emitting potential (54) of the preparation under the experimental conditions chosen.

Measurement of membrane potential. Membrane potential across the inner membrane is a critical parameter in the study of overall mitochondrial function because it represents the balance between reduction potential of the proton pumps in the electron transport system and the rate of ion conductance (e.g., H^+) back across the membrane into the matrix. In essence, it reflects the redox state of the system and therefore the tendency of electrons to leak to O_2 to form $O_2 \cdot^-$. A number of cationic fluorescent probes have been developed for assessing qualitative changes in mitochondrial membrane potential in cultured cells using both confocal microscopy and flow cytometry (55,56). Quantitative measures of membrane potential have typically been performed on isolated mitochondria using electrodes sensitive to potential-dependent probes such as

triphenylmethyl phosphonium cation (1,2). Calculation of membrane potential is based on the Nernst equation and requires an estimate of the matrix volume (per unit protein) over which the probe distributes.

ATP production via bioluminescence. ATP production rate has been used to provide an index of oxidative phosphorylation activity in isolated mitochondria. The ATP produced is detected through the reaction of luciferin with ATP to ultimately generate oxyluciferin, AMP, and light via firefly luciferase, which is detected in a luminometer (57). This method is typically performed on isolated mitochondria and provides a direct measure of ATP production rate rather than relying on the assumption that all ADP added is phosphorylated. This is particularly advantageous if a given experimental treatment may change coupling efficiencies (P/O ratios, reflecting the amount of ATP produced per O_2 reduced to water during oxidative phosphorylation). Hence, a given respiration rate would no longer represent a specific rate of ATP production rendering conclusions regarding ATP synthesis quite difficult based on respiratory kinetics alone. A more detailed overview of this methodology is reviewed elsewhere (57).

Calcium retention capacity. The sensitivity of the permeability transition pore to calcium loading has been used as an index of mitochondrial viability and can be evaluated by determining the calcium retention capacity of mitochondria to Calcium Green-5N (Molecular Probes), a relatively low-affinity impermeable calcium indicator that exhibits an increase in fluorescence emission intensity upon binding calcium. Pulses of calcium are added at defined intervals, each eliciting a spike in signal that quickly dissipates due to the uptake of calcium into the mitochondrial matrix. Calcium concentration is monitored continuously with each addition until opening of the Permeability Transition Pore collapses the membrane potential and releases the accumulated calcium from the matrix, causing a sudden and sustained increase in signal. The amount of calcium retained prior to opening of the Permeability Transition Pore is considered an index of mitochondrial viability, particularly in the context of ischemia-reperfusion injury in the heart (58–60).

SAMPLE PREPARATIONS: IN VITRO AND IN SITU APPROACHES

Isolated mitochondria. Isolation of mitochondria through tissue/cell culture homogenization and differential centrifugation is routinely used for assessments of mitochondrial O_2 consumption, oxidant emission, and membrane potential in a variety of tissues (1,61–65). This preparation is ideal for studying mitochondrial bioenergetics free from the influence of other cellular factors (e.g., cytoskeleton and endoplasmic reticulum). However, comparisons of isolated mitochondria to permeabilized muscle fiber bundles can be a powerful approach for isolating the effects of cytosolic structures in regulating mitochondrial function as highlighted previously (54,66–69). It also permits assessments of distinct subcellular pools of mitochondria, specifically subsarcolemmal and intermyofibrillar fractions of skeletal muscle, which appear to have unique functional properties (70). However, the disadvantages of the isolated mitochondria approach (described in ref. 62) include 1) disruption of mitochondrial structure, which may impact function, especially if fused into a continuous reticulum such as in mature skeletal muscle (62,69,71–75); 2) biased selection of certain mitochondrial populations in a given sample (e.g., less dense mitochondrial populations excluded from the

differential centrifugation step) (62,74); 3) requirement of relatively large sample sizes ($>200 \cdot 10^6$ cells, or ≥ 100 – 150 mg wet weight of tissue) to generate sufficient mitochondrial yields, which at best often amount to $<20\%$ of the total population (61,62); and 4) loss of mitochondrial interactions with other cellular components, thus altering the functional properties (i.e., microcompartmentalization, metabolic channeling, and intracellular energy transfer) known to be present in vivo (62,76–79).

Permeabilized tissue. The scope within which mitochondrial functions can be analyzed in vivo is limited due to the fact that many exogenously added treatments (e.g., effectors such as ADP) do not readily penetrate cell membranes (62). Efforts to both circumvent the limitations associated with isolated mitochondrial preparations, and at the same time maintain the experimental freedoms conferred by the methodology, have led to established protocols involving chemically permeabilized cells (80). This method has also been adapted to assess mitochondria in multiple tissue types including cardiac muscle fibers (62,81), hepatocytes (82), adipose tissue (83,84), and especially skeletal muscle (62), although the application of this approach in each tissue requires careful consideration of unique limitations such as the ability to remove endogenous substrates following permeabilization. The successful permeabilization of cell membranes is achieved by cholesterol-specific detergents such as digitonin, filipin, α -solanine, α -tomatine, and β -escin (62). One of the most widely used permeabilizing agents is quillaja saponin, a relatively mild detergent (e.g., compared with digitonin [85]) derived from the soap bark tree (*Quillaja saponaria*) and referred to in the literature as simply saponin.

Saponins owe their detergent properties to hydrophilic glycoside moieties attached to lipophilic triterpene derivatives (86), which gives saponin its high affinity for cholesterol (62). Because plasma membranes contain approximately seven times more cholesterol (~ 0.5 mol cholesterol per mol phospholipid) than mitochondria (0.07 and 0.01 mol cholesterol per mol phospholipid for the mitochondrial outer and inner membranes, respectively) (87,88), very small concentrations of saponin (e.g., up to $50 \mu\text{g} \cdot \text{mL}^{-1}$) are sufficient to selectively permeabilize the plasma membrane (62). Pores ~ 8 nm in diameter are formed, providing an effective “washout” of soluble cytosolic contents and allowing direct access of exogenously added substrates/reagents to the mitochondria (89,90). Additional intracellular structures containing little or no cholesterol, such as the sarcoplasmic reticulum (SR) and the contractile apparatus will also remain intact upon saponin treatment (78). Furthermore, saponin concentrations as low as $50 \mu\text{g} \cdot \text{mL}^{-1}$ have been shown to increase Ca^{+2} loss from the SR of mammalian cardiac and skeletal muscle (91,92) and to also reduce the ability of the SR to load Ca^{+2} (90). This is thought to result from the effect of saponin on the SR Ca^{+2} release channels, and not SR membrane permeabilization, per se (90). Because mitochondria are known to readily take up Ca^{+2} (93), the loss of Ca^{+2} from the SR during permeabilization is beneficial to studies involving mitochondrial function. Furthermore, normal mitochondrial morphology, including intact inner and outer mitochondrial membranes, has been verified by electron microscopy in saponin-permeabilized cardiac fibers due to a low abundance of cholesterol in these membranes (62,81). Mitochondrial integrity is often verified by adding exogenous cytochrome *c*, such that any additional increase in respiration is indicative of a disrupted

outer membrane (94). A common practice is to accept up to 10% increases in respiration upon addition of cytochrome *c* as evidence of preserved integrity, although this has yet to be validated. Indeed, it has been shown that isolated mitochondria can lose cytochrome *c* independent of outer membrane intactness (95). There is also evidence that a cytochrome *c* effect may depend on the substrates used to support respiration, although the reasons for this observation are not entirely clear (96,97). Moreover, it has been suggested that cytochrome *c* release into the cytosol in vivo is sufficient to maintain oxidative phosphorylation (96,98). In saponin-permeabilized cardiac fibers, the results of a cytochrome *c* test have even been noted to depend on the respiratory steady-state, with mitochondria in state 4 exhibiting greater cytochrome *c* responses than those in state 3 (97). There is an apparent paucity of studies in which the cytochrome *c* test is performed on isolated mitochondria, and those having reported the test results tend to indicate an increase in respiration after cytochrome *c* addition (99,100). Our own experience suggests that at 37°C , a cytochrome *c* response is unavoidable in mitochondria isolated from rat skeletal muscle using standard procedures (unpublished observations by D.A.K.). Thus, the cytochrome *c* test represents a convenient, but clearly imperfect means of testing for outer mitochondrial membrane intactness. When the objective is to establish the intactness of the mitochondria independent of the research hypothesis, the cytochrome *c* test should be performed during state 3 respiration.

When compared with isolated mitochondria, the advantages of the permeabilized fiber approach to study muscle mitochondria (described in 62) include 1) retention of the mitochondrial reticular morphology (66), 2) retention of potential interactions between mitochondria and other subcellular structures/organelles (cytoskeleton, contractile proteins, peroxisomes, endoplasmic reticulum, etc.) (67), and 3) small sample size (1–2 mg wet weight). Limitations include 1) the requirement for fresh tissue, although cryogenic freezing with glycerol-based media to prevent membrane rupturing has been reported (101)—but with limited effectiveness following long-term preservation (102); 2) the potential for diffusion limitations of O_2 and substrates to the mitochondria; 3) the potential for nonmitochondrial-specific cationic probe distribution used in membrane potential measurements; 4) the propensity for permeabilized muscle fibers to contract in response to ADP (54,103); and 5) challenges in normalizing data to mitochondrial content. Regarding the latter, recent evidence suggests it may be possible to quantify mitochondria using protein densitometry in lyophilized samples postexperiment (104) or perhaps mitochondrial DNA or enzyme activities. However, the marker for mitochondrial content must be carefully considered, as not all markers have been shown to strongly correlate with total mitochondrial content (105). Furthermore, standard convention is to express functional data to wet weight, which is often recorded prior to an experiment, or dry weight, which requires retrieval of tissue sample from the reaction vessel. Dry weights avoid the inconsistencies in water contribution to total weight. However, blotting and weighing wet permeabilized muscle fibers prior to experimentation may affect certain respiratory metrics, particularly the apparent K_m to ADP (K_{mapp}), which may be due to contraction in the absence of relaxing solution during weighing (unpublished observations by C.G.R.P. in rodent and human skeletal muscle). Certain tissues are prone to

disintegration during functional assessments in systems with stirring, although this is prevented by myosin ATPase inhibition in the case of skeletal muscle (54).

Cultured cells. Cultured cells provide several advantages, including the obvious relevance to *in vivo* biology, the ability to manipulate the expression of proteins of interest, and the ability to apply optical imaging of subcellular compartment-specific fluorophores targeted to specific proteins or biochemical signal generation. However, most immortalized cell lines are metabolically adapted for rapid growth, often require culture conditions that are nonphysiological (i.e., up to 25 mmol/L glucose), and derive most of their energy from glycolysis rather than mitochondrial oxidative phosphorylation (106,107). Primary cells in culture possess more physiologically relevant metabolic poise but are short lived and more technically challenging. Furthermore, care must be taken when interpreting O₂ consumption data, given that mitochondrial bioenergetics, regardless of the cell type used, is governed primarily by cellular energy demand.

CONTROLLING CRITICAL EXPERIMENTAL PARAMETERS

A number of experimental parameters can affect mitochondrial function and should be considered when designing experiments or clinical diagnostic tests. These include the composition of the respiration buffer, reaction temperature, O₂ concentration, and factors specific to working with permeabilized cells/tissues.

Respiration buffer. The goal of most *in vitro* or *in situ* studies is to replicate, as much as possible, the environment of mitochondria *in vivo*. To this end, assay media have been designed to control the concentration of specific ions and total osmolarity reflective of extracellular and intracellular conditions (Table 2). Isolated mitochondria are generally prepared in media rich in sucrose, a mitochondrial inner membrane-impermeant osmolyte, which is intended to promote stabilization of mitochondrial integrity (108,109). With permeabilized muscle fiber bundles, separation and permeabilization is achieved in the K⁺-based buffer (BIOPS [81]) or similar buffers with slight modifications (62,110). Respiration buffers are generally high K⁺-based media with physiological osmolarities

TABLE 2

Composition of preparation and respiration buffers for assessing mitochondrial respiratory kinetics in permeabilized muscle fiber bundles (all buffers) and isolated mitochondria (MiRO5)

	Buffer Z (ref. 54)	Mitomed (ref. 129)	MiRO5 (ref. 170)
EGTA	1 mmol/L	0.5 mmol/L	0.5 mmol/L
Taurine	0	20 mmol/L	20 mmol/L
Dithiothreitol	0	0.5 mmol/L	0
MgCl ₂	5 mmol/L	3 mmol/L	3 mmol/L
Sucrose	0	110 mmol/L	110 mmol/L
K-MES	105 mmol/L	0	0
KCl	30 mmol/L	0	0
K-lactobionate	0	60 mmol/L	60 mmol/L
KH ₂ PO ₄	10 mmol/L	3 mmol/L	10 mmol/L
K-HEPES	0	20 mmol/L	20 mmol/L
Bovine serum albumin	5.0 mg/mL	2.0 mg/mL	1 mg/mL
Leupeptin	0	1 μmol/L	0
Soybean trypsin inhibitor	0	1 μmol/L	0
pH	7.1	7.1	7.1

(~295 mOsm) that differ largely with respect to sucrose and other stabilizing agents (54,62,110–112).

A special consideration of creatine and phosphocreatine (PCr) concentrations is required for the assessment of ADP-stimulated respiratory kinetics. Energy exchange (ADP/ATP) between matrix and cytosolic compartments can occur through creatine-dependent and -independent mechanisms. Optimal exchange is achieved by phosphate shuttling from matrix-derived ATP to creatine via mitochondrial creatine kinase in the intermembrane space with the PCr product then exported to the cytosol (113). This mechanism is depressed by PCr concentrations typical of relaxed skeletal muscle *in vivo* (54,114). In the absence of creatine, energy exchange is believed to occur less efficiently through direct ADP/ATP transport/diffusion across both mitochondrial membranes (66,113–117). The importance of supplementing assay media with creatine and PCr to control phosphate shuttling must therefore be considered when designing respirometric protocols.

Temperature. Historically, *in vitro* and *in situ* studies of mitochondrial function have typically been performed at 22–30°C for convenience and to maximize the duration of the experiment before O₂ becomes rate limiting. One might assume that respiratory fluxes measured at lower temperatures may be corrected to 37°C based on the Q₁₀ effect (respiration doubles for every 10°C increase). However, the respiratory system represents a balance between opposing forces (i.e., reducing potential of the electron transport system driving proton pumping versus the membrane potential across the inner membrane), and therefore the influence of temperature may not be as straightforward. Indeed, this problem has been addressed for biological systems in general (118). Data using permeabilized skeletal muscle fibers indicate that the actual temperature coefficients between respiration data obtained at 25°C or 30°C versus 37°C are much lower than 2, meaning decreasing temperature does tend to reduce respiration but the effect is much less than predicted (54). A Q₁₀ of less than 10 has also been reported in isolated mitochondria (119,120), which may suggest the diminished Q₁₀ effect relative to *in vitro* extraction-based determinations is due to properties within mitochondria rather than to mitochondrial morphology or the extramitochondrial environment that is largely retained in permeabilized muscle. In fact, increased stability of membranes and/or decreased ATPase activity have been suggested as potential factors to account for the lower rates of respiration at lower temperatures (121,122). Interestingly, isolated mitochondrial H₂O₂ emission has been demonstrated to increase or decrease with temperature, depending on the species and tissue source (121,123). Thus, reaction temperature must be considered when designing experiments as the practical advantages of conducting experiments at lower temperature may compromise extrapolation of findings to physiological temperature.

Permeabilized fiber bundles: contractile state. Although not widely reported, permeabilized muscle fiber bundles tend to spontaneously contract, particularly in response to exogenous additions of ADP in a temperature-sensitive manner (54). This is potentially problematic as contraction may augment diffusion limitations for substrates and O₂. Fortunately, contraction may be prevented through the use of nonspecific (e.g., pyrophosphate) (124,125) or specific (e.g., blebbistatin, *N*-benzyltoluene sulphonamide) (126,127) myosin ATPase inhibitors. In fact, direct comparison of respiratory kinetics in relaxed versus contracted fibers has revealed that contraction can

dramatically increase both the sensitivity (lower K_{mapp}) and maximal responsiveness (V_{max}) to ADP, depending on the muscle type and species (54,128). Disruptions in the interaction between the voltage-dependent anion channel, which participates in regulating ADP/ATP flux in and out of the mitochondria, with the cytoskeletal protein tubulin has recently been suggested as a mechanism by which contraction may alter mitochondrial ADP permeability (67). This is particularly intriguing, as it would represent a physical mechanism for increasing the sensitivity of the mitochondria to ADP at a time when it is most needed.

In the context of an experimental system however, exactly what causes permeabilized fibers to contract in the absence of added ADP/ATP is unresolved. Neither inhibition of potential cycling of residual endogenous ADP/ATP between creatine kinase and myosin-ATPase, nor limiting exposure to calcium (i.e., inclusion of high concentrations of the Ca^{2+} -chelator EGTA up to 5 mmol/L) prevents the spontaneous contraction (54), suggesting that the effect is independent of endogenous adenine nucleotide cycling or calcium. Treatment with trypsin induces a similar rapid shortening of permeabilized cardiomyocytes due to degradation of the cytoskeleton (68), leading Kuznetsov et al. (129) to raise the intriguing possibility that the presence of endogenous proteases may account for the apparent calcium-independent contraction in permeabilized muscle fibers. We recently tested this possibility in both rodent (Fig. 1) and human (115) permeabilized skeletal

muscle fiber bundles treated with protease inhibitors in three different respiration buffers, including Mitomed as recommended by Kuznetsov et al. (129). Inclusion of leupeptin, soybean trypsin inhibitor, and/or a protease inhibitor cocktail did not prevent contraction (see supplementary videos in ref. 115) nor the contraction-induced increase in respiratory sensitivity to ADP (i.e., lower K_{mapp} , Fig. 1). Thus, proteolytic degradation of the cytoskeleton does not appear to account for the spontaneous contraction. This leaves residual myosin-ATPase as the most likely explanation at this time (54), possibly related to rigor resulting from washout of ATP (103), although clearly further work is needed.

Oxygen concentration. It has been reported that O_2 at air saturation ($\sim 220 \mu\text{mol/L}$) rapidly becomes limiting to respiration in permeabilized muscle fibers due to diffusion limitations that are not present in cultured cells and isolated mitochondria in solution (130–132). This has prompted the practice of raising the initial O_2 concentration in the respiration chamber to above air saturation (300–500 $\mu\text{mol/L}$) (133,134), which provides an additional benefit of avoiding frequent resupplementation of oxygen into the chamber during an experiment. A more recent study directly comparing air saturation (~ 150 – $220 \mu\text{mol/L}$) with hyperoxia (275–450 $\mu\text{mol/L}$) failed to find any difference in maximal ADP-stimulated respiration in rodent red or white permeabilized muscle fiber bundles at 30°C or 37°C in high K^+ respiration buffer (Buffer Z) (54). Surprisingly, the lack of

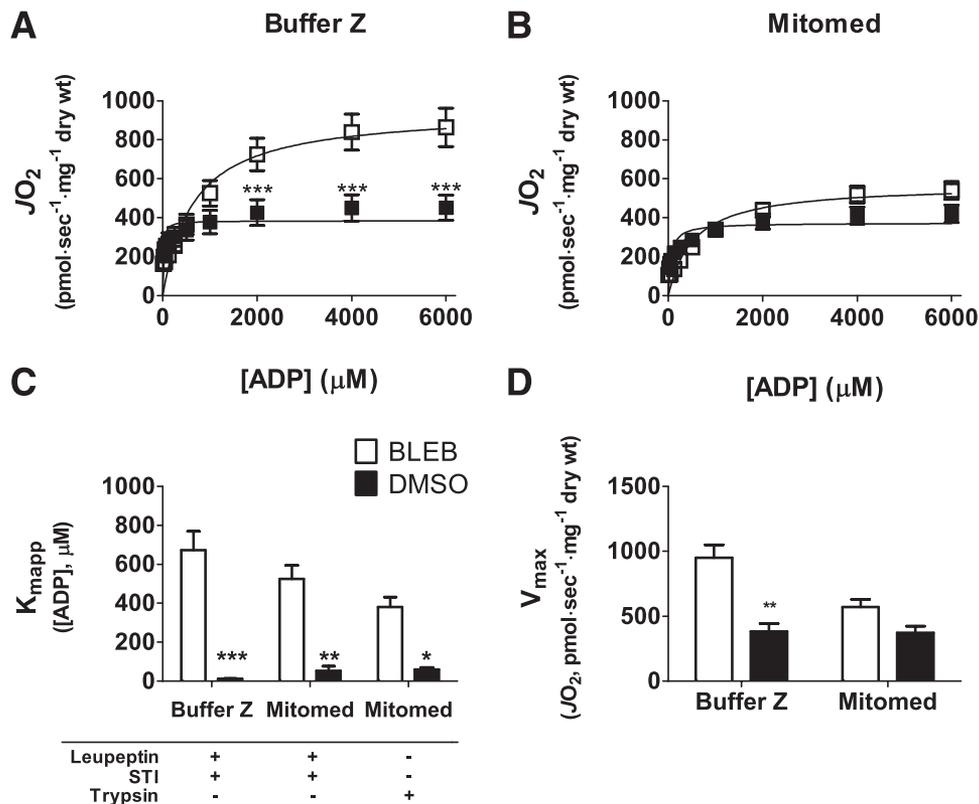


FIG. 1. The effect of protease inhibition or addition on contraction-mediated alterations in respiratory kinetics for ADP in Sprague-Dawley rat permeabilized red gastrocnemius fiber bundles. *A* and *B*: Michaelis-Menten-type line-fitting curves of respiratory O_2 flux (JO_2) in permeabilized muscle bundles, with 5 mmol/L pyruvate + 2 mmol/L malate, as a function of ADP concentration with the myosin II inhibitor blebbistatin (BLEB, 25 $\mu\text{mol/L}$), representing relaxed fibers, or vehicle (DMSO), representing contracted fibers, in two common respirometric assay media (Buffer Z [54,110,173] and Mitomed [129]). Protease inhibitors (1 $\mu\text{mol/L}$ leupeptin and soybean trypsin inhibitor [STI]) were included in wash and assay buffers. *C*: BLEB prevented the contraction-mediated (DMSO; spontaneous contraction) increase in respiratory sensitivity to ADP (i.e., lower K_{mapp}) whether treated with protease inhibitors or protease (5 $\mu\text{mol/L}$ trypsin, 30 min in permeabilization buffer). *D*: The respiratory V_{max} in permeabilized muscle bundles was significantly greater with BLEB in Buffer Z only. $n = 3$, triplicate trials averaged. *Significantly different than BLEB, $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

effect of hyperoxia was independent of whether a contraction inhibitor was present or not (54). Similar data have been obtained in human permeabilized muscle fiber bundles (unpublished observation by C.G.R.P., D.A.K. and P.D.N.), suggesting that O_2 concentrations above air saturation are not requisite for obtaining maximal O_2 flux in permeabilized muscle under these experimental conditions. However, a significant impairment in K_{mapp} to ADP was reported with hyperoxia at 30°C, so care must still be taken when considering the assay O_2 concentrations (54).

IN VIVO APPROACHES: NUCLEAR MAGNETIC RESONANCE

In most species, mitochondrial oxidative capacity is closely matched to maximal O_2 consumption ($V_{O_{2max}}$) measured in vivo. Humans, however, are an interesting exception because mitochondrial respiratory capacity exceeds leg V_{O_2} measured in vivo by arteriovenous O_2 difference (135). This apparent discrepancy between mitochondrial $V_{O_{2max}}$ and muscle $V_{O_{2max}}$ was explained by limitations to O_2 delivery to the contracting skeletal muscle (135). This unique aspect of human physiology has prompted careful scrutiny of methods to evaluate mitochondrial function in human skeletal muscle. As discussed previously, experiments in isolated mitochondria provide critical knowledge at the level of the intact organelle, whereas experiments in permeabilized muscle fibers are somewhat less reductionist by providing a window into the capacity and function of the entire population of mitochondria in the tissue. A well-recognized limitation of these in vitro methods is that measurements of O_2 consumption are made in the absence of the circulatory system, a key limiter of $V_{O_{2max}}$ in humans. For many years, the Fick principle has been applied to measure leg V_{O_2} from measurements of blood flow and arteriovenous O_2 difference, but a variety of noninvasive methods have emerged more recently to evaluate mitochondrial capacity and function in skeletal muscle in vivo. This section of the review will provide a broad-strokes overview of several magnetic resonance-based approaches that are quickly becoming widespread in use.

Oxidative capacity and basal ATP synthesis. A common premise to each method is that a target tissue is positioned within a static magnetic field such as a clinical magnetic resonance imaging scanner. Within this magnetic field, certain atoms (e.g., 1H , ^{13}C , ^{31}P) resonate at unique frequencies when exposed to a second oscillating magnetic field. Nuclear magnetic resonance (NMR) spectroscopy and magnetic resonance spectroscopy (MRS) are one and the same, but the word “nuclear” is commonly dropped to avoid the common misconception that the procedure involves exposure to ionizing radiation. Phosphorous MRS (^{31}P -MRS) is a noninvasive technique that can be used to monitor the levels of high-energy phosphates (e.g., ATP, PCr) as well as other phosphorous-containing metabolites (inorganic phosphate [Pi], phosphomonoesters, and phosphodiester) in human skeletal muscle. A unique aspect of ^{31}P -MRS in skeletal muscle is the high signal-to-noise, which permits rapid acquisition of spectra with temporal resolution of several seconds or less. Chance et al. (136) first applied ^{31}P -MRS to investigate human skeletal muscle bioenergetics in the 1980s. Thirty years earlier, Chance and Williams (137) had established the regulatory role of ADP in controlling mitochondrial respiration. Chance applied the hyperbolic relationship between ADP and mitochondrial respiration to predict skeletal muscle

oxidative capacity from ^{31}P -MRS measurements made during graded, progressive exercise (136). Owing to its micromolar concentration and bound state, it is not currently possible to directly measure free ADP using ^{31}P -MRS in human muscle, but it can be calculated from the concentrations of other measureable metabolites using formulas described elsewhere (138). Chance showed that mitochondrial oxidative capacity could be predicted in vivo from a “transfer function” demonstrating a Michaelis-Menten-type relationship between progressive work (or the rate of respiration) and inorganic phosphate/PCr (ADP) (139). A major challenge of this approach is accurate measurement of muscle work and the critical assumption that the workloads are truly steady state.

A simpler method involves predicting skeletal muscle oxidative capacity using the kinetics of PCr recovery following a brief bout of muscle activity. During intense muscle contractions, the creatine kinase reaction buffers intracellular ATP levels at the expense of PCr, which falls rapidly. During a recovery period after exercise, PCr is resynthesized, and this repletion of PCr is a function of mitochondrial ATP synthesis. Since the ATP generated during recovery is driven primarily by oxidative metabolism, the rate of PCr recovery is a commonly used indicator of oxidative capacity (140–144). The assumption that PCr resynthesis is a function of mitochondrial ATP synthesis is supported by observations that PCr resynthesis is absent when the muscle is kept ischemic following muscle contraction, and PCr recovery occurs only upon reperfusion of the limb (145). More recent studies have shown some transient PCr resynthesis during ischemic recovery following muscle activity (146–148), owing to the ever-improving signal-to-noise and temporal resolution as magnetic resonance technology progresses. Notwithstanding, the transient glycolytic contribution to PCr resynthesis exerts minimal influence on the rate constant that defines PCr resynthesis over a 10-min recovery period. It is important to note that during the recovery period after exercise, the mitochondria are not working at their oxidative capacity; rather, oxidative capacity can be predicted from the rate at which PCr is regenerated. This approach has been shown to be highly reproducible (149–152) and well correlated with in vitro methods for assessing mitochondrial capacity (149,153,154). An advantage of this approach compared with the transfer function is that accurate measurement of muscle work is not necessary, but major assumptions are that ATP concentrations are stable and glycolytic flux is negligible during recovery.

Various approaches have been used to deplete PCr in skeletal muscle, ranging from resting ischemia (155), supramaximal stimulation of motor nerves (144), dynamic voluntary contractions (151), submaximal isometric contractions (150,153), and maximal voluntary contractions (140,149). Transcutaneous nerve stimulation is an attractive approach because the muscle can be maximally activated in a way that overcomes potential limitations in voluntary drive from higher centers. However, supramaximal motor nerve stimulation may not be well tolerated by all patient populations and poses a new set of challenges related to noise injection from the stimulator/electromyography cables breaching the shielded magnet room. Alternatively, maximal voluntary contractions can be performed by most patient populations. With sufficient habituation and visual feedback provided by a computer monitor or series of light-emitting diodes, it is possible to maximally activate skeletal muscles with no limitations of central motor drive (assessed

by superimposing electrical stimulation) (141). Although submaximal contractions are sometimes used to deplete PCr, the calculated oxidative capacity appears to be consistently lower compared with maximal contractions (150,151). The lower values derived from submaximal efforts may result from incomplete motor unit recruitment if the contraction intensity is below the threshold for complete motor unit recruitment. It is also possible that motor unit cycling during submaximal efforts could result in recruitment of new motor units as the muscle begins to fatigue while some initially recruited motor units no longer contribute to force production. Either case is problematic from the standpoint of measuring ^{31}P metabolite levels by MRS. A phosphorous spectrum represents the average signal of all of the tissue within the sampling volume of the radiofrequency coil, regardless of whether the muscle is active or not. Therefore, the changes in ^{31}P metabolites in the active muscle will be essentially "diluted" by the inactive tissue. Although this issue can be avoided by using supra-maximal stimulation or maximal voluntary contractions, one drawback is that PCr recovery is influenced by intramuscular pH. Careful attention is needed to ensure that the muscle contraction is sufficient to deplete PCr without acidosis by optimizing the duration of the exercise. Alternatively, it is possible to model mitochondrial capacity from the recovery rate of the calculated free ADP, which is independent of pH (150,156). In sum, the kinetics of PCr (or ADP) recovery following exercise is a reproducible, valid method to non-invasively evaluate skeletal muscle oxidative capacity. This approach, however, is restricted to skeletal muscle and cannot be used to evaluate mitochondrial capacity in other tissues such as liver, adipose, or brain.

In addition to measuring oxidative capacity, ^{31}P -MRS can be used to measure basal ATP synthesis rates using magnetization-transfer techniques. The basic premise of this approach is that it is possible to calculate metabolic fluxes by perturbing the signal from a specific metabolite (e.g., ATP) and monitoring how this perturbation is transferred to another metabolite (e.g., Pi, PCr) that is linked by an exchange reaction (e.g., F_1F_0 ATPase, creatine kinase). An advantage over the PCr recovery approach is that magnetization transfer methods are not restricted to skeletal muscle tissues. There are several recent reviews that provide excellent detail on the principles and practice of this approach (157,158). An important point of emphasis is that metabolic fluxes measured by magnetization transfer are restricted to the steady-state conditions of resting muscle and cannot be used to evaluate mitochondrial oxidative capacity. Although the magnetization approach has been widely used to evaluate mitochondrial function (159–164), there continues to be lingering controversy over some aspects of the data and its interpretation (158). The main point of concern is that resting $\text{Pi}\rightarrow\text{ATP}$ flux measured by ^{31}P -MRS is ~ 10 -fold higher than all other noninvasive measures of resting oxidative ATP synthesis. As pointed out by Kemp and Brindle (158), the exchange between Pi and ATP at rest is dominated by glycolytic enzymes (glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase) and should not be interpreted as an index of mitochondrial ATP synthesis. Others contend that the glycolytic contribution is minimal at rest (165), and that the $\text{Pi}\rightarrow\text{ATP}$ exchange scales as expected in mouse models in which mitochondrial protein abundance is genetically altered (166,167). The interested reader is pointed to several recent articles in which these considerations are extensively reviewed (157,158,168,169).

Mitochondrial coupling. The functionality of mitochondria is often inferred from their capacity to consume O_2 or generate chemical energy. However, another important aspect of mitochondrial physiology is the efficiency of ATP synthesis by the organelles (i.e., coupling). Various approaches have been used to evaluate bioenergetic efficiency of isolated mitochondria (170) and in human skeletal muscle in vivo (171,172). This section of the review will touch briefly on two distinct in vivo approaches that have been applied to human skeletal muscle. The first approach combines optical spectroscopy and MRS to simultaneously measure O_2 consumption and ATP production, respectively (155,171). The basic premise of this approach is to determine the P/O by quantifying both processes in parallel. Mitochondrial ATP synthesis is determined from the decline in PCr concentrations in resting ischemic muscle by ^{31}P -MRS. During ischemia, mitochondrial ATP synthesis is suppressed and glycolytic ATP flux is minimal ($<8\%$) (155,171). In the absence of aerobic or glycolytic ATP synthesis, intracellular PCr stores are consumed at a rate that reflects the ATP demand of the muscle. Mitochondrial ATP flux is determined using an ATP balance approach that assumes equality between ATP demand and mitochondrial ATP supply in resting muscle. Other assumptions include that ischemia does not change ATP demand, that glycolytic flux is negligible in resting muscle, and that ischemia does not alter glycolytic flux (155,171). Simultaneously, O_2 consumption is measured in vivo by near infrared spectroscopy during the first minute of ischemia. A partial least squares analysis is used to differentiate between hemoglobin and myoglobin desaturation during ischemia. From these independent pieces of data acquired during ischemia, it is possible to determine the ratio of net ATP turnover to O_2 uptake as a measure of mitochondrial bioenergetic efficiency. Although the ^{31}P -MRS component is relatively straightforward, the measurement of O_2 uptake requires substantial technical expertise, in vitro calibration sets, wavelength shift analysis to determine the ratio of different chromophores, and ability to measure deoxyhemoglobin by proton MRS.

Another noninvasive NMR technique to evaluate bioenergetic efficiency of mitochondria involves measuring basal ATP flux by magnetization transfer paired with TCA flux measured by ^{13}C -MRS (157,168,172). This approach deviates somewhat from the conventional definition of mitochondrial coupling at the level of the cytochrome chain; rather, it assesses efficiency from the ratio of substrate oxidation in the TCA to ATP synthesis. Assessment of TCA flux is made by infusing a magnetic resonance-visible tracer, $[2\text{-}^{13}\text{C}]$ acetate, which enters the TCA after it is converted to acetyl CoA. Although the TCA intermediates are not present in sufficiently high concentrations to be directly measured by ^{13}C -MRS, it is possible to monitor the time course of ^{13}C enrichment of glutamate, which exchanges rapidly with α -ketoglutarate. At this point, sophisticated modeling is required to determine the metabolic fate of the labeled acetate molecule. The details of this modeling are beyond the scope of this review, but are nicely detailed elsewhere (157,168,172). By pairing TCA flux with basal ATP synthesis measured by ^{31}P -MRS magnetization transfer, it is possible to evaluate the efficiency of mitochondria, by comparing the level of substrate oxidation at a given level of basal ATP flux. Similar to the P/O measurements described above, this approach also requires substantial technical expertise, mathematical modeling, and careful attention to assumptions. It is important to note that

this estimate of mitochondrial efficiency differs from the more traditional direct measurements of phosphorylation efficiency measured *in vitro* by high-resolution respirometry or *in vivo* by the combination of optical spectroscopy and ^{31}P -MRS described above. Nevertheless, all of the approaches described here represent theoretically plausible methods to evaluate aspects of mitochondrial coupling or efficiency, but as with any method, careful attention must be given to underlying critical assumptions.

PERSPECTIVES AND CONCLUSIONS

To summarize, it is clear that a variety of experimental parameters and/or modeling approaches must be carefully considered in the context of the specific hypothesis being tested. Each method provides a unique set of advantages as well as limitations. The purest way to assess mitochondrial function free from other factors is to isolate mitochondria from the local environment, but this requires extensive disruption of mitochondrial structure and morphology with considerable loss of sample. Mitochondrial structure can be largely retained through simple chemical permeabilization of cell membranes providing access to mitochondrial compartments free from soluble cytoplasmic factors. However, numerous experimental parameters must be carefully controlled, and classic approaches for assessing certain bioenergetic parameters in isolated mitochondria (e.g., membrane potential) have not been fully developed in permeabilized tissue. Interestingly, combining multiple approaches may yield significant insight into the regulation of mitochondrial bioenergetics. For example, comparing the function of isolated mitochondria versus permeabilized samples is a powerful approach to determine the role of mitochondrial morphology or local structures in regulating mitochondrial function. Recent technological advances now permit assessment of respiratory control in intact adherent cultured cells, offering the opportunity for high through-put analyses in this specific model. Lastly, NMR is the most direct method for assessing mitochondrial function *in vivo*, especially in humans. Mathematical models have specifically targeted energetic components of mitochondrial function, with a focus on oxidative capacity, basal ATP synthesis, and coupling, which can be measured in real time. Ongoing developments in algorithms and conceptual assumptions have enhanced the power of this approach. The rapid progress of these technologies and approaches has expanded opportunities to investigate the potential contribution of mitochondrial bioenergetics and redox systems biology to the etiology of type 2 diabetes.

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