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Real-time multiparameter study of mitochondrial functions: Instrumental and analytical approaches

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ABSTRACT

In modern biomedical science, a descriptive study is no longer the major focus of many fields. More researchers are now seeking approaches that will help them obtain maximum information from a single sample or model, which will allow them to make more detailed conclusions than previously about mechanisms that underlie certain phenomena. Clearly, simultaneous measurement of multiple parameters will provide more useful information compared to that which can be assessed through parallel studies with multiple single-parameter measurements. Mitochondria are actively involved in the regulation of a number of biochemical processes that are vitally important for normal cell functioning. Dysregulation of cell metabolism occurs under multiple pathological conditions. While changes in mitochondrial and cellular functioning are related to each other, understanding of the details of most mechanisms underlying these relationships are still unknown. It would be appropriate to have an instrument that will help to uncover sequences of events and temporal links among the parameters that involve functional mitochondrial and cellular integration. The current review is focused on the analysis of these technological limitations, and, based on the combined approach, provides hypothetical suggestion on how possibly to create such an instrument.

Introduction

Mitochondria are of great importance for normal cellular functioning. A key mitochondrial role is energy production through oxidative phosphorylation and oxidation of key biomolecules. Depending on the nature of the tissue, a number of other metabolic functions are also carried out by mitochondria [reviewed in 1]. Impairment of mitochondrial functioning is a well-documented phenomenon that occurs under multiple pathophysiological conditions, including, for example, cancer. Inhibition of the mitochondrial respiratory complexes and ATP synthesis, alteration of the mitochondrial membrane potential ($\Delta\Psi$), production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), dysregulation of the transport of Ca^{2+} and other ions, mitochondrial pH imbalance, and induction of the mitochondrial permeability transition (MPT) will lead either to programmed cell death (apoptosis) or necrosis [1,2]. However, the detailed mechanisms of how the changes in mitochondrial functioning are related to each other are pressing unresolved issues. For many mitochondrial functions in normal cells there is only a partial understanding of the mechanisms involved and their regulation, and even less is known about these mechanisms, for example, in cancer cells.

Scientific knowledge gap

Interest in studying mitochondria was given a significant boost in 1996, after it was discovered that mitochondria play an important role in triggering apoptosis. The mitochondria-associated apoptosis is executed through the release of cytochrome *c* (cyt*C*), which is widely, although not universally, accepted to occur via induction of MPT [reviewed in 3]. Induction of the MPT results in an opening in the inner mitochondrial membrane of a non-specific pore, permeable to solutes and small peptides with molecular masses of up to 1500 Da, which causes numerous changes of mitochondrial parameters. The MPT has been extensively studied, but mostly with isolated mitochondria [1–3].

Because of the importance of mitochondria for cell survival, their physical status may be regarded as an indicator of the overall cellular

Abbreviations: $\Delta\Psi$, mitochondrial membrane potential; ROS, reactive oxygen species; RNS, reactive nitrogen species; MPT, mitochondrial permeability transition; cyt*C*, cytochrome *c*; TPP+, tetraphenylphosphonium; ADC, analog-digital converter; DAC, digital-analog converter; MFFR 680, MitoFluor Far Red 680; BCECF, 2′-7′-bis(carboxyethyl)-5(6)-carboxyfluorescein; SNARF-1, seminaphtharhodafluor; H_2DCFDA, dichlorodihydrofluorescein diacetate; DCF, 2′,7′-dichlorofluorescein; LED, light-emitting diode; PD, photodiode; FOP, fiber optical probe; H/R, hypoxia/reoxygenation

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condition. Taking into account the unique ability of mitochondria to almost immediately respond even to minute alterations in cell physiology, these organelles can serve as reporters for early-stage changes in cell metabolism [1–3]. Key mitochondria-related cellular parameter, such as levels of ATP and NADH, level of H_2O_2, together with key mitochondrial parameters, such as O_2 uptake, $\Delta\Psi$, mitochondrial matrix pH, Ca^{2+} flux, and swelling of mitochondria, should provide important information linking cell function to mitochondrial function and *vice versa* under normal and pathological conditions.

For example, almost a century ago Otto Warburg pointed out that cancer cells, in general, are profligate in their use of energy sources in that they use inefficient anaerobic glycolysis for energy needs, even in the presence of O_2 (The Warburg Effect) [4]. First, we now know that the mitochondria in cancer cells are much less efficient than normal mitochondria as energy producers [4]. Secondly, we also now know that mitochondria in cancer cells are much more resistant to Ca^{2+} and ROS challenges compared to normal cells [4]. Ca^{2+} and ROS (when elevated) have been shown to induce MPT in normal cells, which results in release of cyt*C* from mitochondria and propagation of apoptosis signals within the cell. The resistance of cancer mitochondria against apoptosis-associated permeabilization and the altered contribution of these organelles to metabolism are closely related [4]. It has been pointed out that inhibition of the processes and enzymes that participate in cancer cell metabolism (particularly glycolysis) may have a dramatic effect on tumors, not only by limiting cancer cell-specific bioenergetic flow and anabolic reactions, but also by reversing the neoplastic phenotype and hence stopping growth, inducing apoptosis, and/or blocking angiogenesis and invasion [4]. However, blocking of glycolysis may have an untoward effect on normal tissues, which for energy production rely not only on mitochondrial oxidative phosphorylation, but also on glycolysis (e.g. skeletal muscle). Thus, understanding why cancer mitochondria are so resistant to MPT is very important, because such an understanding may suggest new ways of possibly targeting cancer cells. As a prerequisite at least the following questions should be answered:

1) How are mitochondrial and cellular mitochondria-related physiological parameters linked to each other, and how are they different in cancer cells compared to those in normal cells?
2) Does the degree of mitochondrial functional change correlate with tumor aggressiveness?
3) What is the physiologically-based mechanism that allows mitochondria in cancer cells to become less sensitive to apoptosis-inducing conditions (Ca^{2+} and ROS) than are normal cells?
4) Which mitochondrial function(s) can we manipulate in such a way that the apoptosis cascade will be activated in cancer cells, but will not be activated in normal cells?

Technology gap

Commercially available instruments exist that are capable of simultaneous measurement of several parameters either in mitochondrial suspension or in cells, for example, the OXYG1 Plus or Seahorse XF Analyzer, produced by Hansatech Instruments Ltd, Norfolk, UK and Agilent Technologies, Santa Clara, CA, respectively. However, the number of simultaneously and conveniently measured parameters in a single experimental sample that are permitted by these instruments are usually limited to 2 or 3. The possibility of increasing the number of parameters is limited either by design of the instrument or by its technical capabilities.

We believe that for answering the questions highlighted above it would be beneficial to have an instrument that can help in obtaining data on multiple physiologically relevant mitochondrial and mitochondria-related parameters in isolated mitochondria and intact cells. Translation of the data obtained with such an instrument will help in understanding of how exactly physiological activities of mitochondria are linked to cellular metabolism. The best way to establish such a correlation will be to uncover a temporal link and to determine the sequence of events among relevant mitochondrial and mitochondria-related physiological parameters by simultaneous measurement of multiple parameters in the same sample and in a real-time scale. In our opinion, the reason why such an instrument has not previously been built is that measurement of different mitochondrial parameters involves many diverse technological approaches that are not easy to combine in a single device with minimal interference among them. Nevertheless, we believe that this technological gap can be at least in part narrowed by building a device that is the focus of this review. To briefly illustrate the situation in the field, we present below a short overview of some methods that are currently available to researchers for studying mitochondrial functioning in isolated mitochondria and in whole cell models.

Studies of isolated mitochondria

To evaluate mitochondrial function, classical bioenergetic experiments on isolated mitochondria (here referred to as the *in vitro* model) are usually employed. These experiments are based on the quantitative measurement of such important parameters as O_2 uptake, $\Delta\Psi$, redox state of respiratory substrates, and ATP levels. [Similar mitochondrial parameters can be assessed in intact cells (here referred to as the *in situ* model), but mostly through qualitative or semi-quantitative monitoring.]

Oxygen uptake is established as an integral energy indicator that reflects the energy status of the mitochondria and the whole cell. Respiration of isolated mitochondria is commonly monitored by using an O_2-sensitive Clark-type electrode [5]. Comparison of the rates of O_2 uptake and ADP/O ratio under defined experimental conditions serve as a “gold” standard for characterizing the bioenergetic properties of isolated mitochondria. Generation and maintenance of $\Delta\Psi$ are among the main physiological functions of mitochondria [1,2]. A convenient technique to measure $\Delta\Psi$ in isolated mitochondria makes use of a tetraphenylphosphonium (TPP^+)-selective electrode [6]. The method is based on a potential-dependent redistribution of the lipophilic membrane-permeable cation TPP^+ across the mitochondrial membrane. Mitochondria can modulate cellular [Ca^{2+}] through highly regulated channels: a $\Delta\Psi$–dependent Ca^{2+} uniporter, and pH–dependent Na^+/- and H^+/- Ca^{2+} exchangers [7]. Ca^{2+} ion-selective electrodes are probably the easiest tool that can be used to monitor the [Ca^{2+}] in experimental media as a reflection of Ca^{2+} fluxes in cells or organelles [8]. When isolated or present in the intact cell, the mitochondrion behaves as an osmometer, changing its ion content in parallel with entry or exit of water. Thus, $\Delta\Psi$ (a driving force for ion transport through the inner mitochondrial membrane) determines the distribution of osmotically active ions and water across the inner membrane and hence the mitochondrial volume. Changes in mitochondrial volume (swelling or shrinkage) can be monitored by light absorbance, light transmission, or light scattering [9]. Changes in pH in the mitochondrial incubation media can be screened with a standard pH electrode. Mitochondria are responsible for about 95% of the overall ROS produced in the cell under normal conditions [10]. A common method for detecting ROS production is by using fluorescent probes [11]. $\Delta\Psi$, [Ca^{2+}] fluctuations, as well as pH dynamics can also be assessed with fluorescent probes in *in vitro* models [11].

Studies of mitochondria-related parameters in whole cells

Mitochondrial functioning in intact cells can be assessed in part, for example, by fluorescent microscopy or by flow cytometry techniques. Basic mitochondrial and mitochondria-related cellular parameters such as $\Delta\Psi$, mitochondrial matrix [Ca^{2+}], ROS production and mitochondrial matrix pH, can be evaluated with fluorescent probes [11]. (The redox state of the NADH/NAD^+ pair is usually assessed by measuring