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Assessing bioenergetic function in response to oxidative stress by metabolic profiling

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ABSTRACT

It is now clear that mitochondria are an important target for oxidative stress in a broad range of pathologies, including cardiovascular disease, diabetes, neurodegeneration, and cancer. Methods for assessing the impact of reactive species on isolated mitochondria are well established but constrained by the need for large amounts of material to prepare intact mitochondria for polarographic measurements. With the availability of high-resolution polarography and fluorescence techniques for the measurement of oxygen concentration in solution, measurements of mitochondrial function in intact cells can be made. Recently, the development of extracellular flux methods to monitor changes in oxygen concentration and pH in cultures of adherent cells in multiple-sample wells simultaneously has greatly enhanced the ability to measure bioenergetic function in response to oxidative stress. Here we describe these methods in detail using representative cell types from renal, cardiovascular, nervous, and tumorigenic model systems while illustrating the application of three protocols to analyze the bioenergetic response of cells to oxidative stress.

Introduction

Assessment of mitochondrial function in cultured cells is commonly performed using either measurements of oxygen consumption of the intact isolated organelle or activity measurements of individual enzymes. Although both approaches yield important information regarding mitochondrial performance, measurements of oxygen consumption in intact cells allow a physiological perspective of mitochondrial function to be achieved [1,2]. To date, quantification of oxygen consumption in cultured cells has been largely performed using Clark electrodes with the cells suspended in stirred, buffered solution [3]. These electrodes are more commonly used for assessing mitochondrial function in isolated samples from animal studies or clinical samples. Measurement of oxygen consumption from cultured cells in a Clark-type electrode usually requires removing the cells from their growth substrate and adding them to a stirred solution. In many cells, this detached state may result in anoikis, which is associated with increased reactive oxygen species (ROS) and mitochondrial damage [4]. In addition, nonlaminar shear, which occurs as a result of stirring in the oxygen electrode, will also result in increased oxidative stress [5,6]. This can be partially overcome by seeding cells on microcarrier beads to prevent anoikis; however, these cells are still subject to nonlaminar shear [7], and thus the effects of ROS and reactive nitrogen species (RNS) on mitochondrial function in cells are difficult to dissect using this experimental approach. Notably, recent publications highlight the application of the Seahorse XF24 to the study of isolated mitochondria in a more high-throughput fashion [8,9]. These publications offer a direct comparison to Clark-type electrodes in models normally measured by this method. Importantly, data derived from the Seahorse XF24 instrument compare favorably to those acquired using the Clark-type electrode. Monitoring mitochondrial oxygen consumption while the cells remain adherent is

Abbreviations: 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; 2-DG, 2-deoxy-D-glucose; AUC, area under the curve; Deta NO, Deta NONOate, (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; DMNQ, 2,3-dimethoxy-1,4-napthoquinone; DPI, diphenyleneiodonium; ECAR, extracellular acidification rate; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HNE, 4-hydroxy-2-nonenal; HO-1, heme oxygenase-1; IAA, iodoacetic acid; KA, koningic acid; mito-15d-PGJ_2, mitochondrially targeted 15d-PGJ_2; mito-PGE_2, mitochondrially targeted prostaglandin E_2; NRVM, neonatal rat ventricular myocytes; OA, oxamate; OCR, oxygen consumption rate; PBS, phosphate-buffered saline; PPR, proton production rate; RASMC, rat aortic smooth muscle cells; RNS, reactive nitrogen species; ROS, reactive oxygen species; siRNA, short-interfering RNA; XF, extracellular flux.

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thus preferable in experiments to assess the impact of oxidative stress on cellular bioenergetics as these measurements can be related to other experimental endpoints after the intervention of interest.

This can now be achieved using recently developed fluorimetric systems to detect oxygen consumption in adherent cells in culture [10–12]. These systems allow for highly sensitive and specific measurements of mitochondrial function to be made with greater throughput than possible with electrode-based systems. The most important difference between the Clark-type electrode and these systems is in the maintenance of a cellular context within which mitochondrial dynamics are examined. In this article, several protocols describing the use of a Seahorse Bioscience XF24 extracellular flux analyzer to assess the impact of oxidative stress on bioenergetics are described.

Mitochondria and oxidative stress in health and disease

Beyond the role of mitochondria in ATP production, it is now evident that these organelles are implicated in many chronic pathologies. This is not surprising because the mitochondria are major producers of energy in the cell and deficits in energy production are readily translated to other cellular processes that require energy to function. These include ion pumping [13], which is essential for maintaining ionic homeostasis (especially critical in excitable cells such as neurons and myocytes); molecular motors, such as those that enable muscular function; and the synthesis of molecules for intracellular communication. Importantly, cellular repair pathways, which are activated in response to the accumulation of proteins damaged by ROS/RNS, are also energy consuming. Furthermore, many of the pathways that remove ROS/RNS use NADPH as a source of reducing equivalents, which in turn increases the metabolic demand on the cell.

At nonpathological levels mitochondria integrate signals from ROS/RNS and are, therefore, uniquely poised as both regulators and integrators of intra- and intercellular signaling. Seminal discoveries of the necessary and permissive role of the mitochondria in diverse cellular processes have highlighted a role for mitochondria in responding to viral infection [14], growth signaling [15,16], and death signals [17–21]. In fact, death signaling is a classic example of retrograde signaling to the nucleus [22]. This retrograde signaling is accomplished through the action of noneffector proteins. Control of the Bcl-2 family members Bax and Bak is a typical example of the mitochondrial role in apoptotic signaling [23–25], but it also may include regulation of cell–cell junctions [26–29], proteasomal activation [30], kinase activation [31,32], and oxygen sensing [33]. Thus, it is increasingly clear that mitochondrial function, not only important to the progression of a disease, is now recognized as a therapeutic target [34,35]. This emphasizes the importance of assessing mitochondrial function in response to normal physiological and pathological stressors in a cellular setting.

The impact of oxidative stress on mitochondrial function

As mitochondria are both sources and targets of oxidants, amplification of oxidative damage can occur as the increase in ROS/RNS damages mitochondria and further exacerbates ROS production. For example, damage to mitochondria during the progression of atherosclerosis is an ROS/RNS-dependent process that causes loss of bioenergetic control and eventual vascular dysfunction [36–38]. However, an understanding of mitochondrial responses to ROS and RNS in intact cells is incomplete. Recently, our studies on the effects of ROS/RNS generators on mitochondrial function have been greatly facilitated by the Seahorse Bioscience XF24 technology (Seahorse Bioscience, North Billerica, MA, USA), which enables us to accurately quantify mitochondrial function in intact cells [39–42]. The noninvasive nature of this technique allows for repeated measurements of oxygen consumption and extracellular acidification rates as well as post hoc measurements of cytosolic and mitochondrial proteins by Western blot, total cellular protein levels, and other endpoints. Several models of ROS/RNS generation have now been examined in multiple cell lines. Here, examples of ROS/RNS production are used to illustrate the utility of measuring extracellular flux to monitor mitochondrial and glycolytic function.

Principles and approaches to measuring bioenergetic function by extracellular flux

The cell lines used in these studies (Table 1) have been selected to be broadly representative of the cultured cells used by investigators in the cardiovascular, cancer, neurodegeneration, and renal fields of research. In each of these cell types, an XF24 analyzer from Seahorse Bioscience, which measures O_2 and protons (pH) in cell culture, was used to determine the effects of oxidative stress on cellular bioenergetics [11,43]; all experiments described in this paper use plates with a 7-μl volume, termed V7 plates. Because the assay does not involve direct manipulation of the cells apart from a transient change in oxygen tension, the cells can be harvested at the end of the experiment for the measurement of other endpoints. The small volume and 24-well format of the XF24 allows for high-throughput, real-time measurements of O_2 consumption and pH change. The rate of O_2 consumption (OCR) can be assigned to oxidative phosphorylation and the rate of extracellular acidification (ECAR) to glycolysis. These endpoints are discussed in further detail below. The system is capable of measuring 20 samples at a time and is equipped with four injection ports per well to allow for injection of a compound of interest or to add inhibitors that can aid in the elucidation of defects in individual cellular respiration pathways or enzymes [44]. A full description of the instrumentation and related methodology can be found in Refs. [11,12,43].

Fig. 1 shows three main approaches that can be used to determine the response of cells to the effects of ROS/RNS. Protocol 1 is designed to observe the effects of oxidative stress on bioenergetic function in "real time." It utilizes the injection ports of the XF24 to directly introduce the compound of interest into the experimental wells and follows the response in OCR and ECAR over time. Experiments of this type can be readily compared to other endpoints by harvesting the cells at the completion of the XF assay as demonstrated below. Protocol 2 extends Protocol 1 to include the measurement of a mitochondrial profile in which oxygen consumption attributed to ATP production, proton leak, reserve capacity, and nonmitochondrial sources is assigned. This procedure is performed at a defined time after injection of the stressor of interest. The third protocol is to treat cells in culture *ex machina* and assess the parameters of mitochondrial function either after the desired amount of time of pretreatment or after removal of the compound of interest some time later. Examples utilizing each protocol to determine the response to oxidative stress are discussed in further detail below.

Table 1
Cell lines discussed in this article.

Abbreviated cell name	Full name	Species	Derivation	References
BAEC	Bovine aortic endothelial cells	*Bos taurus*	Aorta	[39]
NRVM	Primary neonatal rat ventricular myocytes	*Rattus norvegicus*	Neonatal hearts	[40,45,46]
RASMC	Rat aortic smooth muscle cells	*Rattus norvegicus*	Aorta	[41,42]
MES13	MES13	*Mus musculus*	Mesangial cells	
SH-SY5Y	SH-SY5Y	*Homo sapiens*	Neuroblastoma	
MB231	MDA-MB231	*H. sapiens*	Breast adenocarcinoma	[47]
H9c2	H9c2 myoblast	*Rattus norvegicus*	Cardiac tissue	