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CHAPTER 31

Metabolic Assays for Cellular Therapies

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CELL METABOLISM IS ESSENTIAL FOR understanding cellular function. It regulates how cells generate and utilize energy to maintain survival, carry out activities, and adapt to changes. Because metabolic processes are highly complex and dynamic, they cannot be fully captured by a single experimental approach. Instead, several complementary assays are used to evaluate different aspects of metabolism, from bulk biochemical measurements to functional analyses at the single-cell level.

In recent years, the field of immunometabolism has experienced remarkable growth, largely due to the introduction of extracellular flux analysis using the Seahorse (Agilent) technology. This tool allows researchers to explore energy metabolism in live cells with high sensitivity, while eliminating the need for cell labeling.

This method has deepened the understanding of cell metabolism by enabling precise measurements of energy consumption across various systems, including cells,[1,2] tissues,[3] three-dimensional structures,[4-6] or isolated mitochondria.[7,8]

EXTRACELLULAR FLUX ASSAY

Principles of Seahorse Analyzer Technology

Adenosine triphosphate (ATP) serves as the primary energy source for cells. Understanding its production and regulation is important not only for unraveling the complexities of cell physiology but also for uncovering the mechanisms behind various diseases. Through simultaneous analysis of glycolysis and oxidative phosphorylation, a comprehensive perspective on cellular energy metabolism is obtainable and metabolic profiling is enhanced.

Mitochondria play a crucial role in a cell's energy production, generating approximately 95% of its ATP. The two main metabolic pathways involved in energy production are glycolysis and oxidative phosphorylation. Each pathway has unique and distinct characteristics that significantly contribute to energy production.[9,10] The details of these pathways are illustrated in Fig 31-1.

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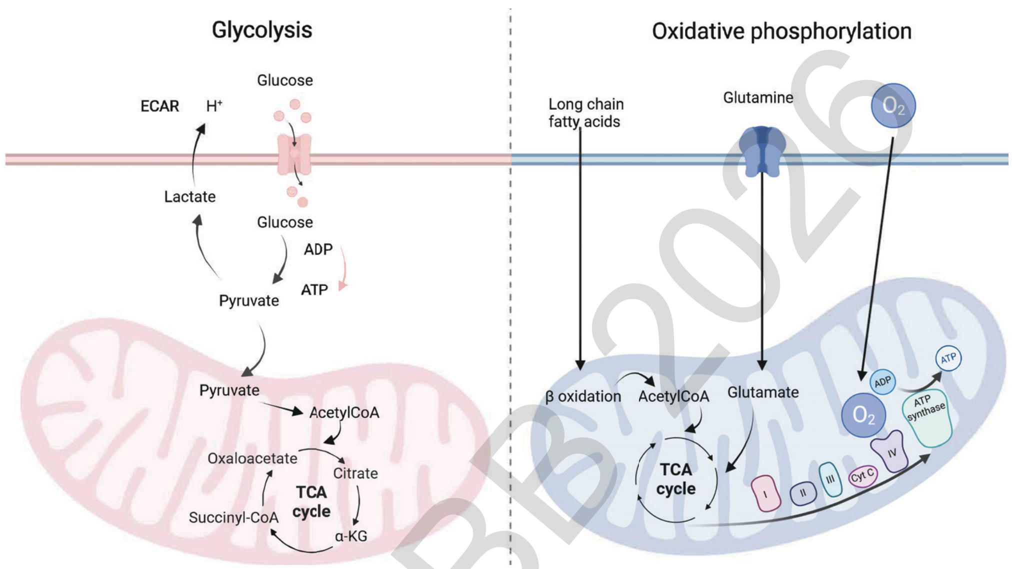

FIGURE 31-1. Two key metabolic pathways crucial for cellular energy production. Glycolysis (left panel) occurs in the cytoplasm, breaking down glucose into pyruvate while producing adenosine triphosphate (ATP). Once pyruvate is formed, it can follow one of two pathways: it can be converted into lactate, or it can enter the mitochondria. Alongside this process, long-chain fatty acids and glutamine enter the TCA cycle. These components are crucial for oxidative phosphorylation (right panel), as they donate electrons to molecular oxygen, resulting in a significant production of ATP. This electron transfer unfolds within the inner mitochondrial membrane, ensuring energy is maximally released. (Created with BioRender.)
α-KG = alpha-ketoglutarate; ADP = adenosine diphosphate; Cyt C = cytochrome C; ECAR = extracellular acidification rate; TCA = tricarboxylic acid.

Glycolysis converts glucose into pyruvate in the cytoplasm. The pyruvate can either be reduced to lactate and protons, which leads to the acidification of the extracellular environment, or enter the mitochondria for further processing in the tricarboxylic acid (TCA) cycle. This process does not require oxygen. Although glycolysis is a rapid metabolic pathway, it is relatively inefficient, producing only two ATP molecules per glucose molecule. Key indicators of glycolytic activity include the proton efflux rate (PER) and the extracellular acidification rate (ECAR).

In contrast, oxidative phosphorylation, also known as mitochondrial respiration, occurs in the mitochondria and requires the presence of oxygen. This process generates ATP via the electron transport chain, making it more efficient than glycolysis, as it can produce up to 36 molecules of ATP. The oxygen consumption rate (OCR) is important in assessing the effectiveness of ATP production during this process.

The Seahorse XFe96 analyzer [Fig 31-2(A)] is an instrument designed for noninvasive, real-time analysis of cellular metabolism by measuring key metabolic parameters. It uses specialized cartridges with fluorophore-based sensors to monitor fluctuations in oxygen and protons in cell culture media [Fig 31-2(B)]. Each sensor tip is equipped with four integrated drug injection ports, which allow precise delivery of inhibitors, stimulants, and substrates during the assay. A typical measurement cycle consists of three main steps: mixing, measuring (which includes three baseline measurements), and the injection of inhibitors or assay modulators. However, these steps can be customized based on the specific assay and research objectives.[11,12]

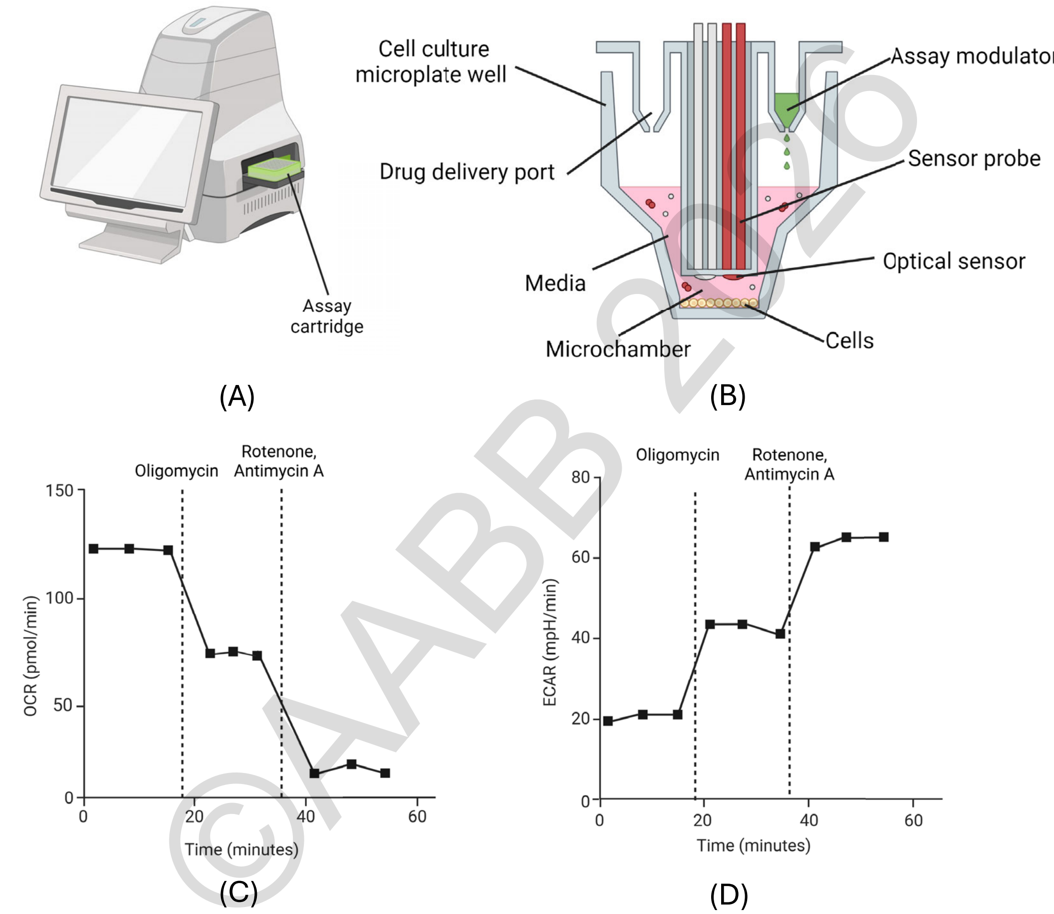

FIGURE 31-2. (A) The Seahorse XFe96 instrument allows for real-time measurement of oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) using a multiwell-plate format designed to effectively accommodate a variety of samples (8-, 24-, or 96-well systems). (B) Every Seahorse XF assay relies on the sensor cartridge, which is crucial for obtaining accurate and effective results. Each cartridge features advanced fluorophores that monitor changes in the oxygen and proton levels of the cell culture media. Additionally, it includes integrated drug delivery ports, allowing for the injection of up to four compounds into the assay media at designated intervals. The assay is useful for measuring total adenosine triphosphate (ATP) production in cells and for differentiating between ATP generated through mitochondrial oxidative phosphorylation and glycolysis. (C) The basal OCR and (D) the basal ECAR are measured three times before oligomycin injection, which leads to a decrease in OCR and an increase in lactic acid formation, resulting in elevated ECAR values. After this step, a mixture of rotenone and antimycin A is added to inhibit complexes I and III of the electron transport chain in the mitochondria. This blocks the flow of electrons through the chain and prevents any remaining oxygen from being used. As a result, there is a further decrease in OCR and an increase in ECAR values. (Created with BioRender.)

Applications

The analyzer offers a wide variety of assays that deliver precise and reliable measurements of the metabolic profiles of the cells of interest.

The Seahorse XF Real-Time ATP Rate Assay is used for a quantitative comparison of ATP production derived from mitochondrial oxidative phosphorylation and glycolysis.[13,14] This assay evaluates key parameters such as the OCR and ECAR. The process starts with three baseline measurements of intact cells, followed by sequential treatments with oligomycin and then a combination of rotenone and antimycin A. Oligomycin is introduced first to inhibit ATP synthase, resulting in a decrease in OCR. Simultaneously, this inhibition promotes the conversion of pyruvate into lactic acid, leading to an increase in ECAR values. After conducting three measurements following oligomycin addition, a mixture of rotenone and antimycin A is administered. These compounds inhibit complexes I and III of the electron transport chain in the mitochondria. This intervention stops the flow of electrons through the electron transport chain, slows the acidification of the intermembrane space, and prevents the use of any remaining oxygen. The results of this assay allow researchers to investigate either the aerobic or glycolytic characteristics of the targeted cells [Fig 31-2(C,D)].

For a deeper understanding of mitochondrial function, the XF Cell Mito Stress Test evaluates mitochondrial activity, while the XF Substrate Oxidation Stress Test examines how cells adapt to the oxidation of mitochondrial substrates, such as long-chain fatty acids, glutamine, or pyruvate. In contrast, the XF Glycolytic Rate Assay kit provides precise measurements of glycolytic activity. Additionally, the XF Hu (human) T Cell Activation Assay can monitor PER related to energy production through glycolysis following T-cell activation. The latest XF T Cell Metabolic Profiling kit examines T-cell metabolic fitness and persistence.

Analysis Workflow

The assay is typically conducted over 2 consecutive days. On the day before the assay, the assay cartridge is hydrated with sterile water and incubated overnight in a non-CO_2 incubator, along with an appropriate volume of XF calibrant. Fresh media is prepared on the day of the assay [eg, pH-calibrated DMEM (Dulbecco modified Eagle medium) or RPMI (Roswell Park Memorial Institute medium]. Before starting the assay, cells are seeded into the cell culture microplate, and various assay modulators are loaded into injector ports. Up to four different substrates can be added to the assay cartridge for automated injection during the assay. Automatic calibration is initiated once the sensor cartridge is inserted into the analyzer. After calibration, the cell culture plate is placed into the instrument. During the assay, baseline OCR and ECAR measurements are taken three times before introducing the compounds of interest, preloaded into the drug delivery ports of the assay cartridge. The total duration of the assay varies based on the selected assay type and the number of drugs injected.

Optimizing Seahorse Assays to Obtain the Most Reliable Data

The analyzer delivers real-time reliable data on the key metabolic pathways used by cells, as well as on their fitness during different stages of their proliferation and differentiation. Its ability to distinguish ATP production from oxidative phosphorylation and glycolysis highlights its importance across all research fields. However, several basic requirements must be met to ensure a successful assay.

- **Cell viability and adherence:** Cells must be viable and firmly attached to the bottom of the plate. Adherence can be ensured by using Cell-Tak adhesive (Corning) or using precoated poly-D-lysine microplates.
- **Cell density optimization:** Adjusting cell concentration is crucial for data quality. Conducting a cell concentration gradient test ensures reliable results. An ideal cell density should range from 50% to 90% confluency, and the formation of a uniform monolayer should be visually confirmed (Fig 31-3). Figures 31-4, 31-5, and 31-6 show varying results seen in peripheral mononuclear cells at different seeding densities.