Application Note
Drug Discovery

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Gaining Insights into Disease Biology for Target Identification and Validation using Seahorse XF Technology

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Abstract

It is now well accepted that dysfunctional metabolism is associated with many different disease states, including cancer, immunological disorders, neurodegeneration, diabetes, and obesity. Therefore, looking at the genes, proteins, and pathways that modulate metabolism is a promising avenue for developing novel therapeutic targets for a broad range of diseases. Agilent Seahorse XF technology measures energy metabolism in live cells, providing critical information that relates directly to cellular function. Here we provide an overview with examples of how key metrics generated by Seahorse XF assays predict cellular phenotype and function in the context of these therapeutic discovery areas.

Introduction

Once thought to be solely for 'housekeeping' functions, energy metabolism has recently come out of the textbooks and back into the forefront of academic and clinical research and drug discovery. This resurgence is because metabolism is now recognized as a critical factor in many important cell functions in both normal and disease states. As such, it is a promising area for finding drug targets related to cancer, immune dysfunction, cardiovascular disease, and neurodegeneration. The intertwined realization of the importance of metabolism in disease, along with the advancement in technological capabilities for measuring metabolism, has presented significant opportunities for discovery in these therapeutic areas. It also provides opportunities in 'traditional' metabolic diseases such as diabetes and obesity.

Conventional assays for investigating metabolism include enzyme activities, protein levels, steady-state ATP levels, and concentrations of metabolic substrates such as glucose and lactate. But these end-point measurements often result in a static view of metabolism, which is a dynamic and rapidly changing cellular process. Agilent Seahorse XF technology measures the kinetic activity (i.e. rates) of two main ATP-producing pathways in live cells: mitochondrial respiration and glycolysis. Mitochondrial respiration is measured by oxygen consumption rate (OCR) and is a quantitative metric of mitochondrial function via oxidative phosphorylation (OXPHOS). Glycolysis is indicated by the Extracellular Acidification Rate (ECAR). The Proton Efflux Rate (PER), a derivation of ECAR, is also easily calculated as a quantitative measurement of glycolytic rate. With judicious application of well-known modulators, standardized assays for interrogating specific aspects of energy metabolism have been developed. The body of literature (>5000 peer-reviewed publications[1]) using Seahorse XF technology has shown that these key XF assay parameters, derived from standard assays, are valuable indicators of metabolic cellular phenotype and function associated with specific processes and disease states:

1. OCR: oxygen consumption rate, a direct measure of mitochondrial respiration. When measured under normal and stressed conditions, it can reveal defects in mitochondrial function and/or respiratory capacity.
2. ECAR: extracellular acidification associated with lactate efflux; increases indicate cellular activation and proliferation. This metric is used to derive glycoPER, a quantitative measure of glycolysis.
3. OCR/ECAR ratio: a measure of metabolic phenotype used to detect metabolic changes such as the Warburg effect in cancer cells as well as normal cellular states such as proliferation, chemoresistance, and fat browning.

This Application Note highlights a few examples of how specific Seahorse XF assay parameters can be used to determine specific metabolic states associated with cellular dysfunction and disease. It also shows how these parameters can be used to validate the function of associated genes and proteins.

Bioenergetic balance reveals cancer cell vulnerabilities and dependencies

A very accessible measure of bioenergetic balance is the OCR/ECAR ratio, which is a qualitative measurement of the relative utilization of mitochondrial (oxidative) versus glycolytic pathways for energy production. The higher the ratio, the more oxidative; the lower, the more glycolytic. Both OCR and ECAR are measured in every well in a Seahorse XF assay and can be plotted as OCR/ECAR ratio or in an XF Energy Map. When comparing cell types or performing experimental interventions (e.g. drug treatment, genetic manipulation) plotting OCR versus ECAR on an energy map and calculating OCR/ECAR ratios reveals a clear bioenergetic picture. The OCR/ECAR ratio is widely variable across different cancer cell lines and can identify cancer vulnerabilities and dependencies. A recent study showed that 11 ovarian cancer cell lines and two immortalized ovarian surface epithelium cell lines had distinctly different bioenergetic profiles (Dar et al. 2017). Not only did the parental cell lines vary greatly in relative oxidative and glycolytic activity (Figure 1A), the authors were able to classify critical cancer vulnerabilities via OCR/ECAR ratios. These ratios revealed that chemosensitive cells are heavily reliant on glycolysis, while in contrast, chemoresistant cells perform more mitochondrial activity for energy production (Figure 1B).

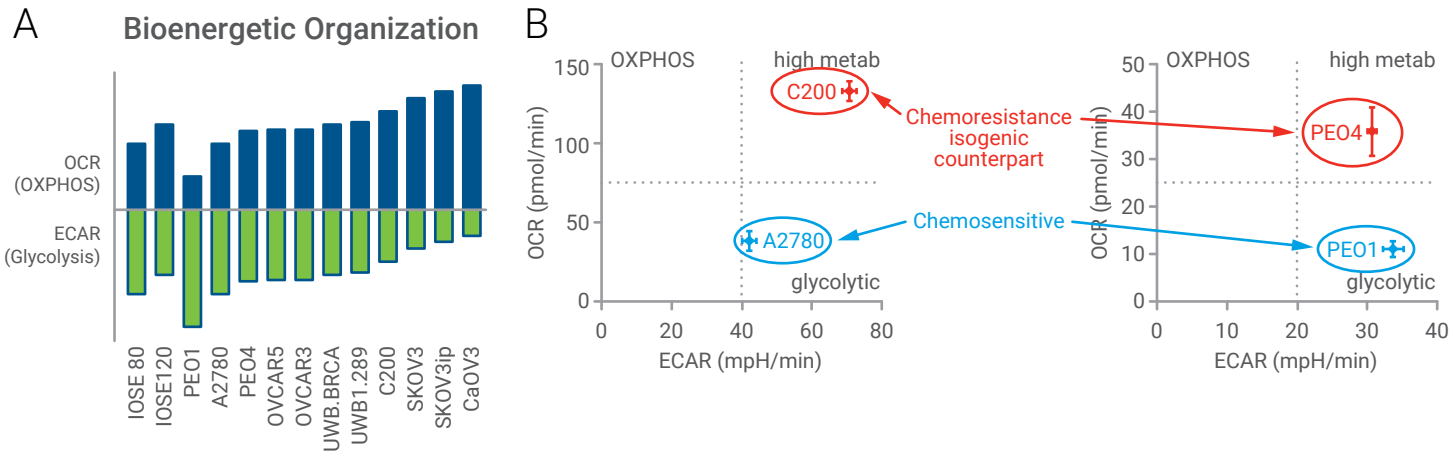

Figure 1. OCR versus ECAR reveals different bioenergetic phenotypes among ovarian cancer cell lines. A) Bar chart of cell lines ranked by OCR to ECAR ratio along the x axis. B) Energy maps of ovarian cancer-derived cell lines. Chemoresistant ovarian cancer cells appear in the upper-right quadrant, reflecting increased mitochondrial activity compared to the analogous chemosensitive cell line (lower right quadrant). (Figure adapted from (Dar et al. 2017)).

Metabolic reprogramming in cancer cells has also been implicated in the creation of a premetastatic microenvironment in stromal cells (La Shu et al. 2018). Here, highly significant changes in both basal OCR and basal ECAR, as measured by the Agilent Seahorse XF Cell Mito Stress Test (MST) and XF Glycolytic Rate Assay (GRA) respectively, indicate that the phenotype of Human Adult Dermal Fibroblast (HADF) cell lines are changed by melanoma exosome microRNA. Modulation of stromal cell metabolism may contribute to the creation of a pre-metastatic niche that promotes the development of metastasis (Figure 2). The correlation of melanoma exosome microRNA and metastasis is so predictive that it has become a standard tool of cancer research biologists (Svedman et al. 2018, Tengda et al. 2018, Bastos and Melo 2018).

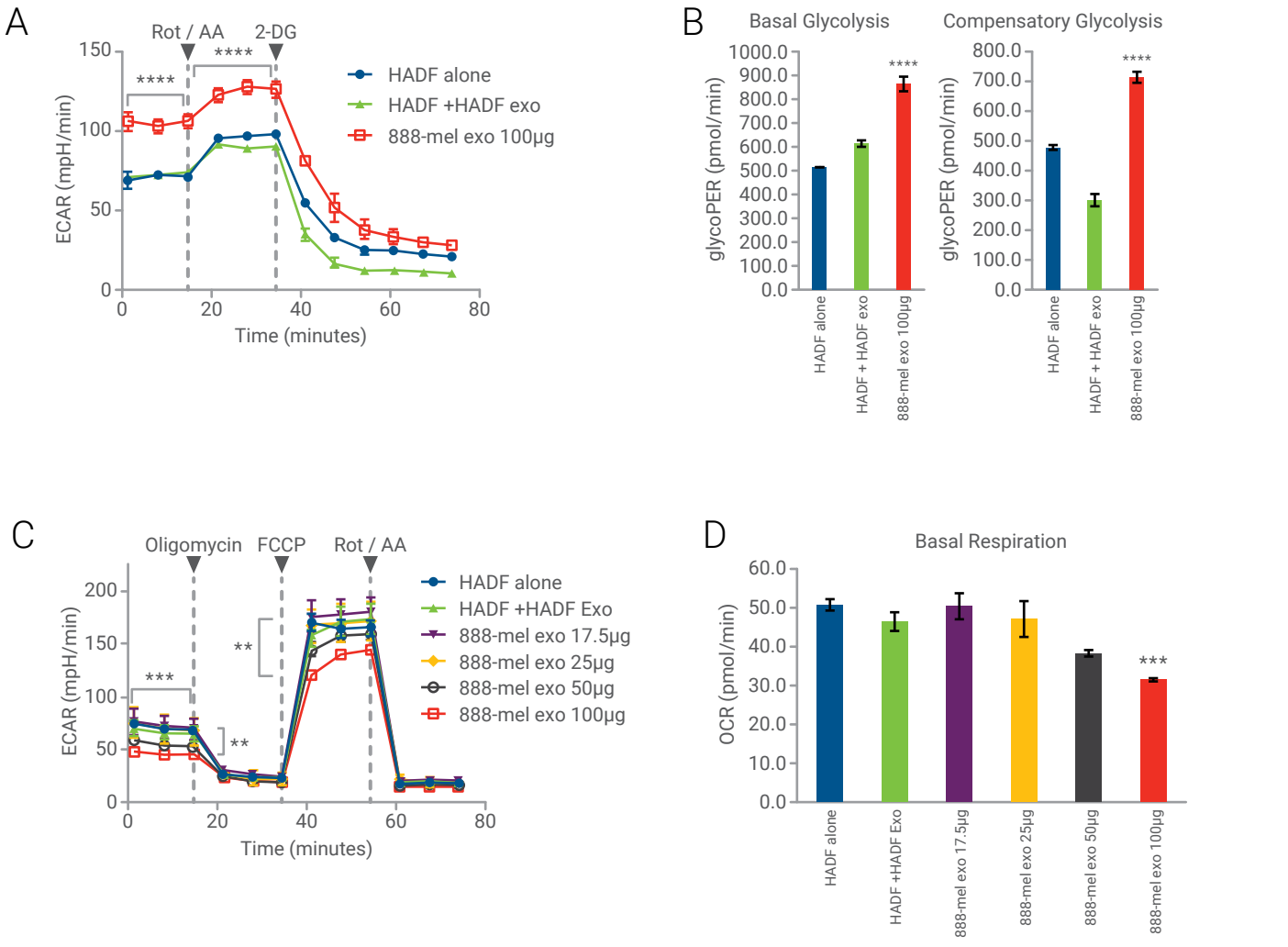

Figure 2. Metabolic changes induced by melanoma exosome miRNA. A, B) The Seahorse XF Glycolytic Rate Assay reveals an increase in glycolysis driven by melanoma exosome miRNA. C, D) The Seahorse XF Cell Mito Stress Test detects a decrease in basal mitochondrial respiration in the presence of melanoma exosome miRNA (Figure adapted from (La Shu et al. 2018)).

Metabolic measurements monitor and predict immune cell fate and function

Functional glycolytic measurements and the OCR/ECAR ratio are particularly relevant for monitoring immune cell function and can serve as an early indicator of immune cell activation. Many cell types that proliferate upon activation increase glycolysis to provide starting materials for macromolecule synthesis (Almeida, L., et al. 2016, Boothby, et al, 2017, Lunt, S.Y, et al. 2011). Immune cell activation provides a well-defined example of energy metabolism driving cell function, as demonstrated by Gubser et al. (2013). Concomitant with cellular activation, ECAR increases within minutes of T cell stimulation with appropriate antibodies (Figure 3).

This activation is easily measured using an Agilent Seahorse XF Real-Time activation assay (Swain et al. 2018). For T cell activation, baseline ECAR rates are established before cells are activated by injecting CD3/CD28 antibodies (Figure 3A). Using this method, a robust increase in acidification (ECAR) is detected upon stimulation of the cell with an appropriate activator, an effect that is detectable in minutes instead of the hours or days needed for typical markers (e.g. CD69, IFN- γ). This increase in ECAR is directly attributable to glycolysis, as this response depends on the presence of glucose (Figure 3B). Gubser, *et. al.* demonstrated that glycolysis is necessary for human T-cell activation as previously shown by well-accepted orthogonal assays (Gubser et al. 2013).

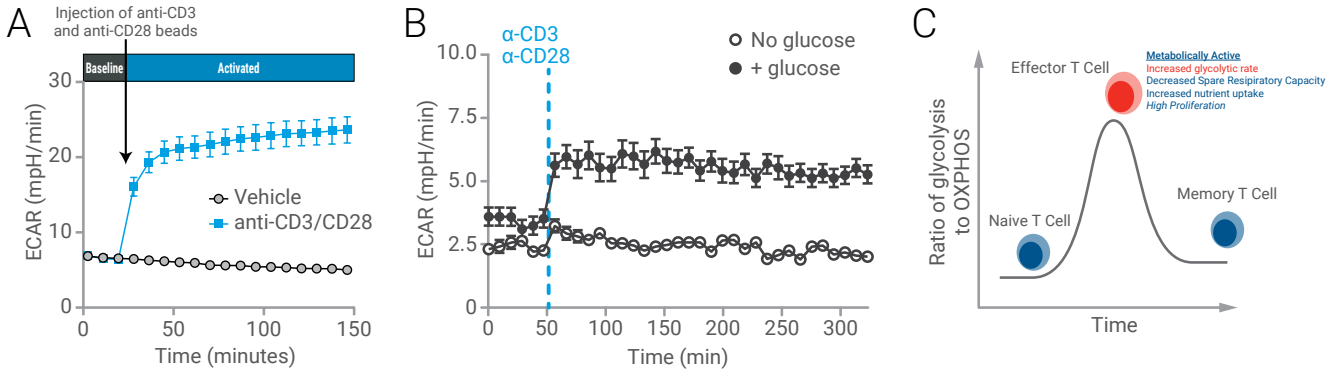

Figure 3. Measuring T cell activation in real time. A) XF T cell activation assay reveals a rapid increase in acidification (ECAR) upon activation with α-CD3 and α-CD28 antibodies. B) Glucose is required for T cell activation, thus linking this increase in ECAR directly to glycolysis (Gubser et al. 2013). C) Metabolic shifts expressed as the ratio of glycolysis (ECAR) to oxidative phosphorylation (OCR) versus time as naïve T cells change phenotype to effector then memory T cells (adapted from (Chi 2012)).

This feature of immune cells appears to be nearly global, as many other immune cell types show similar metabolic programs upon activation, including CD4+ cells, T_{reg} cells, macrophages, monocytes, and dendritic cells (Guak et al. 2018; Dominguez-Andres et al. 2017; Wang et al. 2018). Once activated, the equilibrium between mitochondrial and glycolytic energy production controls T cell fate through proliferation and differentiation to effector cell (increased glycolysis rate) (Almeida, L., et al. 2016, Boothby, et al, 2017, Lunt, S.Y, et al. 2011). T cells also have the potential to revert to predominantly mitochondrial oxidative metabolism (OXPHOS) and remain viable as a memory T cell. (Figure 3C and (Chi 2012; Kim 2018)).

Similar to cancerous cell types, changes in these metabolic programs are typically associated with changes in cellular signaling or function, or both. Gubser et al. also examined effects of cell signal inhibition by pretreating human Effector Memory (EM) CD8+ T cells with inhibitors of PI(3)K (LY294002), Akt (Akti-1/2), or mTORC1 (rapamycin), followed by XF T cell activation experiments (Figure 4).

This study demonstrated that the immediate-early glycolytic switch in EM CD8+ T cells is insensitive to inhibition of mTORC1 but depends on Akt activity and mTORC2 signaling. These findings led to a further study showing that Ndfip1-deficient T_{reg} cells have altered metabolic activity, including elevated levels of mTORC1 expression and significantly increased rates of glycolysis (Layman et al. 2017).

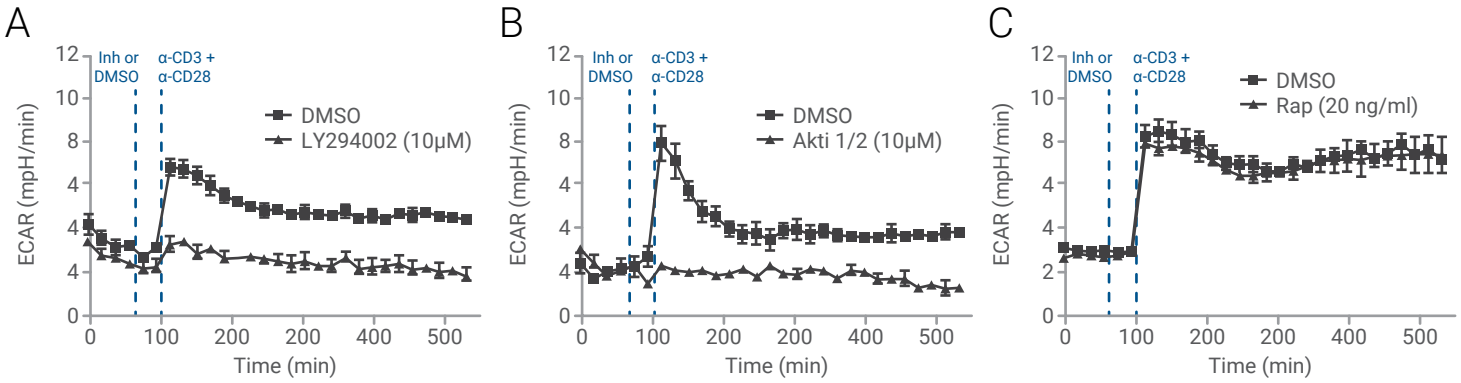

Figure 4. Using the XF Real-Time T cell activation assay to probe upstream signaling events required for activation. T cells pretreated with LY294002, Akti-1/2, or Rap were used in the Agilent Seahorse XF Real-Time T cell activation assay, which showed the immediate-early glycolytic switch in EM CD8+ T cells depends on PI3K/mTORC2 (a) and Akt (b) activity, but insensitive to inhibition of mTORC1 c), figure adapted from (Gubser et al. 2013).

Measuring mitochondrial function provides a window into neuronal cell health

The Seahorse XF Cell Mito Stress Test and respective parameters have been widely adopted as robust metrics for studying neurodegenerative disorders *in vitro*. In neurons, mitochondria are critical for maintenance of membrane ion (Na^+ and Ca^{2+}) gradients and for neurotransmission and synaptic plasticity (Raefsky and Mattson 2017). Neurons have a limited glycolytic capacity; therefore, proper mitochondrial bioenergetics are critical for the many different ATP-dependent processes that enable neurons to function and respond adaptively to environmental challenges (Herrero-Mendez A., et al. 2009). Thus, the rate of mitochondrial respiration, as measured by OCR, is a very sensitive indicator of neuronal cell function and health (Oliveira, J.M.A., 2011). By employing the XF Cell Mito Stress Test, several standard key parameters of mitochondrial health and function including basal, ATP-linked, maximal respiration, and spare respiratory capacity can be assessed quickly in the same cells (Figure 5. Left).

Mitochondrial changes have long been implicated in the pathogenesis of Parkinson's disease (PD). The glycine to serine mutation (G2019S) in leucine-rich repeat kinase 2 (LRRK2) is the most common genetic cause for PD and has been shown to impair mitochondrial function and morphology in multiple model systems (Ryan, B.J, et al. 2015, Yue, M. et al. 2015). Using the XF Cell Mito Stress Test, Schwab and colleagues demonstrated that mitochondrial respiration is decreased in LRRK2 G2019S iPSC-derived dopaminergic and glutamatergic neurons (Schwab et al. 2017). Specifically, decreases were most evident in the ATP-linked, maximal, and spare respiratory capacity parameters (Figure 5. Right). In the context of LRRK2 G2019S, decreases in these parameters point to distinct sirtuin and bioenergetic deficiencies intrinsic to dopaminergic neurons, which may underlie dopaminergic neuron loss in PD.

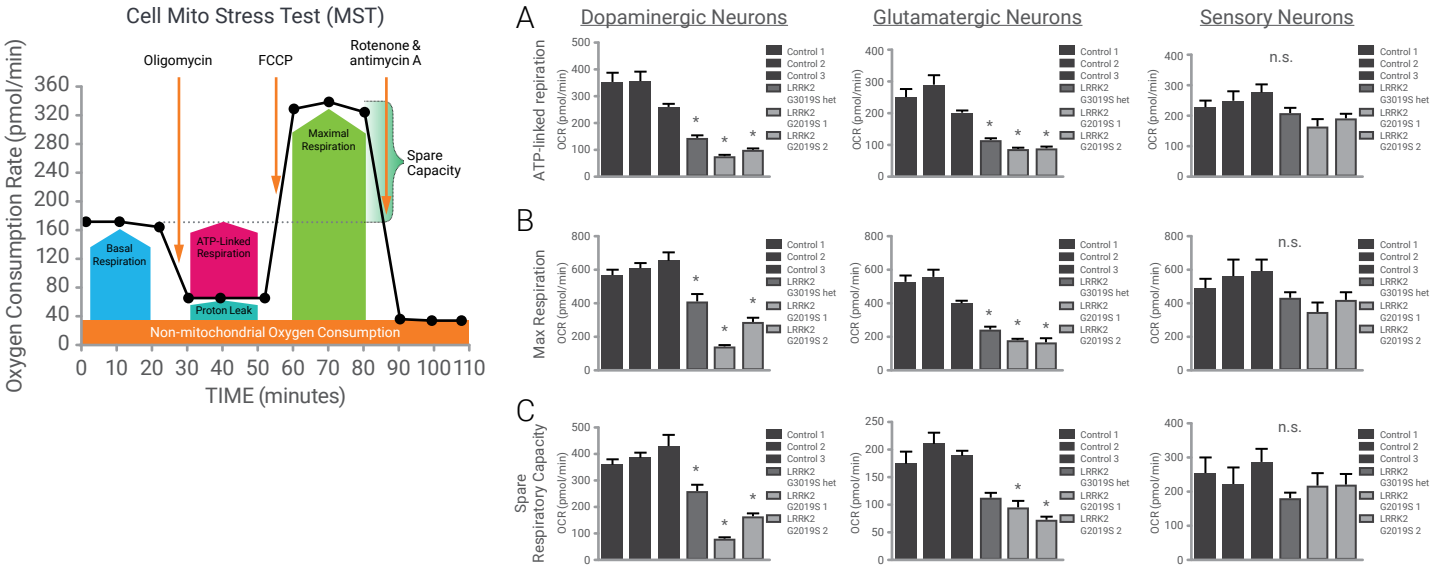

Figure 5. The Agilent Seahorse XF Cell Mito Stress Test detects mitochondrial defects in neurons. Left) XF Cell Mito Stress Test assay design and standard output parameters. Right) Using the XF Cell Mito Stress Test shows that LRRK2 G2019S iPSC-derived dopaminergic and glutamatergic cultures display diminished (A) ATP-linked, (B) maximal, and (C) spare respiratory capacity compared with respective control cultures. Note LRRK2 G2019S iPSC-derived sensory neurons are unaffected by the mutation (Schwab et al. 2017).

Measuring mitochondrial dysfunction in diabetes and cardiovascular disease models

AMPK is a master sensor of cellular bioenergetics, and thus is a potential drug target for the treatment of type 2 diabetes mellitus (T2DM) and other related metabolic diseases (Hardie, Schaffer, and Brunet 2016). In a recent investigation of novel synthesized compounds against AMPK, treatment of L6 cells caused a reduction in basal OCR (Figure 6), which was further correlated to increased glucose consumption, reduced gluconeogenesis, and resulted in indirect activation of AMPK (Zhou et al. 2017).

Diabetic cardiomyopathy has also been attributed to changes in mitochondrial function (Galloway, C.A., et al, 2015). Typically, cardiac cells are metabolically flexible, oxidizing both fatty acids and carbohydrates to generate energy. However, this flexibility is lost with T2DM, with the heart exclusively utilizing fatty acids, promoting diabetic cardiomyopathy. In the following example, the XF Cell Mito Stress Test was used to demonstrate that a decrease in pyruvate-supported respiration (OCR) and a shift to a preference for fatty acid oxidation occurred in the model systems used (Vadvalkar et al. 2017). These changes were linked to the degree of acetylation of the mitochondrial pyruvate carrier 2 (MPC2) protein. Using the XF Cell Mito Stress Test, the authors demonstrated that the double acetylation mimetic K19Q/K26Q (QQ) decreased the pyruvate-dependent cellular basal and maximal respiration rates (Figure 7).

	Oxygen consumption rate[a] (%)	
DMSO	**100.0 ± 1.6**	
Compd.	10μM	20μM
4aa	92.9 ± 0.9*	90.4 ± 1.4*
4bq	54.0 ± 1.5***	47.5 ± 2.3***
4bv	56.9 ± 1.8***	54.6 ± 2.1***
Berberine	91.9 ± 4.4*	75.0 ± 2.8***

Figure 6. Known AMPK modulators inhibit oxygen consumption rate (OCR) in L6 myotube cells (Zhou et al. 2017).

Summary

- Changes in the balance of oxidative phosphorylation and glycolysis in cancer cells are measured by the OCR/ECAR ratio and this metric is an easy-to-use indicator of changes in cell phenotype or activity. Assays that can be used include the Seahorse XF Cell Energy Phenotype Test, the XF Cell Mito Stress Test, and the XF Glycolytic Rate Assay.
- Immune cell activation is characterized by an acute increase in glycolytic function (ECAR or PER) and can be monitored in real time with XF Real-time immune cell activation assays.
- The rate of oxidative phosphorylation (OCR) is an exquisitely sensitive indicator of mitochondrial function and health and can be quantitatively measured using the Seahorse XF Cell Mito Stress Test.
- Basal, maximal, and spare respiratory capacity are key metrics of mitochondrial function reported by the Seahorse XF Cell Mito Stress Test and can detect dysfunction in signaling, enzyme activity, substrate transport, and ETC/OXPHOS activities.

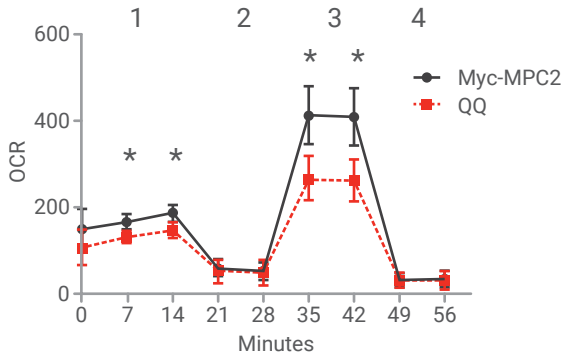

Figure 7. Evaluation of MPC2 variants using the Seahorse XF Cell Mito Stress Test. The double acetylation mimetic (QQ) decreases the pyruvate-dependent mitochondrial respiration, as measured by OCR. (Vadvalkar et al. 2017).

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