

Defining metabolic phenotypes in cancer cells

Kinetic assay reveals the dynamic interplay of oxidative phosphorylation and glycolytic pathways

RESEARCH AREA

Cancer

ASSAY TYPE

Energy expenditure:
Cancer cell metabolic
pathway analysis

KEYWORDS

Cancer, glycolysis, mitochondria, oxidative phosphorylation, OXPHOS, Warburg effect, H460

Malignant transformation of cells is often characterized by aerobic glycolysis, the conversion of glucose into lactate, despite the presence of oxygen. This metabolic shift, known as the Warburg effect, [1] can occur as a result of the activation or mutation of oncogenes or tumor suppressor genes, or as an adaptive response to hypoxia [2]. In contrast, normal differentiated cells rely on oxidative phosphorylation (OXPHOS) to generate ATP [3]. These bioenergetic alterations associated with malignant transformation are being exploited to identify potential cancer-selective therapeutics.

Cancer cell bioenergetic analyses have been limited to sequential, independent measurement of cellular respiration, glucose uptake, lactate production and mitochondrial function. While these assays measure parameters of bioenergetic function, they are time consuming, may require the use of radiolabeled reagents and often do not provide real-time data. XF extracellular flux assays measure both mitochondrial respiration (OXPHOS) and glycolysis simultaneously enabling a rapid and more comprehensive assessment of cellular bioenergetics. XF assays quantify the dynamic interplay between the two dominant energy-yielding pathways, the balance of which may be diagnostic for a particular cancerous phenotype.

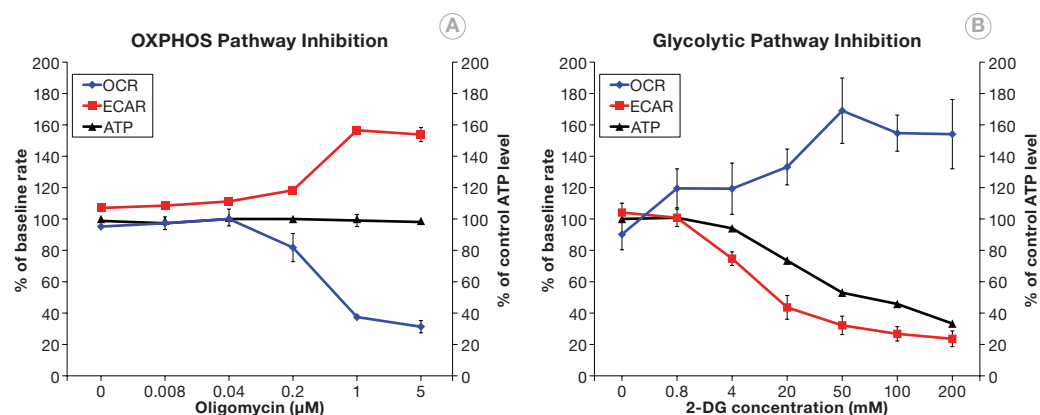
In a previously published paper, Wu *et al* described the use of XF assays to define metabolic phenotypes and measure alterations in cancer cell bioenergetics [4]. The H460 human non-small cell lung carcinoma line was used as a model system to pharmacologically probe mitochondrial respiration and glycolysis.

To assess the cellular response to perturbations of OXPHOS, H460 cells were exposed to oligomycin, an inhibitor of the F_1F_0 ATP synthase that blocks oxidative phosphorylation. In the presence of increasing doses of oligomycin, the cellular respiration rate declined while glycolysis increased, as shown in Figure 1A. Cellular ATP levels, determined later from sequential aliquots, was maintained after exposure to oligomycin suggesting the existence of an effective compensatory up-regulation of glycolysis to sustain cellular ATP levels in H460 cells.

Figure 1 | Metabolic Dependencies in H460 Cancer Cells

Figure 1A shows the decline in cellular respiration rate (OCR) as the glycolysis rate (ECAR) increases in response to increasing doses of Oligomycin. ATP turnover was sustained.

Figure 1B illustrates the changes in response to increasing levels of 2-deoxyglucose (2-DG). The figure shows ECAR decreases with an accompanying increase in OCR in response to increasing 2-DG concentrations. ATP turnover decreased markedly as glycolytic rates declined.



The H460 cells' bioenergetic dependency on glycolysis was then investigated. Figure 1B illustrates the response of cellular respiration and glycolysis rates after administration of 2-deoxyglucose (2-DG) which blocks the first step of the glycolytic pathway. The glycolysis rate decreased with an accompanying increase in respiration in response to increasing 2-DG concentrations. ATP level decreased markedly as glycolytic rates declined, even though there appeared to be a small compensatory rise in cellular respiration.

These observations suggest that H460 cells critically depend on ATP generation through glycolysis. Cellular ATP concentrations could not be maintained despite the observed increase in the respiration rate suggesting that the OXPHOS capacity may be impaired or insufficient to meet the increased energy demand. Prolonged exposure to 2-DG resulted in cell death, confirming dependency on glucose for survival (data not shown).

In summary, these results demonstrate a bioenergetic phenotype in H460 human lung carcinoma cells characterized by a high capacity for glycolysis and a linked attenuation in OXPHOS capacity. These metabolic alterations provide a possible mechanistic explanation for the growth advantage, especially in hypoxic environments, and apoptotic resistance due to decreased aerobic respiration of H460 tumor cells.

Discussion

XF assays provide simultaneous measurements of mitochondrial respiration (OCR) and glycolysis (ECAR), allowing analysis of the alterations and interplay of these pathways in cancer cells. XF assays also enable mechanistic studies of cancerous cellular pathways in response to oncogene activation, or suppression or alterations in signaling pathways, as well as the effects of pharmacologic modulators on these energy pathways.

Recently, Xie *et al* [5] investigated the relationship between malignant transformation and bioenergetics in an *in vitro* model of hereditary leiomyomatosis and renal cancer by downregulating expression of fumarate hydratase using shRNA (FH). XF analyses showed FH inhibition results in OXPHOS reduction (OCR decrease) requiring that cells resort to glycolysis, indicated by an increase in ECAR. Xie then asked whether these cells over-express lactate dehydrogenase-A (LDH-A), the enzyme that converts pyruvate to lactate, as an indication of their increased dependence on glycolysis, and whether LDH-A blockage would result in increased apoptosis and decreased tumorigenicity. The authors concluded that LDH-A inhibition in the context of FH deficiency results in increased apoptosis, as well as a reduction in tumor growth in xenographic mouse models.

de Groot *et al* [6] described increased OXPHOS activity prior to the rise in glycolysis seen in H-RasV12/E1A transformed fibroblasts that develop the Warburg phenotype. As determined by XF assays, glucose to lactic acid flux increased with passage number as cellular oxygen consumption decreased. The authors propose the shift of cells toward a Warburg phenotype occurs as a step-wise adoption process associated with augmented tumorigenic capacity and improved survival characteristics of the transformed cells.

Weinberg *et al* [7] demonstrated the essential function of mitochondrial metabolism in Kras-induced tumorigenesis. Performing XF assays, they demonstrated these cells increase mitochondrial respiration as indicated by increased OCR when galactose substituted for glucose in the cell culture medium. The OCR to ECAR ratio increased from 5 to 15 as the cells grew in galactose medium. The increased OCR resulted in part from active glutamine oxidation as evidenced by increased OCR upon glutamine addition. This study indicate K-Ras-transformed cells depend on mitochondrial metabolism for their growth and survival beyond the Warburg effect highlighting different tumor cells have distinct metabolic phenotype requirements.

XF extracellular flux assays provide simultaneous measurements of mitochondrial respiration (OCR) and glycolysis (ECAR), allowing analysis of the alterations and interplay of these pathways in cancer cells. XF assays also enable potential mechanistic studies of cancerous cellular pathways.

MATERIALS AND METHODS

Cells and compounds: H460 cells were seeded in XF24 V7 cell culture microplates at 2.0×10^4 cells/well (0.32 cm^2) in 200 ml growth medium and incubated at 37°C / $5\% \text{ CO}_2$ for 20 to 24 hours. Growth medium was removed from each well and replaced with 600 ml of assay medium at 37°C and pH 7.4. A low-buffered RPMI 1640 medium containing 1 mM phosphate (Molecular Devices) was used as standard medium in the XF assays and is referred to as “assay medium.”

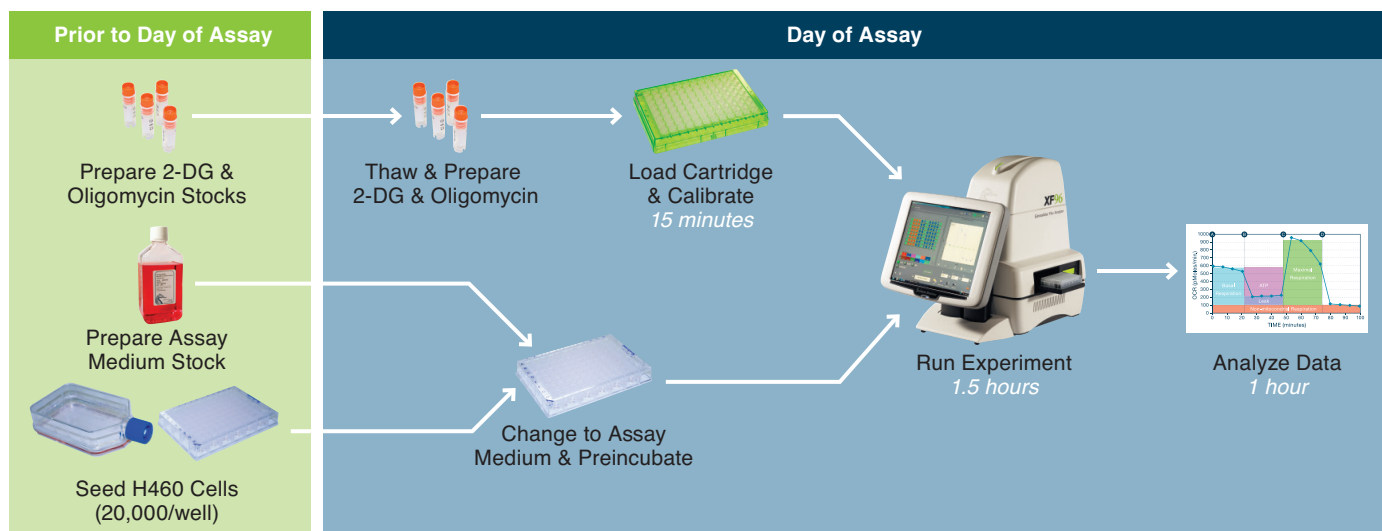
2-DG and oligomycin were from Sigma. Concentrated stocks of 1,000 mM 2-DG were prepared in assay medium immediately prior to the assay. Concentrated stocks of 10mM oligomycin were prepared in DMSO and stored at -20°C .

XF Analysis

XF analyses were performed in the Seahorse XF Extracellular Flux Analyzer a fully integrated, multi-well instrument that measures the uptake and excretion of metabolic end products in real-time. OCR and ECAR were measured using XF assay kits. The disposable assay kits contain an XF24 or XF96 cell culture plate and a solid state sensor cartridge, embedded with 24 or 96 dual-florescent biosensors (O_2 and H^+). Each sensor cartridge is also equipped with four drug delivery chambers per well for injecting agents into wells during an assay. OCR is reported in the unit of pmoles/minute and ECAR in mpH/minute.

To prepare for the assay as illustrated in Figure 2, after the H460 cells were switched from culture medium to assay medium, the sensor cartridge was loaded with experimental compounds, calibrated, and placed over the culture plate containing the prepared H460 cells. Following mixing, OCR and ECAR were measured simultaneously for 3 minutes to establish a baseline rate. Then, 75 μl of testing agent prepared in assay medium was injected into each well to the final working concentration. This was followed by mixing for 5 minutes to expedite compound exposure to cellular proteins after which OCR and ECAR measurements were made. 3 baseline rates and 3 response rates after compound addition were measured in triplicate and averaged. The values of OCR and ECAR observed reflect both the metabolic activities of the cells and the number of cells being measured. The quantity of ATP present in the test cells in each well following compound or vehicle exposure for 30 minutes was subsequently determined using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) in a plate reader.

Figure 2 | Flow Chart of XF Assay



References

1. Warburg O. *On the origin of cancer cells*. Science. (1956); 123: 309-314.
2. Vander Heiden MG, Cantley LC, Thompson CB. *Understanding the Warburg effect: the metabolic requirements of cell proliferation*. Science. (2009); 324 (5930):1029-33.
3. Rolfe DF, Brown GC. *Cellular energy utilization and molecular origin of standard metabolic rate in mammals*. Physiol Rev. (1997); 77 (3):731-58.
4. Wu, M., et al: *Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells*. Am J Physiol Cell Physiol (2007); 292:C125-C136.
5. Xie, H., et al: *LDH-A inhibition, a therapeutic strategy for treatment of hereditary leiomyomatosis and renal cell cancer*. Mol Cancer Ther. (2009); 8 (3):626-635.
6. de Groof AJ, et al: *Increased OXPHOS activity precedes rise in glycolytic rate in H-RasV12/E1A transformed fibroblasts that develop a Warburg phenotype*. Mol Cancer (2009); 8 (1):54.
7. Weinberg F, et al: *Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity*. Proc Natl Acad Sci USA. (2010); 107 (19):8788-93.

Suggested Reading

1. Dang CV. *Rethinking the Warburg effect with Myc micromanaging glutamine metabolism*. Cancer Res. (2010); 70 (3):859-862.
2. Fan, Y., et al. *Akt and c-Myc differentially activate cellular metabolic programs and prime cells to bioenergetic inhibition*. J. Biol. Chem. (2010); 285 (10):7324-7333.

For Research Use Only. Not for use in diagnostic procedures.

This information is subject to change without notice.

© Agilent Technologies, Inc., 2016
Published in the USA, August 1, 2016
5991-7129EN

 **Seahorse Bioscience**

A part of **Agilent Technologies**