Metabolic Pathway Determination Using the XF24 Analyzer

Research Area

Cancer Metabolism

Application

Energy Expenditure: How to measure the contribution of mitochondrial respiration and glycolysis to cellular bioenergetics

Cell Type

Human non-small cell carcinoma (A549)

Work Of

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Tool Box:

- 2,4-DNP
- 2-DG
- rotenone

Keywords:

- A549 cells
- aerobic respiration
- glycolysis
- metabolic pathway
- mitochondria

Energy Expenditure

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The XF24 Analyzer non-invasively measures physiological changes in cellular bioenergetics and metabolic pathways "in vitro" using optical biosensors in a 24-well format. Shifts between mitochondrial respiration and glycolysis as a result of genetic, pharmacologic or environmental manipulation can be measured in a single assay. XF24 assays provide increased throughput in a drug discovery format.

Measure Mitochondrial Respiration and Glycolysis Simultaneously

Determination of the contribution of mitochondrial respiration and glycolysis to cellular bioenergetics is not possible in most assay formats. However, by employing two sensors, one that measures oxygen consumption rate (OCR, an indicator of mitochondrial respiration) and another that measures extracellular acidification rate (ECAR, an indicator of lactic acid production or glycolysis), the XF24 can simultaneously determine both the aerobic and glycolytic components of cellular bioenergetics.

In Figure 1, A549 cells are treated with a variety of metabolic pathway inhibitors to alter the relative mix of aerobic and glycolytic metabolism. Untreated wells (dark circles) show a normal basal mixture of both aerobic and glycolytic metabolism as indicated by an average rate of 125 pMoles/min OCR and 70 mpH/min ECAR, respectively. When exposed for 60 minutes to 100 mM of the glycolysis inhibitor 2-deoxyglucose (2-DG, turquoise circles), the cells shift to an almost exclusive aerobic phenotype as indicated by a low ECAR (approximately 20 mpH/min). Conversely, when incubated for 30 minutes in 1μM of the Complex I inhibitor, Rotenone (orange circles), the cells shift to a more glycolytic phenotype with an average OCR of 20 pMoles/min and an average ECAR of 75 mpH/min. Lastly, incubation with both 2-DG and Rotenone (blue circles) results in a precipitous drop in both pathways leading to a state of low energy yield that endangers the viability of the cell.

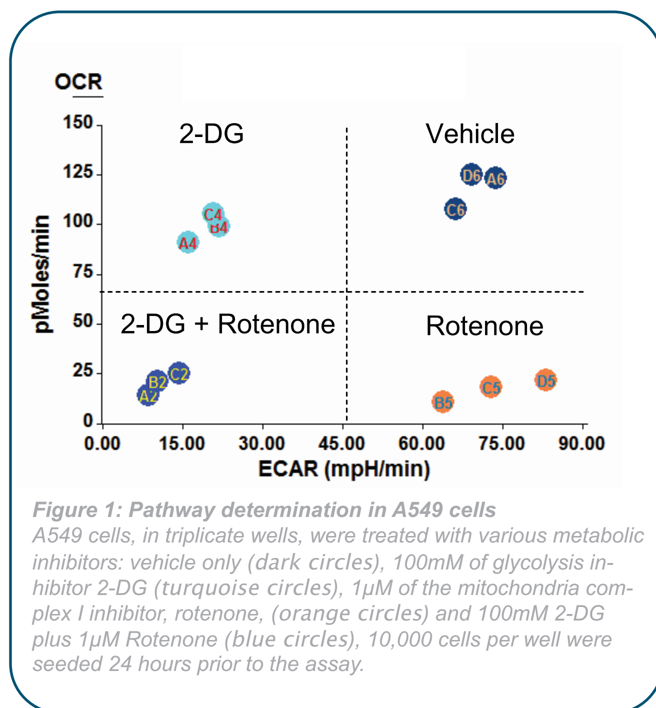

Figure 1: Pathway determination in A549 cells
A549 cells, in triplicate wells, were treated with various metabolic inhibitors: vehicle only (dark circles), 100mM of glycolysis inhibitor 2-DG (turquoise circles), 1μM of the mitochondria complex I inhibitor, rotenone, (orange circles) and 100mM 2-DG plus 1μM Rotenone (blue circles), 10,000 cells per well were seeded 24 hours prior to the assay.

A great advantage of the XF24 system is the ability to add up to four compounds/reagents during an experiment and determine their influence on both mitochondrial respiration and glycolysis. For example, using the same metabolic inhibitors as above and the mitochondrial respiration uncoupler, 2,4-DNP, one can detect shifts in metabolic pathway utilization in real-time.

Materials & Methods

Cell Culture.
Human alveolar basal epithelial cell line, A549 was obtained from ATCC. Cells were cultured in growth media consisting of RPMI 1640, 10% FBS, 100 units of penicillin and 100 µg/ml streptomycin. All cultures were maintained at 80-90% confluence at the time of subculture.

Test Compounds.
2-deoxyglucose (2-DG), 2,4-dinitrophenol (2,4-DNP) and rotenone were obtained from Sigma (St. Louis, MO). Concentrated stocks of 2-DG were prepared in assay medium, 2,4-DNP and rotenone in DMSO. All stocks were diluted in assay media and the pH adjusted to 7.4.

Note: A more detailed protocol for the data presented in this case study can be found in Wu et al. (2007) *Am J Physiol Cell Physiol* **292:C125-136.**

References

Wu, M et al. (2007). 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* **292:C125-C136**

As shown in Figure 2, 2,4-DNP administration invoked a concomitant increase in OCR and ECAR indicating an overall increase in cellular metabolism. Subsequent addition of 2-DG to the same wells elicited a rapid decrease of the 2,4-DNP-stimulated ECAR to well below the baseline rate while OCR remained unaffected. This demonstrated a decrease in glycolysis. Finally, addition of the mitochondrial respiration inhibitor Rotenone, after exposure of the same cells to 2,4-DNP and 2-DG, completely diminished aerobic respiration as measured by decreased OCR. This low energy state is not sustainable by the cell and would eventually result in decreased viability.

To correlate these observed changes in OCR and ECAR directly to ATP production, we also measured cellular ATP concentration in these cells: a) vehicle, b) 2,4-DNP, c) 2,4-DNP plus 2-DG , and d) 2,4-DNP plus 2-DG plus rotenone (data not shown). We observed that the 2,4-DNP treatment reduced ATP content by approximately 40% compared to vehicle treated cells due to the uncoupling of oxidative phosphorylation from respiration. 2-DG in combination with 2,4-DNP caused a dramatic decrease in ATP turnover to less than 10% of the control cells as glycolytic ATP production was now also blocked. And finally, the combination of the mitochondrial respiration inhibitor, rotenone, along with the glycolysis inhibitor, 2-DG, and uncoupler, 2,4-DNP, effectively depletes the cells of their ATP content. Since the ATP concentration of a cell population is determined by both the number of viable cells present and their relative metabolic rates, the cell viability of each population must be determined to ensure similar viability of control and testing cells if the changes in ATP concentration are to be attributed to alterations in their metabolic rate. Therefore, cell viability was also measured in the parallel experiment using Calcein AM staining. The cell viability remained essentially constant among all the experimental samples, thus indicating that the reduced ATP levels in the A549 cells was due to metabolic changes and not cell loss as a result of compound toxicity.

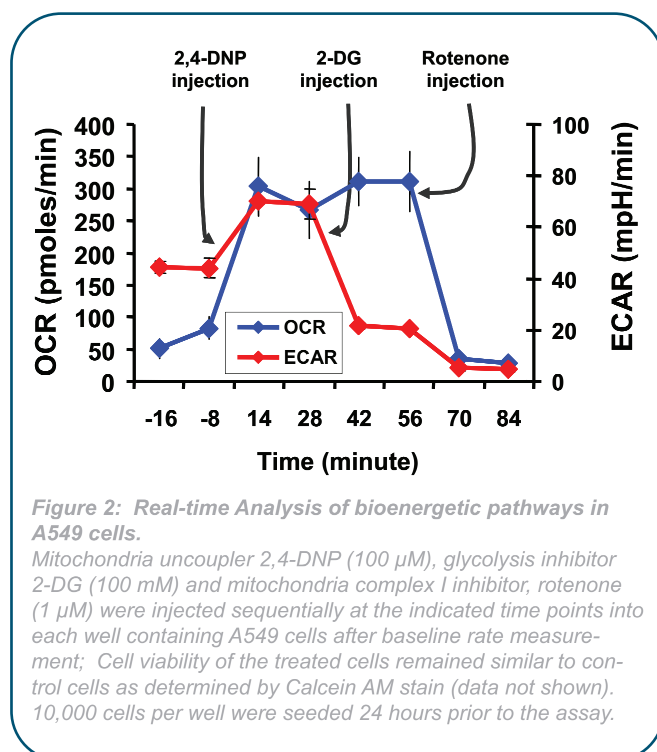

Figure 2: Real-time Analysis of bioenergetic pathways in A549 cells.
Mitochondria uncoupler 2,4-DNP (100 µM), glycolysis inhibitor 2-DG (100 mM) and mitochondria complex I inhibitor, rotenone (1 µM) were injected sequentially at the indicated time points into each well containing A549 cells after baseline rate measurement; Cell viability of the treated cells remained similar to control cells as determined by Calcein AM stain (data not shown). 10,000 cells per well were seeded 24 hours prior to the assay.