

Extracellular Flux Enables Real-time, Non-invasive Measurements of Cellular Metabolism

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Abstract

Metabolic changes in cells are very sensitive, early indicators of processes such as proliferation, differentiation, activation, and apoptosis. Seahorse Bioscience has developed an *in vitro* real time, bioenergetic assay for pathway determination and quantification.

The XF24 Extracellular Flux Analyzer simultaneously measures cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of cells in 24 well microplates in minutes. OCR and ECAR can be used to profile both metabolic rate and the relative contributions of aerobic and anaerobic respiration. Extracellular Flux (XF) assays do not disturb cells and can therefore be repeated frequently to acquire time resolved data, or to compare basal and drug-stimulated metabolic rates.

XF24 assays of two cancer cell lines demonstrated an increased dependency on glycolysis that can be modulated with Topotecan.

XF24 assays of C2C12 myotubes demonstrated a rapid and dramatic increase in OCR, indicative of Fatty Acid Oxidation, following the addition of Palmitate. The response was inhibited by the CPT-1 inhibitor Etomoxir.

Introduction

Mammalian cells are able to consume a variety of substrates to produce ATP by utilizing one or more metabolic processes. For example, cells may consume glucose, fatty acids, or amino acids. Glucose may be consumed through an anaerobic process or through a combination of anaerobic and aerobic processes.

Insight into a cell's choice of substrate and metabolic pathway can be gained from measurements of the flux rates of substrates, gasses, ions, and other analytes between the cell and the media. Glucose, for example, can be used to produce ATP through glycolysis:



Glycolysis consumes no oxygen while producing a significant amount of lactate and free protons as a byproduct of an *atp* ATP. The free protons acidify the surrounding media, causing an increase in ECAR.

In the presence of oxygen, glucose can be converted through the more efficient aerobic process:



The OCR of cells performing glucose oxidation exceeds that of cells performing anaerobic glycolysis, while ECAR (from lactic acid and proton production) is lower.

Certain substrates such as fatty acids can only be converted to ATP through an aerobic process such as the following:



In comparison with glucose oxidation, fatty acid oxidation produces approximately 30% fewer protons due to the lack of carbonic acid production (e.g. during synthesis of acetyl CoA from pyruvate).

Therefore, OCR and ECAR can be used to profile a shift between aerobic and anaerobic respiration, or a shift from glucose to fatty acid oxidation.

Materials and Methods

Cell Culture and Chemical Reagents

Human LNCaP cells, and the derivative C4-2 line were obtained from ATCC (Manassas, VA) and ViroMed laboratories (Minneapolis, MN) respectively. LNCaP cells were maintained in Modified RPMI 1640 (ATCC) supplemented with 10% FBS (Hyclone, Logan, UT) and 100 µg/ml penicillin-Streptomycin (Invitrogen, Carlsbad, CA). C4-2 cells were maintained in T medium (Invitrogen). Mouse muscle myoblast cell line C2C12 was obtained from ATCC and cultured in DMEM (ATCC) supplemented with 10% FBS and 100µg/mL penicillin-Streptomycin.

Materials and Methods (continued)

ATP assay

LNCaP cells were seeded in white 96-well tissue culture microplates at indicated cell density per well 24 hours prior to compound treatment. Cell Titer-Glo luminescent ATP assays (Promega, Madison, WI) were performed at the indicated treatment time using a FLUOstar Optima plate reader (BMG Labtech, Durham, NC).

Calcein AM stain

LNCaP cells were seeded in black 96-well tissue culture microplates at the indicated cell density per well. Calcein AM (2 µM) staining was performed in cells treated with the indicated compounds and at the indicated times. Calcein AM was obtained from Invitrogen and prepared according to the manufacturer's instructions.

Cancer Metabolic Profiling Assay

LNCaP and C4-2 cells were seeded onto poly-D-lysine (PDL)-coated Seahorse cell culture microplates to facilitate complete and rapid adhesion. Approximately 45 minutes prior to the assay, culture medium was exchanged with a low-buffered RPMI assay medium (Molecular Devices, Sunnyvale, CA). Baseline OCR and ECAR were measured for five minutes using the XF24 instrument to determine basal activity. Compound solutions were then added and measurements were repeated. At the end of each assay, viable cells were counted using a ViCell automated trypan blue counter (Beckman-Coulter, Fullerton, CA).

Myxothiazol, 2-deoxyglucose (2-DG), 2,4-dinitrophenol (2,4 DNP) and Topotecan were obtained from Sigma (St. Louis, MO). All compounds were prepared according to the manufacturers' instructions. For experiments involving Topotecan, cells were exposed for 24 hours prior to XF assay.

Fatty Acid Oxidation Assay

C2C12 myoblasts were seeded at a density of 30,000 cells/well in 24 well Seahorse cell culture microplates containing DMEM. To initiate differentiation, cells were grown for 7 days in medium with 2% FBS.

Palmitic acid and Etomoxir were obtained from Sigma. A 100mM stock solution of Etomoxir was prepared according to the manufacturers' instruction. To prepare palmitic acid, 0.4mM FAF-BSA/KHB solution was dialyzed against KHB to remove calcium from the FAF-BSA (EMD Bioscience, San Diego, CA). Palmitic acid (20mM) was then dissolved in 100% ETOH (Sigma) and an aliquot placed in a 16x100mm glass tube (VWR Scientific, W. Chester, PA). The ethanol was then removed under nitrogen. 0.5ml of dialyzed FAF-BSA was added to the 20mM dried aliquot and the resulting mixture was heated to 37°C for 1 hour with frequent mixing. All XF measurements were done in KHB containing (NaCl at 111 mM, KCl at 4.7mM, MgSO4 at 2.0mM, Na2HPO4 at 1.2mM, MgCl2 at 0.24 mM, Glucose at 5.5mM, L-Carnitine at 0.5mM and bovine insulin at 100nM).

Simultaneous measurements of OCR and ECAR were made on myocytes incubated with either vehicle (FAF-BSA alone), palmitate or palmitate with Etomoxir using a pre-production Seahorse XF instrument.

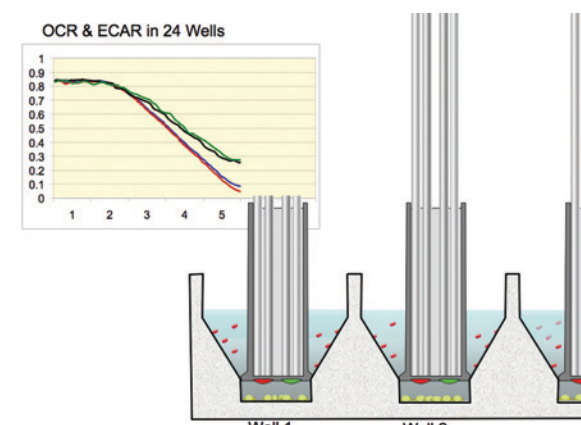
Figure 1. Seahorse XF Instrument Overview

The XF24 Extracellular Flux Analyzer measures the rate of change of analyte concentration (currently oxygen and pH) in media immediately surrounding living cells cultured in a microplate. Changes in the extracellular media are caused by the consumption or production of analytes by the cells.

A unique feature of XF technology is the ability to make accurate and repeatable measurements in as little as five minutes. This is accomplished by temporarily isolating an extremely small media volume (7µL) above the monolayer. Cellular metabolism causes rapid, easily measured changes to this "microenvironment".

During a typical measurement cycle (2-5 minutes) analyte levels are measured every 12 seconds until oxygen concentration drops approximately 10% and pH declines approximately 0.1 unit. Baseline metabolic rates are typically measured twice, and are reported in nmol/min for OCR and mpH/min for ECAR. Compound is added, mixed for 2 minutes, and OCR and ECAR measurements are repeated. As cells shift metabolic pathways, the relationship between OCR and ECAR changes.

Because XF measurements are non-destructive, cells can be profiled over a period of minutes, hours or days.



Results

Figure 2. Real-time Measurement of Changes in Bioenergetic Pathways in LNCaP cells in Response to the Metabolic Modulators.

A. The mitochondrial uncoupler 2,4-dinitrophenol (2,4-DNP) (20µM), glycolysis inhibitor 2-deoxyglucose (100 mM) and mitochondrial complex III inhibitor, myxothiazol (0.1 µM) were injected sequentially into wells containing LNCaP cells. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured before and after compound injection. Mitochondrial uncoupler 2,4-DNP, stimulated OCR and ECAR within ten minutes of administration. After 25 minutes, the elevated ECAR was reduced by glycolysis inhibitor 2-deoxyglucose (2-DG). Addition of myxothiazol 25 minutes after 2-DG administration abolished cellular oxygen consumption.

B. Cellular ATP level (columns) diminished after cells were exposed to Myxothiazol. Cells remain viable under all conditions as shown by Calcein AM stain (green line). 15,000 cells per well were seeded 24 hours prior to the assay.

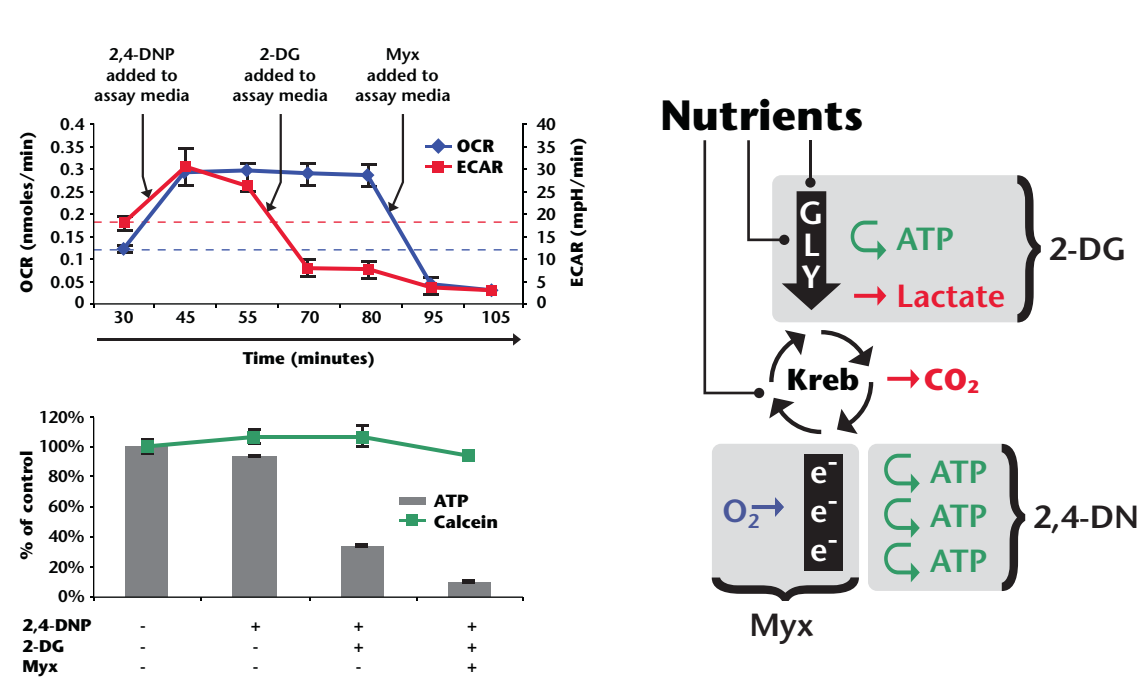


Figure 3. Bioenergetic Pathways and Substrate Utilization (glucose and palmitate oxidation versus anaerobic glycolysis)

Shifts in substrate utilization can be detected by measuring changes in oxygen consumption and acid output by cells. Cells primarily utilizing glucose oxidation produce proportionately more acid per unit oxygen consumed than cells which are engaged in palmitate oxidation. A shift towards palmitate oxidation is reflected as an increased mols of oxygen consumed per mols protons produced. Cells utilizing glycolysis as their primary metabolic pathway will exhibit significantly higher rates of proton production than cells undergoing either oxidative process due to the inefficient ATP yield of glycolysis.

The Seahorse assay is used to measure metabolic profiles of tumor cells and is based on their oxygen consumption (OCR) and extracellular acidification rates (ECAR). When cells are generating ATP by glycolysis they consume no oxygen and produce lactic acid. This low OCR to ECAR ratio is indicative of highly metastatic and invasive cancers. Cells engaged in aerobic metabolism generate higher ratios of OCR to ECAR (~5-8) and are indicative of normal cells and tumors that are non-invasive.

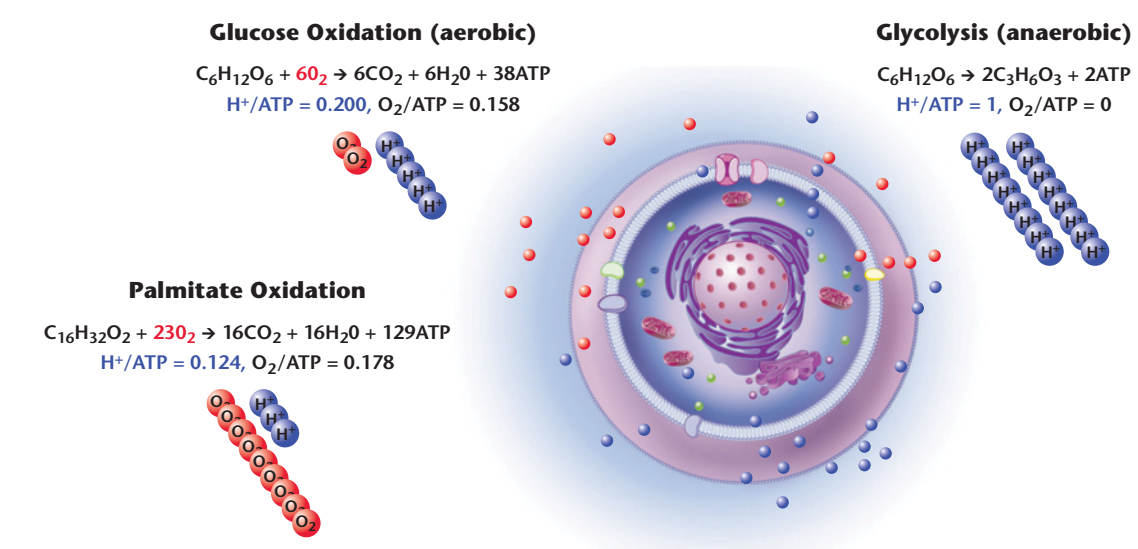


Figure 4. Real-time Measurement of OCR and ECAR

Fatty Acid Oxidation Assay — Cells are seeded in XF24 culture plates, grown overnight, and differentiated into myocytes in 2% FBS media containing 100nM insulin. On the day of the assay, growth media is removed and replaced with KHB with 0.5mM Carnitine + 5.5mM Glucose + 100nM Insulin. Compounds are loaded into the XF24 biocartridge, the biocartridge and plate are placed in the instrument and 2 baseline measurements are made. Palmitate or palmitate and Etomoxir are added to wells and a series of measurements are made following compound addition. The culture plate is removed from the instrument, used for another assay, saved for another later time point, or discarded.

Cancer Cell Bioenergetic Profiling — Cells are seeded in XF24 culture plates and grown overnight in complete RPMI media. On the day of the assay, growth media is removed and replaced with bicarbonate-free RPMI. Compounds are loaded into the XF24 biocartridge, the biocartridge and plate are placed in the instrument and 2 baseline measurements are made. Compounds are added to the wells and a series of measurements are made following compound addition. The culture plate is removed from the instrument, used for another assay, saved for another later time point, or discarded.

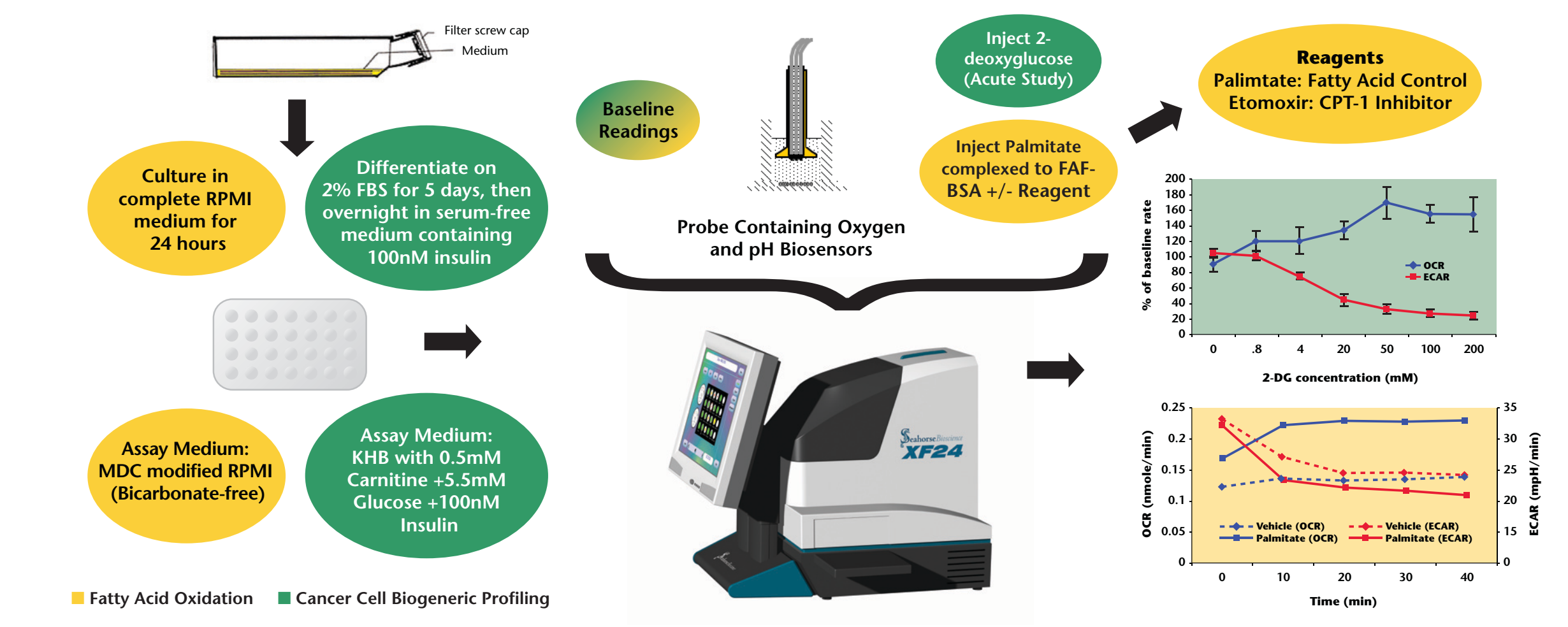


Figure 5. Topotecan (HIF-1α antagonist) Changes the Metabolic Phenotype of C4-2 Cells

C4-2 is a highly metastatic and invasive tumor cell line derived from the non-invasive LNCaP tumor cell line. Basal OCR to ECAR ratios differ substantially between these cell types. A lower OCR/ECAR ratio implies a dependence on glycolysis, and has been observed to correlate with increased metastatic potential.

C4-2 cells were exposed to increasing doses of Topotecan, for 24 hours before XF assays. Topotecan causes rapid degradation of HIF-1α; HIF-1α is known to promote glycolysis over aerobic respiration. As expected, Topotecan decreased ECAR, indicative of reduced glycolysis. Cellular proliferation slowed, but cells remained viable as assessed by Trypan blue staining.

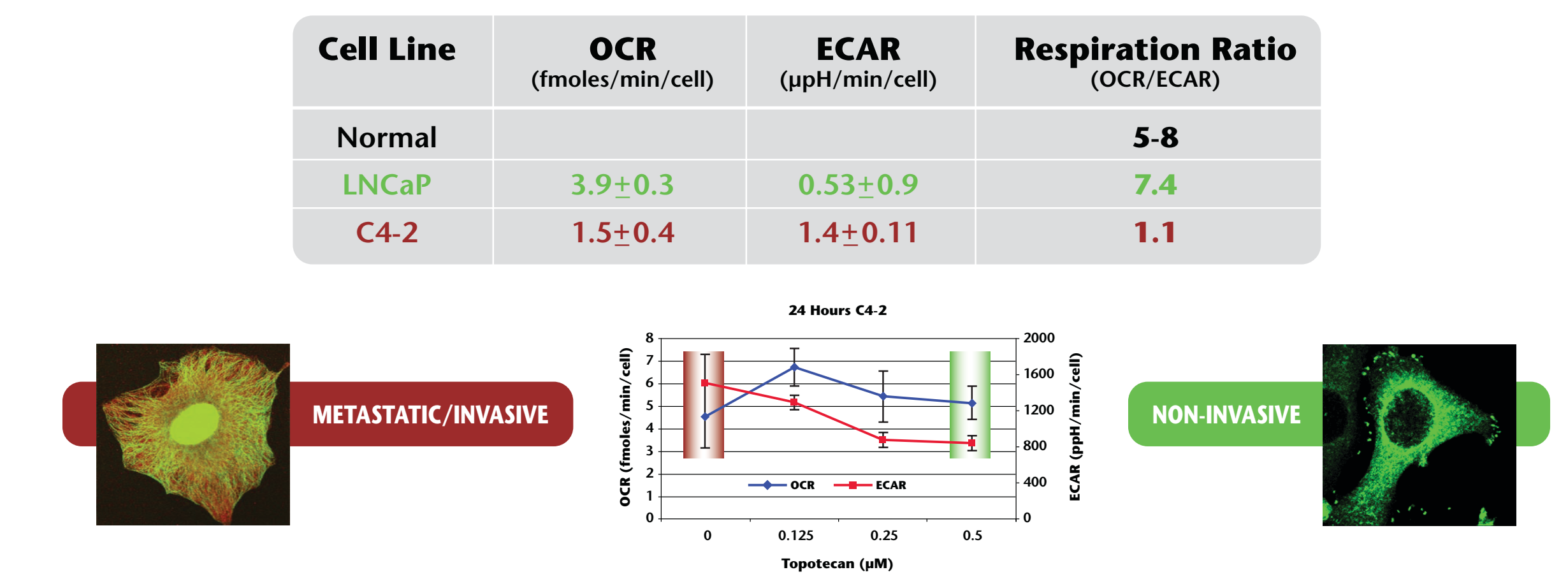
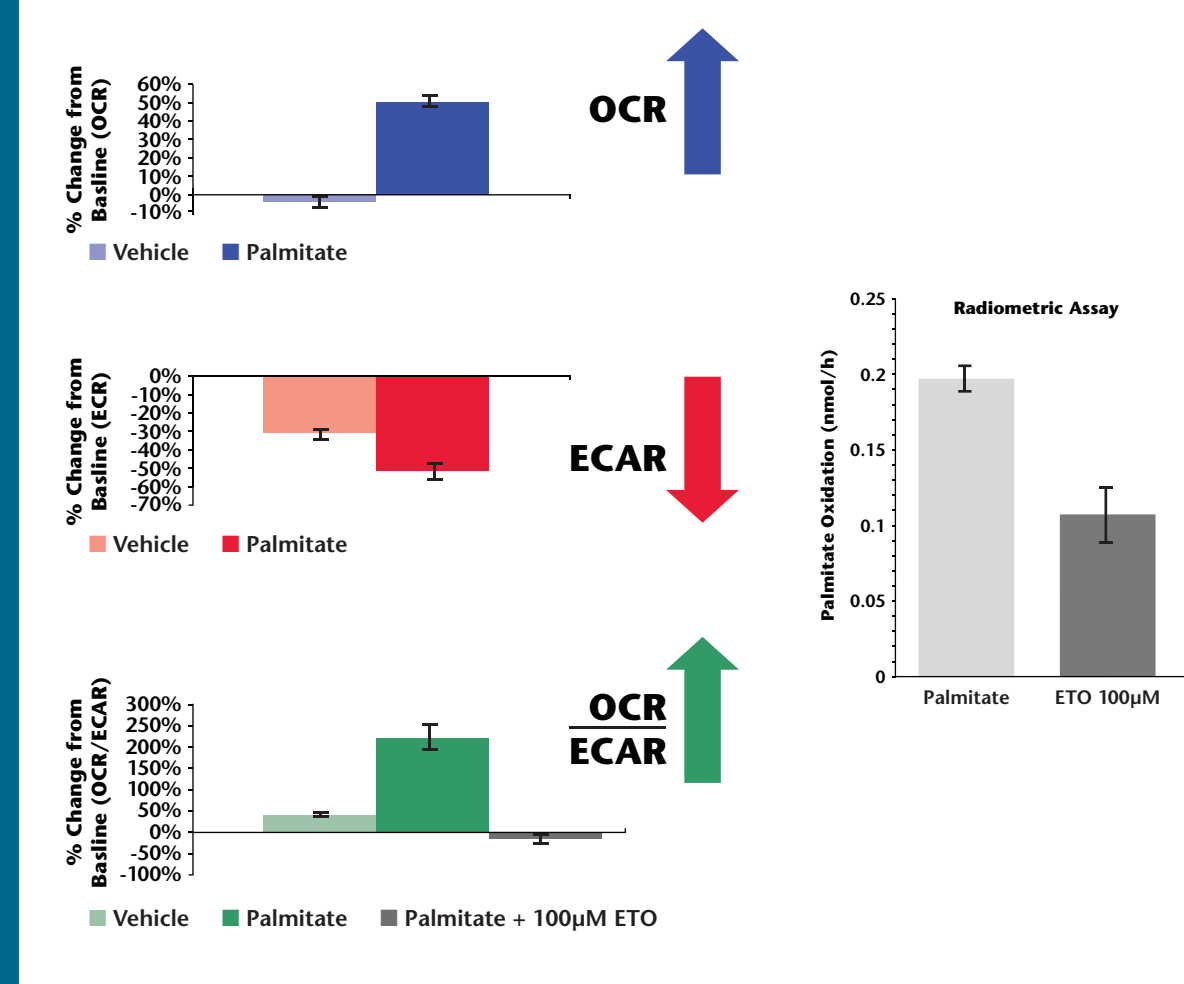


Figure 6. C2C12 Myocytes Challenged with 200µM Palmitate +/- the CPT-1 Inhibitor Etomoxir

C2C12 myocytes were incubated with palmitate or palmitate plus the CPT-1 inhibitor Etomoxir. Measurements of OCR (top left) and ECAR (middle left) were made forty minutes post addition of 100µM palmitate. An increased OCR:ECAR ratio indicates a shift in cellular metabolism to fatty acid oxidation (bottom left). All data were normalized against vehicle prior to calculating percent change from baseline. Comparatively, a radiolabeled ³H-palmitate assays (below) shows decreased fatty acid oxidation.



Summary

- Extracellular Flux assays provide a method for determining metabolic pathways in drug discovery.
- XF assays can differentiate compounds that enhance or inhibit the aberrant glycolytic bias of tumor cells.
- XF assays can identify compounds that modulate fatty acid oxidation.

References

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