

A Novel, Non-Radioactive Method for Measuring Fatty Acid Oxidation and Cellular Metabolism in C2C12 Myocytes

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Abstract

Assays to identify metabolic pathways and quantify cellular bioenergetics are few and not well suited for drug screening. The protocols that exist require the use of radioactivity or expensive low-throughput technologies, such as Mass Spectrometry. Seahorse Bioscience has developed a novel microplate based technology which measures the Extracellular Flux (XF) of molecular oxygen and protons simultaneously. By measuring the rate of oxygen consumption (OCR), a measure of mitochondrial respiration, and extracellular acidification (ECAR), a measure of glycolysis, the XF24 Analyzer provides a means for both quantifying cellular bioenergetics and differentiating metabolic pathways for drug discovery.

We have previously shown that OCR and ECAR are valid measurements for examining cellular metabolism in a variety of cell types including C2C12 myoblasts and cancer lines. To validate these parameters for use in a fatty acid oxidation (FAO) assay we simultaneously measured OCR and ECAR in C2C12 myocytes over a 40 minute time period. Knowing the analytes that should be consumed and excreted during FAO we anticipated that the OCR values should increase and the ECAR values decrease (due to the decreased amount of protons released per ATP in the oxidative state). Using the ratio of OCR to ECAR to represent the relative amount of FAO we found that upon stimulation with palmitate there was a significant increase (>100%) over baseline. To confirm that the increase in the ratio was specific for oxidation of palmitate and not glucose we added the CPT-1 inhibitor Etomoxir which is known to block FAO by preventing the translocation of palmitate into the mitochondria. Etomoxir significantly reduced the OCR:ECAR ratio by greater than 50% compared to palmitate alone. In parallel, we performed these studies using a radiometric assay which measured ³H-water accumulation and found equivalent results. Data is presented in which we challenged C2C12 myocytes with varying doses of the AMP Kinase agonist AICAR (5-Aminoimidazole-4-carboxamide-1-β-ribose Z-ribose). Upon palmitate addition AICAR increased oxygen consumption over control indicating an increase in FAO at the lower doses compared to the highest dose which appeared to inhibit the response. These findings suggest that this approach will have utility in identifying modulators and elucidating mechanisms of metabolic pathways.

Introduction

The use of radioactivity is a mainstay to scientists interested in studying and profiling cellular bioenergetics and metabolism. We have developed a novel real-time, non invasive method for profiling metabolic pathways which does not require the use of radioactivity and allows the cells to be re-used. The XF24 Extracellular Flux Analyzer measures rates of change of two analytes, oxygen and protons, simultaneously and in real-time to give a readout of the metabolic phenotype of cells *in vitro* in a microplate.

Mammalian cells are able to utilize a number of substrates (glucose, fatty acids or amino acids) to produce ATP. Fatty acids, such as palmitate, are consumed through an oxidative process to produce the required intermediates for ATP production. Alternatively, glucose can be consumed through pathways either requiring oxygen in aerobic catabolism or no oxygen such as in glycolysis. In comparison with glucose oxidation, fatty acid oxidation (FAO) produces approximately 30% fewer protons due to lower carbonic acid production and/or glycolysis.

The XF assay measures oxygen consumption rates (OCR) defined as the rate of change by which a cell consumes oxygen and extracellular acidification rates (ECAR) as the rate of change in proton excretion from the cell. If one examines the impact of palmitate oxidation on OCR and ECAR, the theoretical result would be an observed increase in OCR due to the increase in oxygen demand, and the subsequent decrease in ECAR due to the decrease in acid production. The result from glucose oxidation would be different, in aerobic oxidation, OCR and ECAR would increase, while in glycolysis, which does not require any oxygen, OCR would decrease while ECAR would increase.

The purpose of the following study was to utilize the XF24 Analyzer to measure FAO without the use of radioactivity. To determine what fraction of OCR was specific for FAO we used the carnitine palmitoyl transferase-1 inhibitor Etomoxir, and to test if we could enhance FAO we used the agonist AICAR, an AMP mimetic which activates AMP Kinase.

Materials and Methods

Cell Culture and Chemical Reagents

Mouse muscle myoblast cell line C2C12 was obtained from ATCC and cultured in DMEM (ATCC) supplemented with 10% FBS, 2mM GlutMax, 1mM Sodium Pyruvate and 100μg/mL penicillin-Streptomycin (Invitrogen, Carlsbad, CA). Palmitic acid, Etomoxir and 2,4-Dinitrophenol (2,4-DNP) were obtained from Sigma (St. Louis, MO) and prepared according to the manufacturer's instructions. AICAR and fatty acid free bovine serum albumin (FAF-BSA) was obtained from EMD Bioscience (San Diego, CA) and diluted according to manufacturer's instructions in KHB, (containing 111mM NaCl, 4.7mM KCl, 2.0mM MgSO₄, 1.2mM Na₂HPO₄, 0.24 mM MgCl₂, 2.5mM Glucose (unless specified differently), 0.5mM L-Carnitine and 100nM bovine insulin.)

Fatty Acid Oxidation Assay

For differentiation into myocytes, C2C12 myoblasts were seeded at a density of 30,000 cells/well in 24-well Seahorse cell culture microplates containing DMEM maintenance medium with 2% Horse Serum. After 7 days, myocyte formation was visible microscopically.

A 100mM stock solution of Etomoxir was prepared in water. To prepare the palmitic acid-FAF-BSA complex, 0.4mM FAF-BSA/KHB solution was dialyzed against KHB to remove calcium from

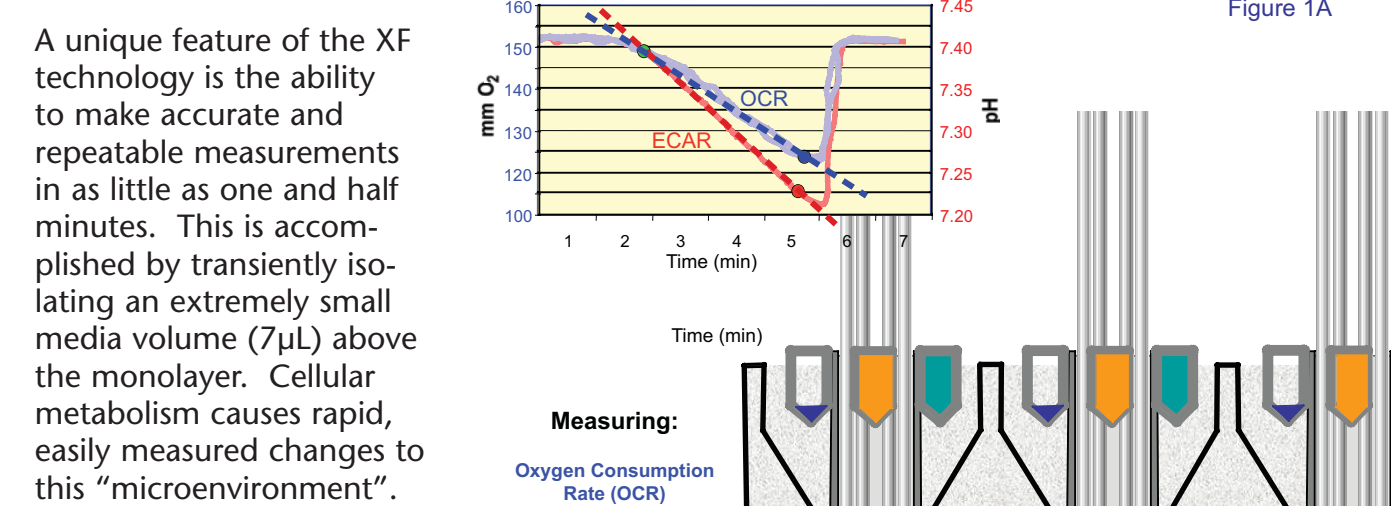
the BSA. Palmitic acid (20mM) was then dissolved in 100% ETOH (Sigma) and a 400μl aliquot placed in a 16x100mm glass tube (VWR Scientific, W. Chester, PA). The ethanol was then removed under nitrogen. 4mls of dialyzed FAF-BSA was added to the 20mM dried aliquot and the resulting mixture was sonicated for 30 minutes with frequent mixing. Once in solution the pH was adjusted by the addition of 5-7μl of a 1M NaOH solution (Sigma). All cellular measurements, except when specified differently, were done in KHB.

Radiometric Fatty Acid Oxidation Assay

C2C12 myoblasts were differentiated into myocytes as previously described. At the start of the assay differentiation media was replaced with KHB buffer, 0.1% FAF-BSA with 5 μCi 3H-Palmitate/1 μL, 100 nM Insulin, 0.5 mM L-Carnitine and 0.15 mM unlabeled Palmitate. Aliquots were taken every thirty minutes after the first hour. At each time interval 75μL was removed from each well and placed into a 96-well plate. After the collection period was complete, 30 μL of a slurry of C-18/methyl alcohol was added to each sample plus 75 μL of chilled TCA (Trichloroacetic Acid). After 30 minutes of incubation on ice, 150 μL aliquot of the sample was transferred to a 96-well Multiscreen™ HV filter plate and filtered into a new 96 well plate. The filtrate was then transferred to scintillation vials containing scintillation fluid. The samples were rested for 24 hours prior to counting.

Figure 1. Seahorse XF24 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 (Figure 1A). Therefore, a sensitive measurement of the media can be used to determine rates of cellular metabolism with great precision and in a non-invasive, label-free manner.

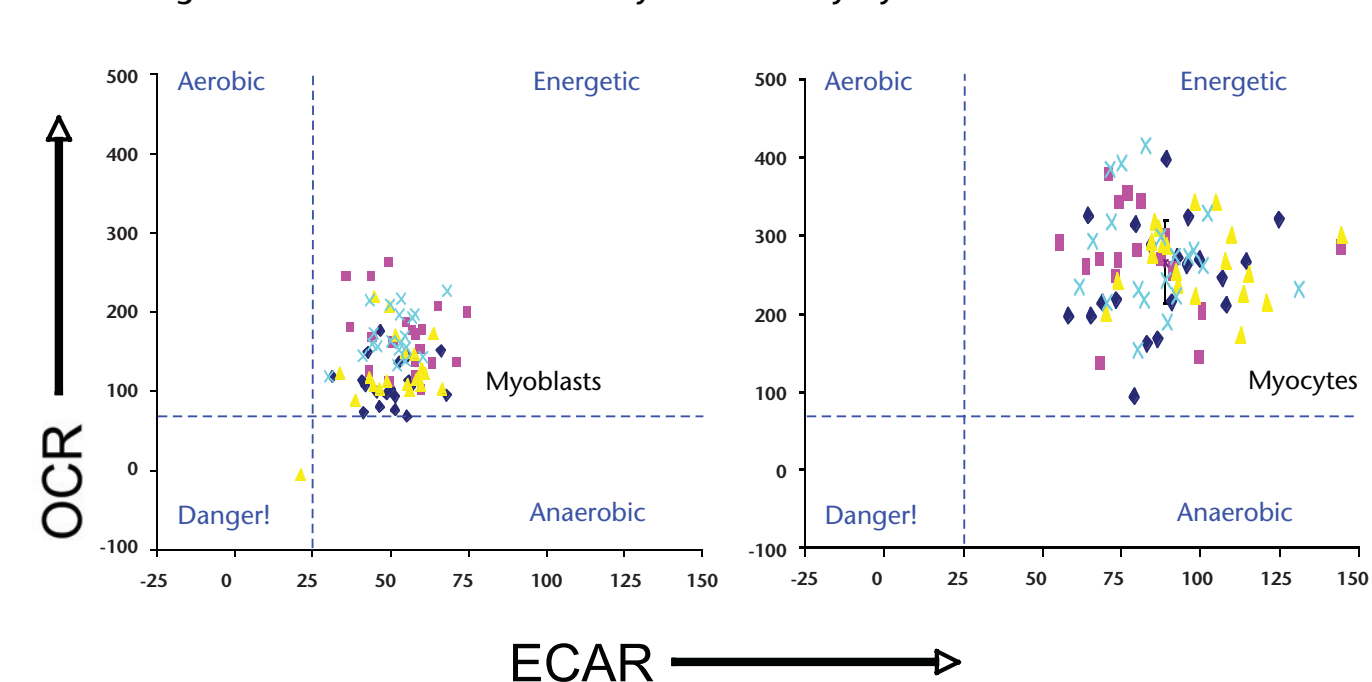


A unique feature of the XF technology is the ability to make accurate and repeatable measurements in as little as one and half minutes. This is accomplished by transiently isolating an extremely small media volume (7μL) above the monolayer. Cellular metabolism causes rapid, easily measured changes to this "microenvironment". During a typical measurement cycle (1.5-4 minutes) analyte levels are measured every 22 seconds until oxygen concentration drops approximately 10-30% and pH declines approximately 0.1-0.6 units. The measurement is performed using fluorescent biosensors embedded in a sterile disposable cartridge that is placed into an XF 24 cell culture microplate. Baseline metabolic rates are typically measured three times, and are reported in nmol/min for OCR and mpH/min for ECAR. Compound is added, mixed for 2 minutes, and OCR and ECAR 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.

Figure 1B shows the sensitivity of the XF24 assay when comparing the bioenergetic profile between two cell types, in this case comparing C2C12 myoblasts versus myocytes. The four quadrants depict the relative metabolic phenotype of cells. Cells which consume relatively high rates of oxygen and release relatively low levels of protons are classified as aerobic; this is an example of a cell line oxidizing fats rather than glucose. Cells in the energetic quadrant are characterized by the increased consumption of both glucose and oxygen to produce ATP, and, as a result, release relatively higher levels of protons into the surrounding medium. Cells in the anaerobic quadrant utilize glucose for glycolysis releasing relatively high amounts of protons but do not consume any oxygen. Cells found in the "danger zone", are lacking respiratory ability and are in poor health.

Figure 1B. Basal rates for C2C12 Myoblasts vs. Myocytes



Results

Figure 2. 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 proton output by cells. Cells primarily utilizing glucose oxidation produce proportionately more protons per unit oxygen consumed than cells which are engaged in palmitate oxidation. A shift towards palmitate oxidation is reflected as an increased moles of oxygen consumed per moles protons produced. Cells utilizing glycolysis as their primary metabolic pathway will exhibit significantly higher rates of proton production than cells undergoing either oxidative process.

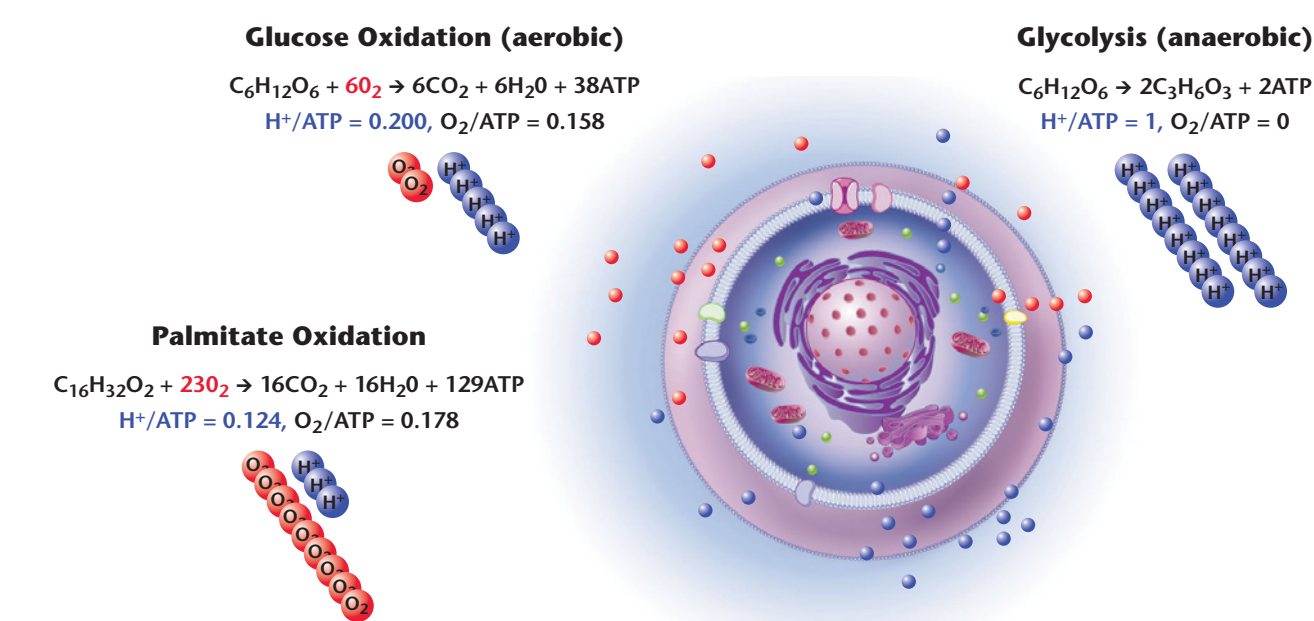


Figure 3. Palmitate and AICAR Induction of Fatty Acid Oxidation in C2C12 Myocytes

C2C12 myocyte OCR and ECAR baseline readings were measured 3 times with the XF24 prior to injection of either 150μM palmitate complexed to FAF-BSA (●) or FAF-BSA alone (○). Two further measurements were taken after which 50μM of the CPT-1 inhibitor, Etomoxir was injected to confirm specificity of the assay. The rise in OCR (Figure 3A, % from Baseline) upon palmitate addition (●) signifies a shift in cellular metabolism to fatty acid oxidation, this observation is confirmed by the decrease in OCR with the addition of Etomoxir. The slower ECAR (Figure 3B, % from Baseline) rate observed with palmitate (●) is potentially due to a decrease in carbonic acid and/or glycolysis compared to the FAF-BSA (○) sample where glucose is the primary substrate rather than palmitate.

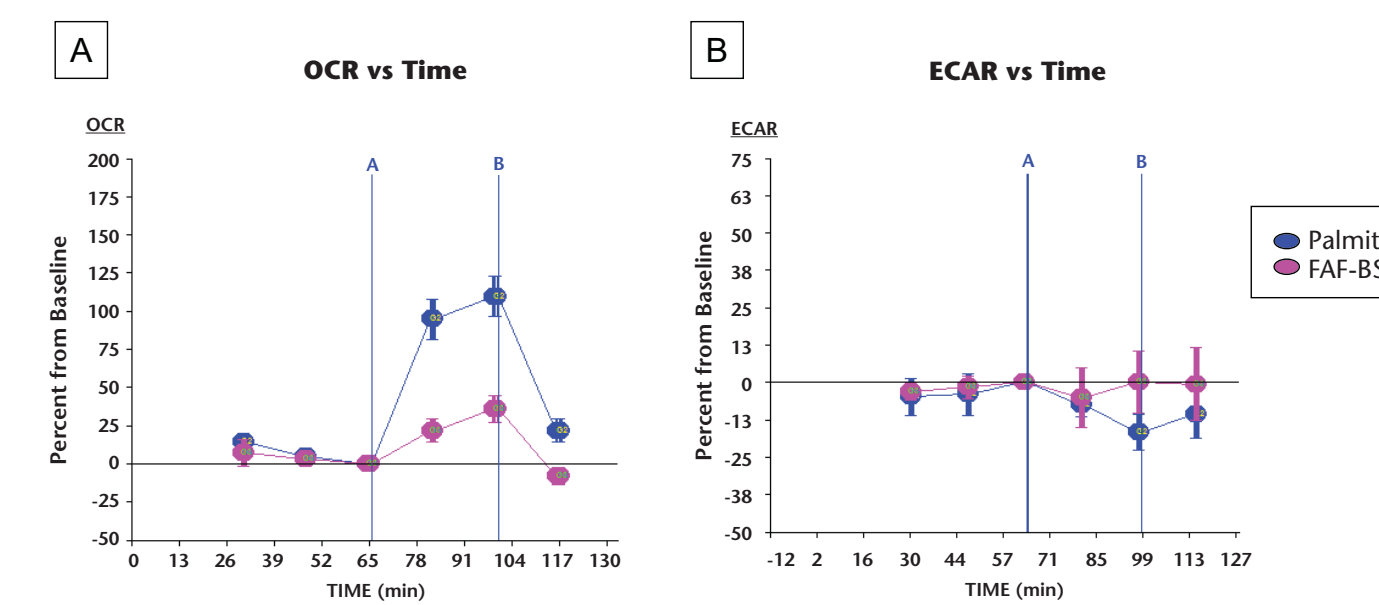


Figure 4. Comparison of XF24 and Radiometric Fatty Acid Oxidation Assays

Results from a parallel radiolabeled substrate assay (³H-palmitate) utilizing the same conditions as the XF24 assay produced similar results for both the induction and inhibition of FAO.

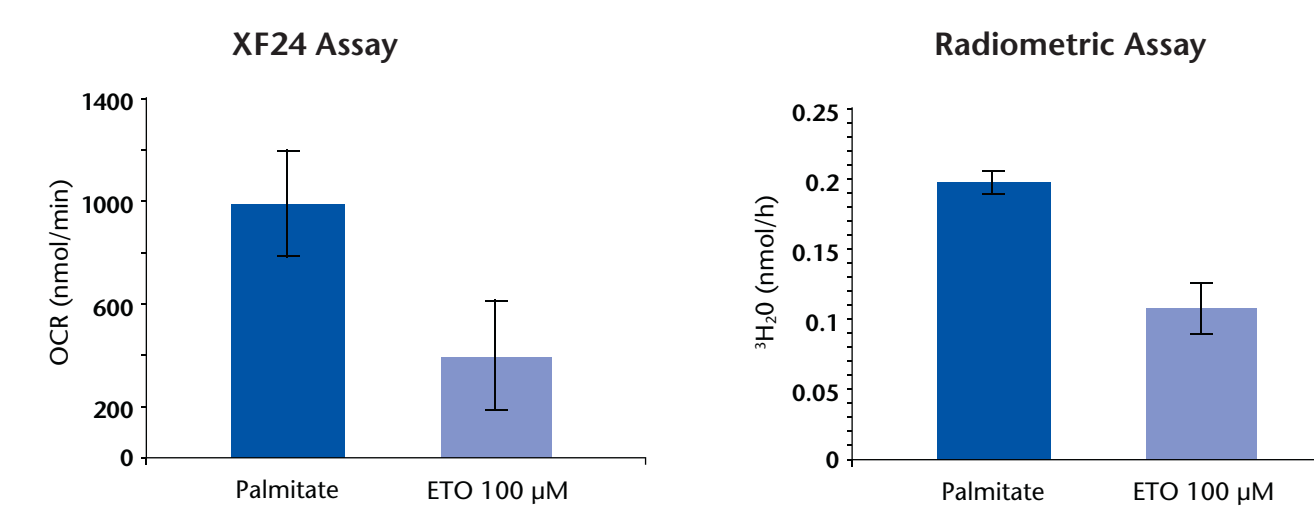
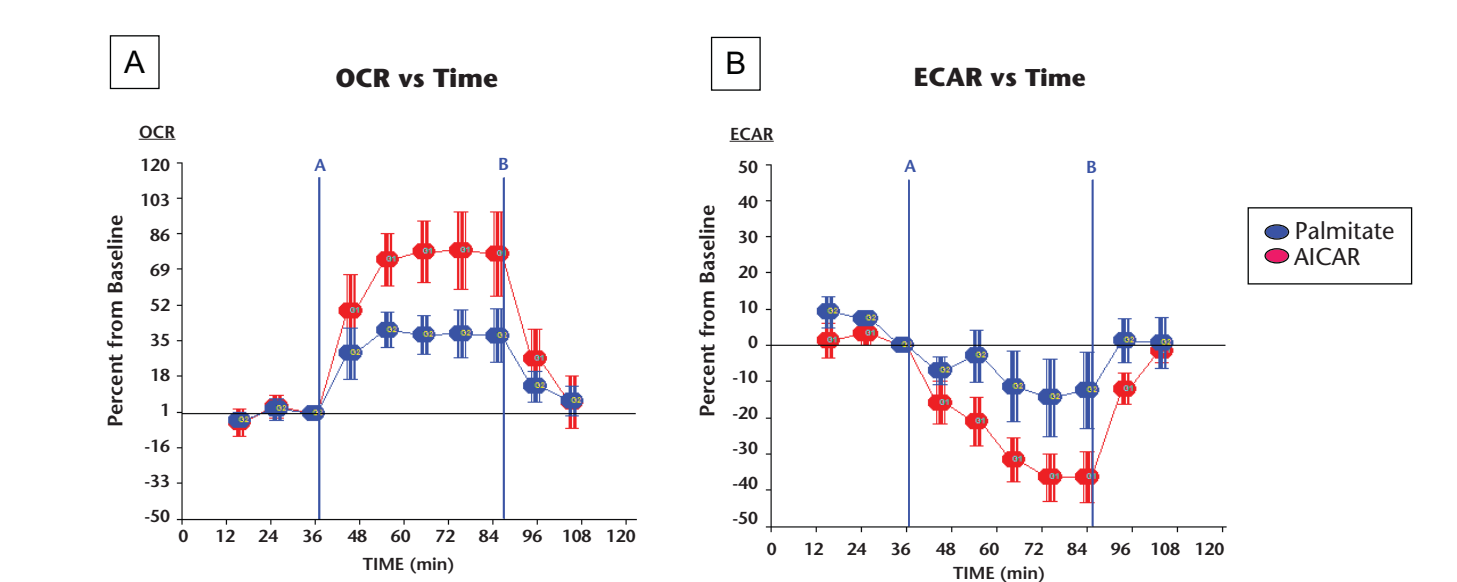


Figure 5. Fatty Acid Oxidation after Exposure to AICAR (5-Aminoimidazole-4-Carboxamide-1-β-Riboside Z-Riboside). Figure 5A and 5B



C2C12 myocytes were preincubated in KHB containing either 0mM AICAR (●) or 0.5mM AICAR (○) for two hours. Prior to the 2 hour mark, 3 baseline measurements were taken after which 150μM palmitate complexed to FAF-BSA was injected into each of the wells.

Subsequently, five additional OCR (Figure 5A) and ECAR (Figure 5B) measurements were taken (77.5min) upon which 50μM of Etomoxir was injected (rate 9) to confirm the OCR specificity for FAO. The observed rise in OCR (Figure 5A, % from Baseline) is indicative of an oxidative response to the palmitate substrate, which is enhanced under AICAR stimulation. The ECAR response (Figure 5B, % from Baseline) shows comparatively lower rates in the AICAR treated versus the palmitate only sample. This observation is consistent with our current view on the biochemistry of the FAO pathway, which is that the higher rate of oxidation in the presence of fatty acids results in a lower production rate of protons.

Figure 5C shows a partial dose curve using 0mM (●), 0.25mM (○), 0.5mM (●) and 1mM (●) AICAR. The AICAR induced a dose dependent increase from low to high concentrations. These data show that this line of C2C12 myocytes is more responsive to the lower doses of the AMPK mimetic rather than the higher (1mM) dose which appeared to inhibit the FAO pathway.

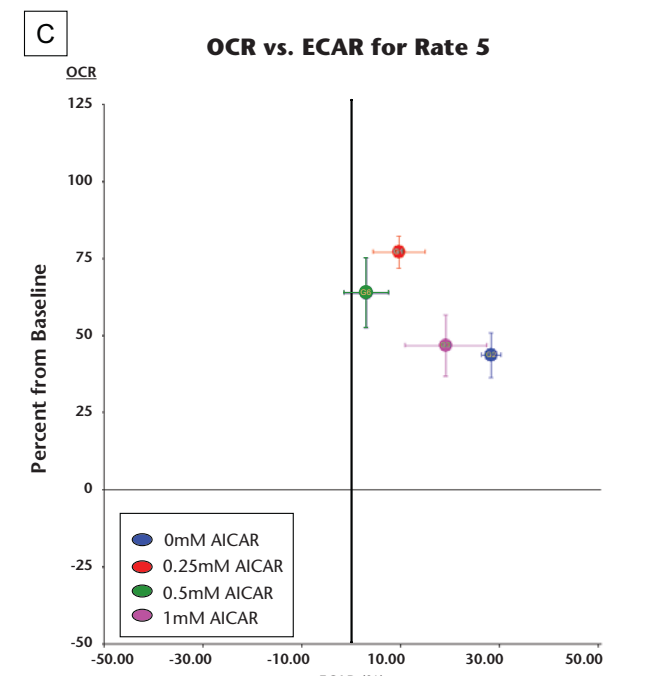
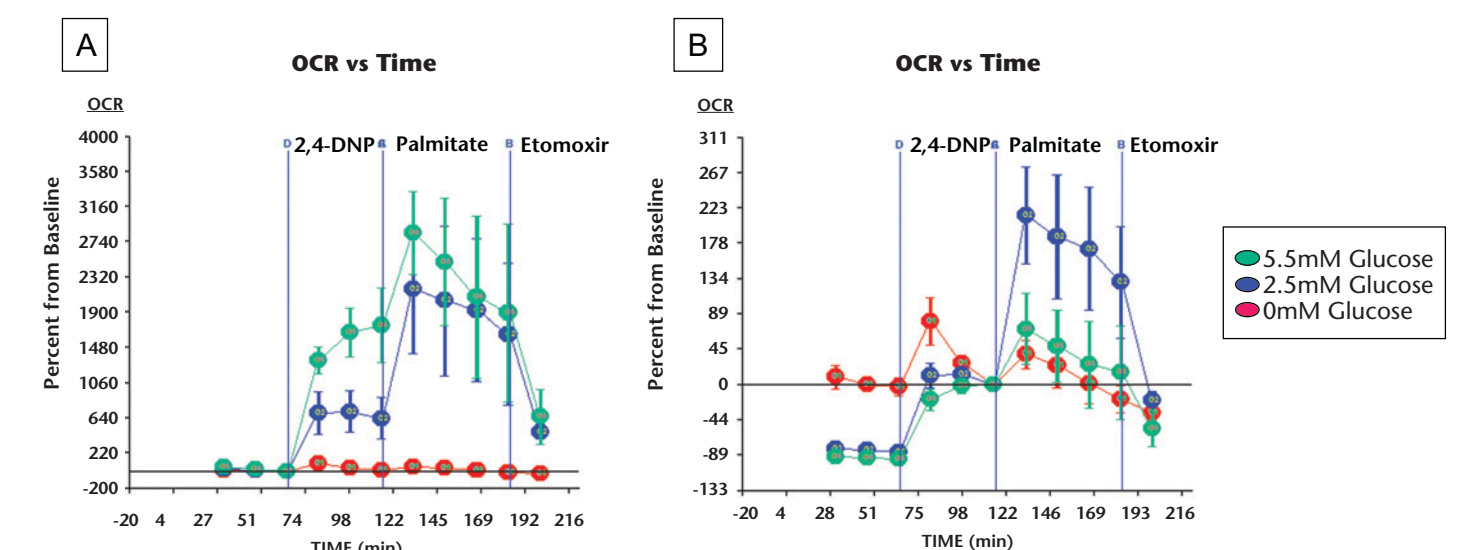


Figure 6. Impact of Palmitate and Three Concentrations of Glucose on 2,4-DNP (2,4-Dinitrophenol) Stimulation in C2C12 Myocytes.

KHB containing either 0 (●), 2.5 (○) or 5.5mM (●) glucose along with 0.5mM carnitine and 100nM insulin was added to appropriate wells. Figure 6A shows the increase in OCR (% from Baseline), in a glucose dose dependent manner, after injection of the mitochondrial uncoupler, 2,4-DNP. In Figure 6B the percent from baseline is calculated just prior to 150μM palmitate injection. The OCR response was enhanced after the injection, with a switch in the maximal OCR response (from 5.5 to 2.5mM glucose) indicating a shift in preference in glucose concentration when the FAO pathway is activated.



Summary

The XF Analyzer is able to detect and measure small differences in metabolism allowing the user to observe changes in substrate utilization.

Using agonists and antagonists of fatty acid oxidation, one can determine specificity and measure the changes in kinetics of a specific metabolic pathway.

In conclusion, we have developed a non-radioactive method for performing fatty acid oxidation and metabolic profiling assays using the Seahorse XF24 Extracellular Flux Analyzer. XF assays will enable investigators to use conventional drug screening approaches rather than relying primarily on radioactive and animal studies, to discover and validate drugs to aid in the treatment of obesity and diabetes.