Fatty Acid Oxidation in C2C12 Myocytes and Agonist Response to an ACC Inhibitor and Metformin

Research Area
Metabolic Disease

Application
Substrate Utilization:How to measure fatty acid oxidation without using radioactivity

Cell Type
Muscle Cell Line (C2C12 myotubes)

Work Of

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Tool Box
- palmitate
- etomoxir

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Substrate Utilization

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The Seahorse Extracellular Flux (XF) assay measures the effect of compounds on Fatty Acid Oxidation (FAO) in mammalian cells.This fast, sensitive, and label-free assay detects the increased aerobic cell metabolism associated with FAO. XF assays provide comparable performance to radiometric methods, with better throughput and without the use of radioactive materials.

Identifying FAO Agonists

Using the XF24 Analyzer, the impact of two agonists on FAO – Metformin and the ACC inhibitor CP-640186 – were determined in the murine skeletal muscle cell line C2C12.

C2C12 myocytes were differentiated for 6 days, then preincubated for 24 hours with either 10µM ACC inhibitor or 1mM Metformin in 24-well microplates.

Using the XF24 Analyzer, three baseline measurements of Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) were made at 8-minute intervals to establish the basal metabolic rates and pathways.

150µM palmitate complexed to fatty acid free BSA was then injected into three replicate wells of treated and untreated C2C12 myocytes, and readings were taken at 8-minute intervals to detect a shift toward aerobic respiration, indicative of FAO.

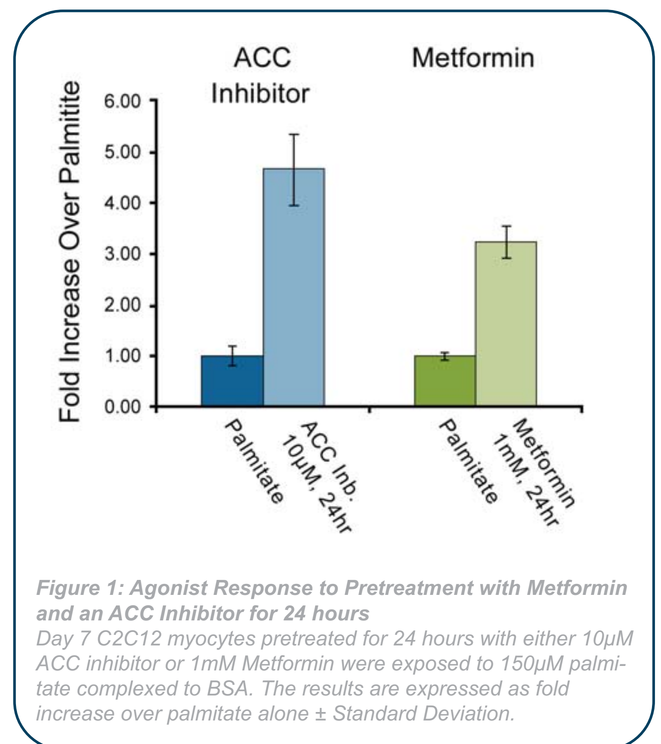

Figure 1: Agonist Response to Pretreatment with Metformin and an ACC Inhibitor for 24 hours
Day 7 C2C12 myocytes pretreated for 24 hours with either 10µM ACC inhibitor or 1mM Metformin were exposed to 150µM palmitate complexed to BSA. The results are expressed as fold increase over palmitate alone ± Standard Deviation.

Figure 1 shows that overnight treatment with 10µM ACC inhibitor or 1mM Metformin caused 4.6 fold and 3.2 fold increases in OCR, respectively.

To confirm that the rise in OCR was due to palmitate oxidation versus glucose, we added 50µM etomoxir, a CPT-1 inhibitor. After the addition of etomoxir, the OCR values decreased confirming specificity for FAO (data not shown).

The protocol for the FAO assay in Figure 1 can be found in Wu et al. *Am J Physiol Cell Physiol* **292:C125-C136**.

Materials & Methods

Cell Culture.
Mouse muscle myoblast cell line C2C12 was obtained from ATCC and cultured in DMEM supplemented with 10% FBS, 1mM sodium pyruvate and 100 μg/mL penicillin-Streptomycin. Cells were seeded at 10,000 cells per well in 24-well XF plates and incubated for 48 hours in 37°C/10% CO2 incubator. For differentiation, growth media was replaced with differentiation media (2% horse serum in DMEM). Well differentiated myocytes were observed at day 3 and thereafter. Day 4 through day 6 myocytes were used for all assays.

Test Compounds.
CP-640186, an ACC inhibitor, was obtained from Pfizer Inc. (Groton, CT). Palmitic acid, metformin and Etomoxir were obtained from Sigma (St. Louis, MO).

References

Watanabe, W et al. (2006). Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. *Nature* **439:484-489**.

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**

Sridharan, V et al. (2007). The prolyl hydroxylase oxygen-sensing pathway is cytoprotective and allows maintenance of mitochondrial membrane potential during metabolic inhibition. *Am J Physiol Cell Physiol* **292:C719-C728**.

A Physical Measurement

FAO is tightly linked to the mitochondrial respiratory chain and requires molecular oxygen to produce ATP. The XF24 Analyzer measures and compares the rates of oxygen consumption (OCR) – a measure of aerobic metabolism, and extracellular acidification (ECAR) – a measure of glycolysis, both key indicators of cellular energetics.

The XF24 instrument uses optical biosensors to measure the rate of change in the oxygen content and pH of the media in proximity to cells and is therefore entirely non-invasive.

In contrast to traditional radiometric assays, XF assays do not require radioactive materials, strong acids and alkalines, long assay times, or scintillation counting steps. Cells may be measured multiple times to profile kinetics of FAO induction, and may be re-used after testing.

A variety of cells, both primary and immortalized, derived from skeletal muscle, liver and adipose have been used successfully to measure the effect of compounds on FAO.

Comparison to Predicates

Radiometric FAO assays measure the quantity of radiolabeled CO_2 produced by the metabolism of fatty acids such as palmitate or oleate containing C^{14}, or the quantity of radioactive water produced by the metabolism of these substrates labeled with H^3.

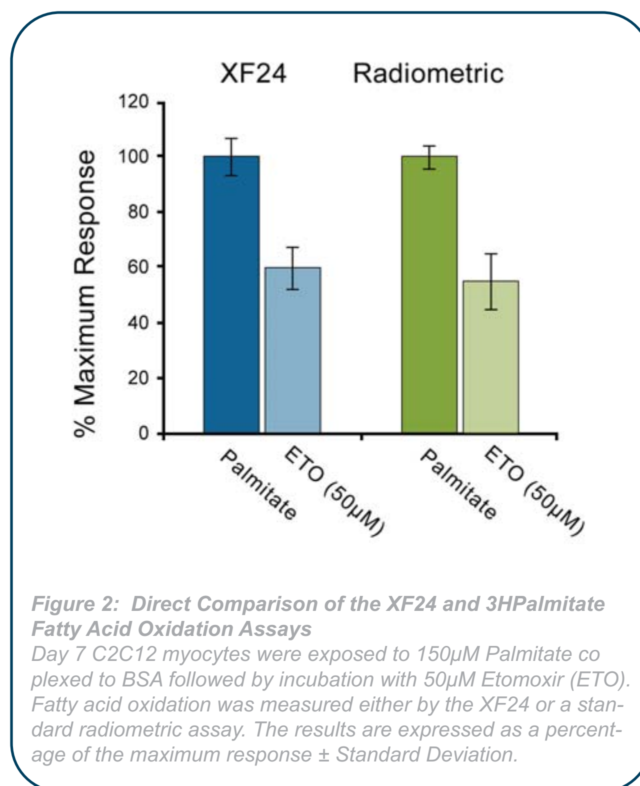

Figure 2: Direct Comparison of the XF24 and 3HPalmitate Fatty Acid Oxidation Assays
Day 7 C2C12 myocytes were exposed to 150μM Palmitate co plexed to BSA followed by incubation with 50μM Etomoxir (ETO). Fatty acid oxidation was measured either by the XF24 or a standard radiometric assay. The results are expressed as a percentage of the maximum response ± Standard Deviation.

To compare the data quality and relative sensitivities of XF and radiometric assays, we measured the inhibition of FAO with the CPT-1 inhibitor, etomoxir. Parallel C2C12 myocyte cultures, exposed to 150μM palmitate, were treated 60 minutes later with 50μM of etomoxir (Figure 2). FAO was assessed using the XF or standard radiometric assay. The kinetic XF24 rate and the single endpoint radiometric data were made comparable by normalizing to the percent maximal response of palmitate.

Both XF24 and radiometric assays observed a significant and comparable inhibition of FAO in the presence of etomoxir. The data quality was very comparable between the two methods.