Direct Comparison of Radiometric versus XF24 Flux Assays to Measure Substrate Utilization

Research Area
Obesity, Diabetes, Metabolic Syndrome

Application
Substrate Utilization: Switch from glucose to fatty acid oxidation in L6 myoblast cells

Cell Type
Muscle cell line from rat thigh muscle (L6 myoblasts)

Work Of

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Tool Box:
- metformin
- palmitate
- etomoxir

Keywords:
- mitochondria
- radiometric assay
- L6 myoblasts
- metformin
- fatty acid oxidation
- oxygen consumption rate (OCR)

Substrate Utilization

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With accuracies comparable to those obtained with radiometric assays, the Seahorse XF24 Analyzer measures substrate utilization via extracellular fluxes of metabolites such as oxygen and protons to estimate metabolic fluxes due to mitochondrial respiration and glycolysis, respectively. The advantages of the XF24 assays are that they are non-radiometric, higher throughput, reduced samples sizes and significantly improved kinetic resolution with rates that are typically obtained in minutes.

Background
Traditionally, metabolic fluxes associated with energy metabolism are measured as the time-dependent accumulation of radionuclide in a metabolite that is derived from a labeled substrate. For example, accumulation of ^{3}HOH water from ^{3}H-labeled palmitate is used as a measure of fatty acid utilization in cells. Radiometric assays are highly specific, but they are also laborious, can lack precision and often require large quantities of tissue or cells. Perhaps even more significant is the limited kinetic resolution as rates are often measured over an hour or longer, unless high levels of radioactivity are used. Extracellular flux measurements of metabolites such as oxygen and total acid can provide estimates of intracellular fluxes with accuracies comparable to those obtained with the radiometric assays (Wiley, 2002). The potential advantages to the extracellular flux assays are higher throughput, reduced sample sizes and high kinetic resolution - with rates in the order of minutes.

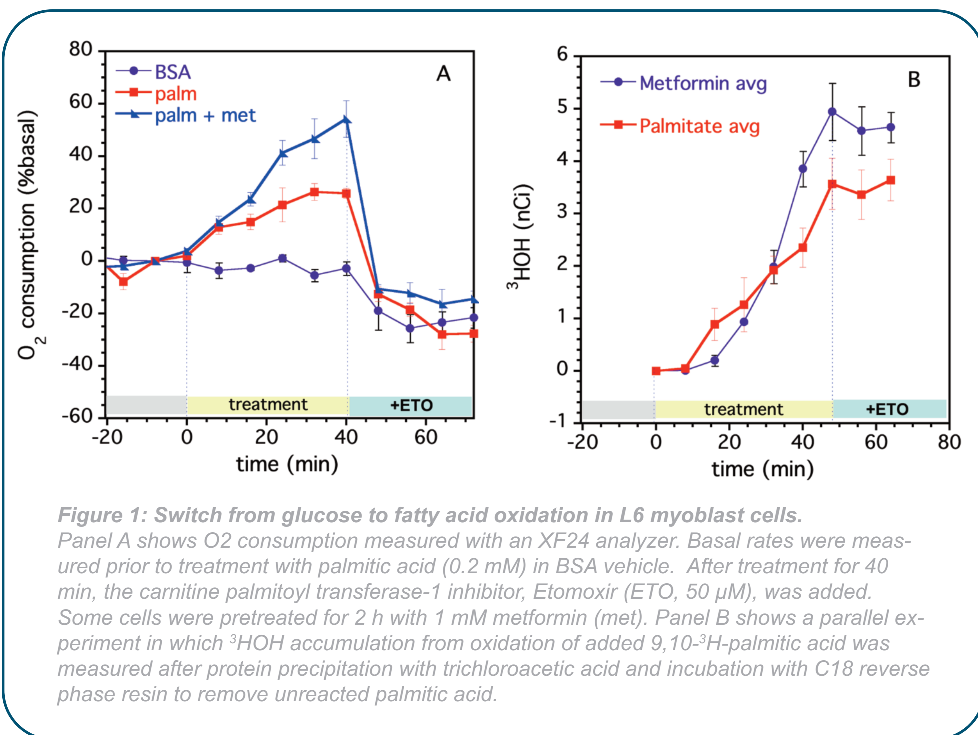

Figure 1: Switch from glucose to fatty acid oxidation in L6 myoblast cells. Panel A shows O2 consumption measured with an XF24 analyzer. Basal rates were measured prior to treatment with palmitic acid (0.2 mM) in BSA vehicle. After treatment for 40 min, the carnitine palmitoyl transferase-1 inhibitor, Etomoxir (ETO, 50 µM), was added. Some cells were pretreated for 2 h with 1 mM metformin (met). Panel B shows a parallel experiment in which ^{3}HOH accumulation from oxidation of added 9,10-^{3}H-palmitic acid was measured after protein precipitation with trichloroacetic acid and incubation with C18 reverse phase resin to remove unreacted palmitic acid.

Materials & Methods

Cell Culture.
Cell Culture: Rat thigh muscle cell line L6 was obtained from ATCC and cultured in DMEM supplemented with 10% FBS and 100 µg/mL Penicillin-Streptomycin. Cells were seeded at 4 x 10^5 cells per well in 24-well XF plates and incubated overnight in The convention for scientists would be 37C at 5% CO_2 incubator. The assay running buffer contained 5.5 mM glucose.

Test Compounds.
Metformin, palmitic acid and etomoxir were obtained from Sigma (St. Louis, MO).

References

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XF24 versus Radiometric Assays

The conceptual basis for an XF24 substrate assay is that a change in the media substrate will induce changes in extracellular fluxes that reflect the differential amount of oxygen used to oxidize a given substrate. Figure 1 compares an XF24 and radiometric assay by looking at the response of L6 myoblasts, untreated or treated with metformin, to exogenous palmitate in both assay formats performed in parallel. In Figure 1A, note that the increase in oxygen consumption rate (OCR, red line) upon addition of palmitate (first dashed vertical line) follows similar kinetics as ^{3}HOH accumulation from ^{3}H-labeled palmitate (Figure 1B, red line). Both assays clearly show a rapid switch from glucose utilization to fatty acid oxidation. However, a basic difference is that the XF assay is measuring the oxygen consumption rate in nearly real time and it increases as the cells switch to fatty acid oxidation, which requires more oxygen per ATP than does glucose. During the radiometric assay, aliquots were taken at the indicated time points but the accumulated ^{3}HOH was measured separately, hours later, after processing. The radiometric assay is highly specific as the ^{3}HOH arises only from oxidation of ^{3}H-labeled palmitate. Although OCR is a less specific measure of palmitate oxidation, using modulators like etomoxir (discussed below) allows one to assess the specificity of the OCR changes, which underscores its ability to measure total oxidative metabolism from not only fatty acids but other substrates such as glucose and amino acids.

In Figure 1A, metformin, a marketed drug that is used to treat type II diabetics is known to upregulate AMPK and as shown here can enhance oxidation of the fatty acid palmitate (Figure 1A blue line, Figure 1B purple line). In fact, if one uses the drug injection ports on the XF24 to inject glucose or palmitate into wells containing either metformin treated or untreated C2C12 myotubes, one can measure in real-time the shift of substrate preference away from glucose oxidation to palmitate (manuscript in preparation).

Another feature of the XF assay not shown here is the corresponding effect of palmitate addition on glycolysis as measured by ECAR. The simultaneous measurement of ECAR and OCR reveals the dynamic interplay between glycolysis and oxidative metabolism as their equilibrium is invariably altered by changes to either or both pathways. A second assay using ^{14}C-labeled glucose would be needed to follow the same interplay with a radiometric assay.

The difference between rate measurements of XF versus accumulation of radioactive metabolite is, perhaps, most easily observed in Figure 1 by looking at the effect of etomoxir addition on the palmitate responses of the L6 cells. Etomoxir is a fatty acid oxidation inhibitor that specifically blocks the carnitine palmitoyl transferase I (CPT-1) that is required to transport fatty acids into the mitochondria. When etomoxir is added (second dashed vertical line in Figure 1A) during the XF assay a decrease in the rate of OCR can be seen. By contrast, the radiometric assay shows a plateau in the accumulation of ^{3}HOH (Figure 1B).

In this report, we have compared and contrasted the XF24 and radiometric assay formats. The XF24 data presented here is an example of the type of assay that can be performed in secondary and tertiary screens to determine the specificity and selectivity as well as mechanism of action of identified compounds.

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