ELSEVIER

Advances in measuring cellular bioenergetics using extracellular flux

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Cell-based assays have become a favored format for drug discovery because living cells have relevant biological complexity and can be highly multiplexed to screen for drugs and their mechanisms. In response to a changing extracellular environment, disease and/or drug exposure, cells remodel bioenergetic pathways in a matter of minutes to drive phenotypic changes associated with these perturbations. By measuring the extracellular flux (XF), that is the changes in oxygen and proton concentrations in the media surrounding cells, one can simultaneously determine their relative state of aerobic and glycolytic metabolism, respectively. In addition, XF is time-resolved and non-invasive, making it an attractive format for studying drug effects *in vitro*.

Introduction

Scientists strive to optimize two opposing aspects of drug discovery; the need to maximize compound throughput and the desire to model human disease, which is inherently low throughput. The compromise that works for many is cell-based assays, because living cells have sufficient biological complexity and their autonomous nature allows them to be multiplexed to screen for drugs and identify their mechanisms of action. This has led to widespread adoption of cell-based assays in drug discovery; however, since the majority of these assay formats depend on intracellular labels, their usefulness is limited as they are often not physiologically relevant, generate only a single time point, are susceptible to artifact and are almost always destructive.

By contrast, by measuring the extracellular flux (XF) (see Glossary) or flow of nutrients, organic molecules and elements in the media, one can directly determine the bioenergetic state and physiology of cells without adding labels or touching them in anyway [1–15]. This has several advantages: first and foremost, the analytes moving in and out of the cell are sensitive indicators of changes in cellular physiology or pathophysiology; second, since XF is label free and non-destructive; the cells can be queried repeatedly over time to generate kinetic data, while retaining the possibility of using the same cells in other applications or in chronic studies.

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Abnormal cellular bioenergetics is always associated with, and often central to, the pathophysiology of diseases such as obesity, diabetes, cancer, neurodegeneration and cardiomyopathy [1–5,16,17]. In essence, all phenotypic changes are driven by underlying adjustments of cellular bioenergetics which often, if not always, have unique profiles. These unique and quantitative signatures identify and discriminate changes in cell physiology because of drugs, agonists, antagonists, and so on.

Measurements of cellular respiration and acidification are among the most fundamental experimental techniques that have formed the basis of our understanding of bioenergetics [5–12]. At the practical level, these types of measurements are also fundamental to process biotechnology and control of cell culture [13–15]. Although these measurements of extracellular flux continue to make important contributions to both basic and applied cell biology, the techniques used have changed little in the past few decades and they continue to be laborious and/or poorly designed for the modern laboratory and workflow. In this review, we will discuss advances in the format and design of assays that overcome many of these deficiencies making this approach more amenable to drug discovery [1–4].

Quantifying bioenergetics using XF

Cellular metabolism is the process of substrate uptake (oxygen, glucose, fatty acids and so on) and energy conversion through a

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GLOSSARY

AMPK AMP-activated protein kinase
ATP adenosine triphosphate
cAMP cyclic adenosine monophosphate
CPT-1 carnitine palmitoyl transferase I
2-DG 2-deoxyglucose
ECAR extracellular acidification rate
FCCP carbonylcyanide-p-trifluoromethoxyphenylhydrazone
GAPDH glyceraldehyde 3-phosphate dehydrogenase
OCR oxygen consumption rate
PKC protein kinase C
XF extracellular flux

series of enzymatically controlled oxidation/reduction reactions. These reactions are executed through a series of intracellular biochemical processes (glycolysis, Kreb Cycle, Electron transport and oxidative phosphorylation) resulting in the production of ATP (see Glossary), the release of heat and chemical byproducts (lactate and CO_2) into the extracellular environment.

Valuable insight into the physiological state of living cells and the changes of those cells in response to experimental intervention has been gained through monitoring, independently, the rate of oxygen consumption, using Clark and Clark-type electrodes [8,9,18,19], or the rate of extracellular acidification, using the Cytosensor® microphysiometer [20,21]. Under typical *in vitro* cell culture conditions, the rate of oxygen consumption (OCR) (see Glossary) is an indicator of mitochondrial respiration and the rate of acid efflux (ECAR) (see Glossary) is predominantly a measure of lactic acid formed during glycolytic energy metabolism.

Although the conventional microphysiometer measured only extracellular acidification, two groups have modified the instrument with the inclusion of additional sensors for oxygen and lactate [22,23]. The key feature of the technology that enables flux measurements is a small, ~10 μl sensor chamber in which the cells are maintained as they are perfused with buffer. During a rate measurement, the flow is momentarily stopped to cause a significant change in extracellular metabolites as the cells respire within the small volume. The pump is then turned back on to prevent significant changes in oxygen tension or pH.

Measuring both OCR and ECAR simultaneously, enables a more comprehensive assessment of cellular energetics and the ability to determine the relative contribution of these two dominant energy yielding pathways [2,22,23]. As shown in Figure 1a (D. Ferrick, unpublished results), drugs that inhibit glycolysis, such as 2-deoxyglucose (2-DG) (see Glossary) lower ECAR, those that inhibit mitochondrial redox reactions of the Krebs cycle or electron transport system lower OCR, whereas those that uncouple electron flow from oxidative phosphorylation such as FCCP (see Glossary) increase OCR and ECAR. Clearly, measuring these two fluxes simultaneously allows one to identify changes in bioenergetic states associated with drug-, genetic- or environmental-induced alterations in cell physiology or pathophysiology.

It has been shown that measurements of OCR and ECAR can provide good estimates of glucose utilization in cells and tissues [22]. In brief, the ratio of oxygen consumed to carbon dioxide excreted is 1:1 for cells oxidizing only glucose. Because the limited diffusion region formed around the cells approximates a closed system, the respired CO_2 is mostly hydrated to proton and bicarbonate. Thus, the measured OCR and ECAR should be approximately equal for 100% glucose oxidation. The pentose phosphate pathway will also contribute CO_2, but in most tissues the contribution is less than 10% of the total CO_2 respired [22] and it is essentially zero in most non-proliferating cells. Under these conditions, any excess, sustained acidification can be attributed to lactic acid production and extrusion.

In contrast to XF, radiometric rates are calculated from the accumulation of ^{3}HOH produced from 3-^{3}H-D-glucose and the difference between ^{14}C incorporation into CO_2 from C1- and C6–^{14}C-labeled glucose [22,24]. Although similar results are produced with both assay formats, the radiometric rates take two days to obtain from a 1-h experiment while the OCR and ECAR rates are generated in 90 s every 5 min during a 1-h experiment.

More recently, a multiwell plate-based assay platform has been developed [1–4]. As illustrated in Figure 1b, the XF24 Extracellular Flux Analyzer uses fluorescent sensors to measure extracellular fluxes of oxygen consumption (OCR) and extracellular acid release (ECAR) from cells in custom microplates. The fluorescent sensors reside on a plastic sleeve, or biocartridge, that fits over the cell culture plate (see cutaway view in Figure 1b).

When the biocartridge is in the raised position, the fluorescent sensors are approximately 5 mm above the cells within about 1 ml of buffer. A measurement under these conditions would take several hours to detect a significant change in oxygen or proton concentrations. As well, the chamber would have to be sealed to prevent atmospheric oxygen from dissolving into the media as this would disrupt the oxygen gradient formed by the cells. To speed up the measurement, the biocartridge is lowered to approximately 200 μM above the cells, creating a temporary 7 μl microchamber with limited diffusion (a 'virtual chamber'). The depletion of oxygen or decrease in pH is then measured in a period of 1.5–4 min, and then the fluorescent sensor returns to its original position where it mixes with the much larger reservoir to re-equilibrate the media.

The rate measurements can be made every few minutes without any significant depression of the oxygen tension or acidification of the media. Four injector ports surrounding the fluorescent sensor of each well can be used automatically to deliver any combination of pharmacological agents, agonist, antagonists or substrates during an experiment. Multiple injections of different reagents can be achieved in a single experiment or escalating doses of a single drug can be delivered to generate dose curves in just a few wells. However, although the XF24 has many advantages over the Clark electrode and microphysiometer in terms of throughput, compound injection and ease of use, they all achieve high sensitivity in the measurement of extracellular metabolic fluxes.

XF versus radiometric assays

Traditionally, metabolic fluxes associated with energy metabolism are derived from time-dependent accumulation of radionuclide in a metabolite derived from a labeled substrate [25,26]. For example, accumulation of ^{3}HOH water from 3-^{3}H-D-glucose is a measure of glycolytic flux through GAPDH (see Glossary). These types of radiometric assays can be not only highly specific, but they are also very labor intensive, can lack precision and often require substantial

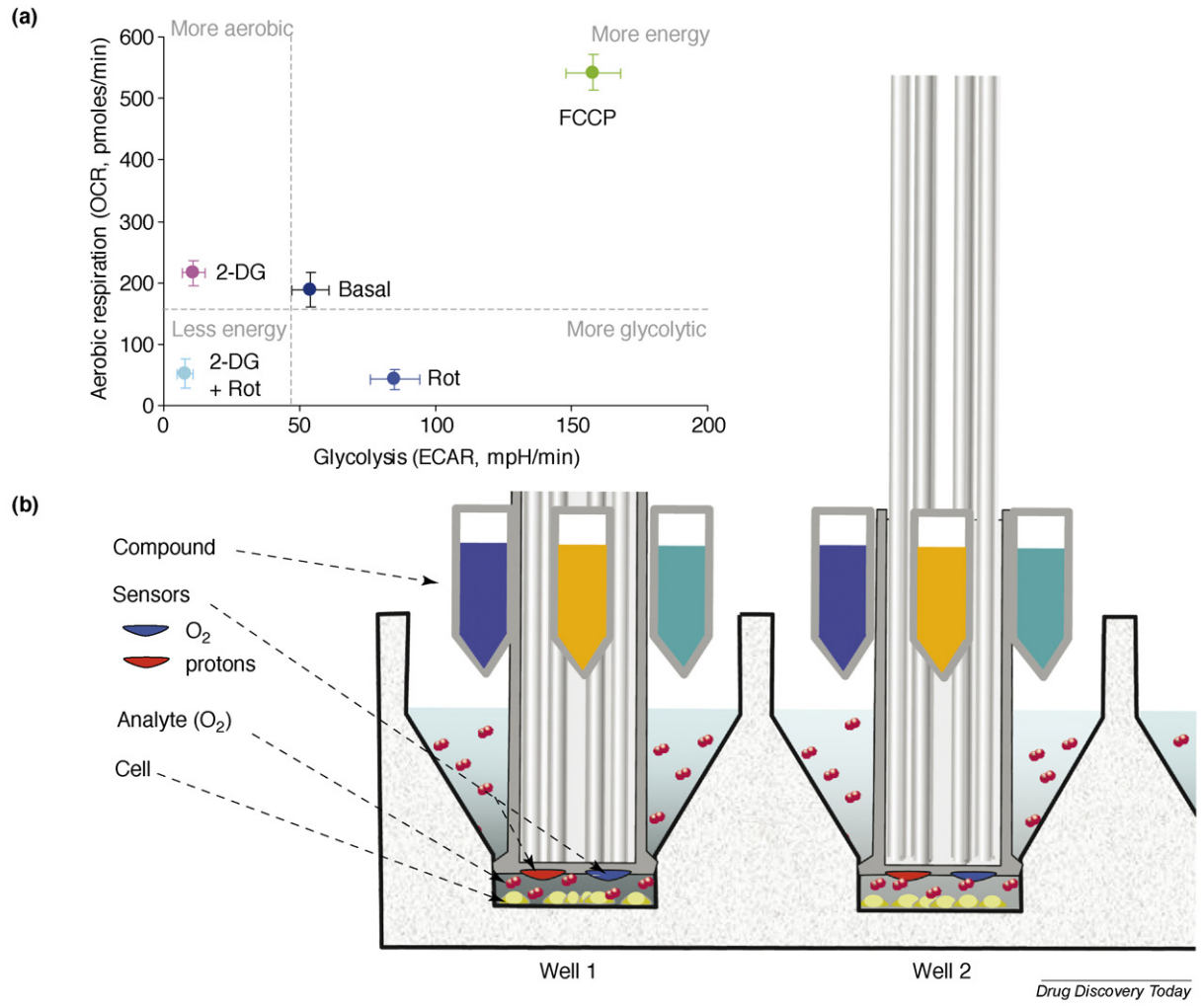

FIGURE 1

Measuring bioenergetics using XF. In **(a)**, overnight C2C12 blasts were seeded at 20,000 cells/well and treated with either 1 μM FCCP, 100 mM 2-DG, 1 μM rotenone (Rot) or 100 mM 2-DG plus 1 μM rotenone. Data are expressed as the average OCR or ECAR ± standard error of three replicates. In **(b)**, a cutaway view of the XF24 microplate and sensor biocartridge is illustrated.

quantities of tissue or cells. Perhaps an even more deleterious drawback is the limited kinetic resolution as rates are often measured over an hour or longer, unless very large sample sizes or high activities are used. Recent work has shown that measurement of extracellular fluxes of metabolites such as oxygen, lactate, and total acid can provide estimates of metabolic fluxes with accuracies comparable to those obtained with the radiometric assays [22,23]. The advantages to the extracellular flux assays are higher throughput, reduced sample sizes and substantially improved kinetic resolution with rates that are typically obtained within several minutes. In many ways these extracellular flux assays recapitulate the classical methods used to study isolated mitochondria with the added advantage of using whole cells or tissue to retain intact cytoplasmic processes and signaling.

In Figure 2 (C. Beeson, unpublished results), we illustrate the similarities and differences between an XF 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 2a, note that the increase in OCR upon addition of palmitate (first blue vertical line) follows similar kinetics as ^{3}HOH accumulation from radiolabeled palmitate (Figure 2b). Thus, both assays clearly show a rise in oxidative respiration because of exogenous addition of fatty acid in the form of palmitate. However, a fundamental difference is that the XF assay is measuring the rate of oxygen consumption in nearly real time. For the radiometric assay, aliquots were taken at the indicated time points but the accumulated ^{3}HOH was measured separately, hours later, after processing. Because the ^{3}HOH arises only from beta oxidation of the labeled palmitate, the radiometric assay is highly specific. Although OCR is a less specific indicator 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 glucose and amino acids as well. Another feature of the XF assay that is not shown was 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 metabo-

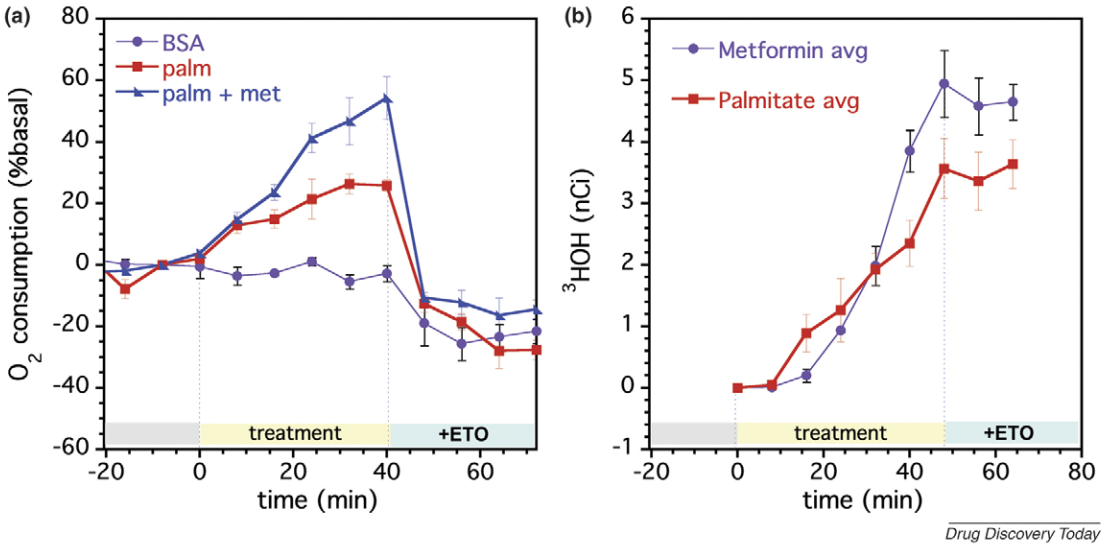

FIGURE 2

Switch from glucose to fatty acid oxidation in L6 myoblast cells. Panel **(a)** shows show O_2 consumption measured with an XF24 analyzer. Basal rates were measured before 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) to activate the AMP-kinase, which stimulates fatty acid oxidation. 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.

lism as their equilibrium is invariably altered by changes to either or both pathways. A second assay or use of a ^{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 2 by looking at the effect of etomoxir addition on the palmitate responses of the cells. Etomoxir is an inhibitor of carnitine palmitoyl transferase I (CPT-1) (see Glossary) which is required to transport fatty acids into the mitochondria to be catabolized and so acts to inhibit fatty acid oxidation. When etomoxir is added (second blue vertical line in Figure 2a) during the XF assay a decrease in the rate of oxygen consumption (OCR) can be seen. By contrast, the radiometric assay shows a plateau in the accumulation of ^{3}HOH (Figure 2b).

There are many drug discovery efforts aimed at identifying compounds that affect fatty acid oxidation and eventually understanding their mechanisms of action. The experiment illustrated in Figure 2 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 such identified compounds. As discussed above, their affect not only on fatty acid oxidation, but also on overall cellular bioenergetics can be assessed with the multiparameter capability of the XF assay format. In Figure 2a, metformin, a marketed drug that is used to treat type II diabetics is known to upregulate AMPK (see Glossary) and as shown here can enhance oxidation of the fatty acid palmitate. 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). This preferential shift is related to the ability of metformin to inhibit mitochondrial respiration at complex I [27,28].

Bioenergetic relevance of OCR and ECAR

The OCR and ECAR for a cell is related to the flux through catabolic pathways used to generate ATP. During steady state, the ATP synthesis rate is counterbalanced against ATP consumption and, thus, the OCR and ECAR are related to ATP turnover. Although quantitative estimates of ATP turnover are technically challenging, it can be shown that the changes in extracellular fluxes show a concordance with changes in ATP turnover rates. For example, inhibition of cytochrome *c* oxidase in primary cardiomyocytes, via subsequent addition of 2 mM potassium cyanide, decreases the basal OCR rates of about 250 pmoles/min to about 20 pmoles/min. By contrast, the basal ECAR rates are about 7 mpH/min. If the respired CO_2 were hydrated to carbonic acid, the basal ECAR would have been comparable to the basal OCR (250 pmoles/min). This confirms that any respired CO_2 is lost to the atmosphere and that the ECAR is primarily a measure of lactic acid production. When potassium cyanide is added to the cardiomyocytes the ECAR initially increases to about 330 mpH/min. The profound ECAR increase arises from a very large increase in glycolytic flux as the cells attempt to recover the mitochondrial ATP lost because of inhibition of the electron transport chain as indicated by the dramatic decrease in OCR. As demonstrated in this relatively simple example, the changes in extracellular flux reflect intracellular flux changes associated with ATP utilization.

The ability to monitor the fluxes through bioenergetic pathways is of obvious value to drug discovery in metabolic diseases. In a more general sense, ATP utilization has much broader application in measuring phenotypic changes in cells. For example, activation of muscarinic receptors on cardiomyocytes will trigger several

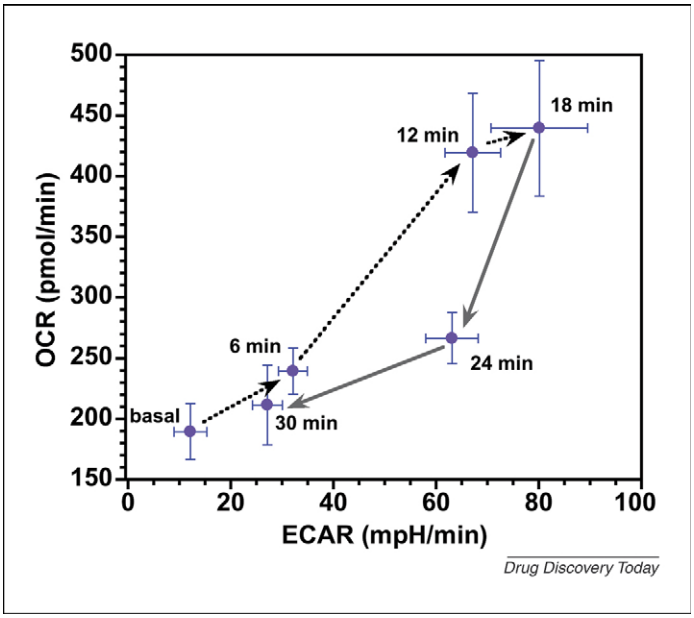

FIGURE 3

Bioenergetics associated with cell signaling. OCR and ECAR values measured with adult feline cardiomyocytes before (basal) and after muscarinic receptor activation with 1 μM carbachol. Shown are the averages of four wells measured every 6 min.

signaling pathways [29]. The Gq-coupled pathways will trigger rapid increases in intracellular calcium ions and activation of PKC (see Glossary) isoforms. The Gi- and Go-coupled pathways activated by muscarinic receptors will trigger accumulation of cAMP (see Glossary) and activation of PKA. Collectively, these processes will mobilize ions that will then require activation of additional ion channels to restore homeostasis, translocation of proteins to different organelles and cytoskeletal modifications. Most, if not all, of these processes will affect ATP utilization either directly or indirectly, which is typically observed as a transient measurable change in energy metabolism as demonstrated in Figure 3 (C. Beeson, unpublished results). In this experiment feline cardiomyocytes are activated through their muscarinic receptor by the agonist carbachol. This is an example of how activation of signaling pathways that do not directly regulate bioeneregtic processes involved in ATP production will still cause at least a transient change in metabolism that can be measured as a change in extracellular flux. Indeed, this observation is the entire basis of the Cytosensor® microphysiometer that is well cited for its ability to measure a wide variety of physiological changes [30–33].

More specifically in the example shown in Figure 3, the initial burst of activity because of signaling ultimately results in some downstream processes such as transcriptional activation, mRNA and protein synthesis and so on. Each of these also has ATP costs but the repercussion of those are often lost in the noise of basal metabolism. The phenotypic change as a result of signaling at 12–24 h could be a change in basal energy metabolism if that was a target but in many cases it will not be. Thus, one could argue that these extracellular flux measurements can be used to 'read-out' early signaling events in a manner similar to the use of other parameters such as calcium signaling, and contact impedance for cell based assays (i.e. filling in the gap left by the loss of the microphysiometer). However, unlike the previous instrument, the XF instrument also provides a measure of OCR, which is more directly related to mitochondrial function, and this organelle is a frequent target of damage associated with drug toxicity. Thus, while an agent that causes a transient change in a cell's metabolism associated with signaling might be viewed as a potentially useful drug, should a drug candidate cause a long-lived measurable change in a cell's basal metabolism that could easily represent a liability.

For example, in Figure 4 (C. Beeson, unpublished results) we present OCR data for primary human hepatocytes exposed to dantrolene, a muscle relaxant known to exhibit clinical hepatotoxicity [34]. Although the mechanistic basis for dantrolene's hepatotoxicity is not known, it does not exhibit significant cytotoxicity in primary hepatocytes *in vitro* [35]. Because dantrolene bears structural similarity to the mitochondrial toxicant nitrofurantoin, it has been suggested that the clinical toxicity of dantrolene arises from mitochondrial oxidative stress [36,37]. It was found that an acute exposure of cyropreserved human hepatocytes to 50 μM dantrolene (dark bars) caused an immediate decrease in OCR that was sustained after 1 h. Although the decrease was relatively moderate, the loss of oligomycin inhibition of respiration (light bars) indicates that much of the respiration at 1 h is uncoupled from ATP production. Although these results do not *a priori* suggest a mechanism for hepatotoxicity, the observation that dantrolene causes a sustained mitochondrial dysfunction in these cells would warrant further investigation into the agent's potential for organ toxicity. Thus, in addition the obvious use in cell based assays for metabolic therapies; the measurement of extracellular

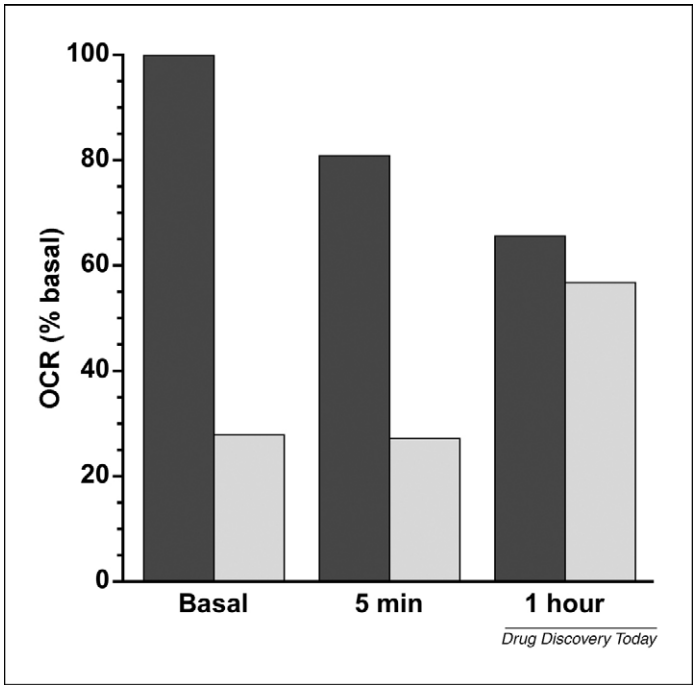

FIGURE 4

Dantrolene causes sustained mitochondrial dysfunction in primary hepatocytes. Shown are OCR values normalized to basal before injection with 50 μM dantrolene (dark bars) and measured at 5 min and 1 h, post injection. Also shown are OCR values measured after 10 min exposure to 10 μg/ml oligomycin (light bars). Cells are cryopreserved, plateable human hepatocytes (Celsis In Vitro Technologies, Baltimore, MD) measured 24 h post thaw. Treatment with 5 μM dantrolene caused no appreciable change in OCR at either time point.

TABLE 1

Comparison of methods for measuring bioenergetics

	XF24	Radiometric substrate	Cytosensor	Electrode	O_2 fluorescent sensor	O_2 fluorescent dye
Format	Microplate	Microplate	Flow chamber(s)	Stirred chamber(s)	Microplate	Microplate
Assay type	Label free	Radio-labeled substrate	Label free	Label free	Label free	Fluorescent dye
Measures	OCR and ECAR	Substrate catabolism	ECAR	OCR and/or ECAR	OCR	OCR
Type of measurement	Kinetic	Serial	Kinetic	Serial	Serial	Serial
Suitable for adherent cells	Yes	Yes	Yes	No	No	No
Average number of cells per well/chamber	40,000	1,000,000	40,000	1,000,000	2,000,000	N/A[a]
Automated injection	Yes	No	Yes	No	Yes	Yes

[a] Primarily used with isolated mitochondria.

fluxes could have more general utility in drug discovery for screening of possible toxicant activities.

Summary

One of the challenges of drug discovery is to develop cell-based assays that can detect relevant phenotypic changes in response to compounds and other modulators. The ultimate goal is, of course, to emulate human disease as closely as possible. In this day of powerful 'omic' tools, the need for highly physiologic assays is even more profound.

There is a growing awareness of the value of measuring bioenergetics to understand and target disease phenotypes. Assays that measure cellular bioenergetics are rapidly spreading from the obvious indications such as obesity and diabetes to oncology, cardiovascular and neurodegenerative indications. In this article, we have described several methods for measuring cellular bioenergetics. Although not exhaustive, we have summarized in Table 1 key features of these approaches that are important to most researchers. Many of these features are embodied in the XF24 extracellular flux analyzer [1–4]. Key benefits of this novel system are:

1. The ability to simultaneously measure OCR and ECAR to assess the two major bioenergetic pathways of the cell.
   a. This provides a relative measure of ATP turnover.
   b. The ability to determine changes in the contribution of both oxidative phosphorylation and glycolysis to total cellular energy expenditure.
   c. A means to look at metabolic responses to a variety of experimental manipulations.
2. The ability to measure kinetics of drug responses which often differentiates compounds into functional classes.
3. Because XF assays are label-free and non-invasive, cells are unaltered which reduces artifacts and makes possible their use in secondary assays or chronic experiments.
4. Up to four compounds or reagents can be automatically added per well during the experiment.

While methods for measuring primary bioenergetic pathways have been available for some time, these recent improvements to the XF format will allow investigators to more broadly apply cellular bioenergetics in cell-based drug discovery research and development.

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