Monitoring Intracellular Oxygen Concentration: Implications for Hypoxia Studies and Real-time Oxygen Monitoring

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Abstract

The metabolic properties of cancer cells have been widely accepted as a hallmark of cancer for a number of years and have shown to be of critical importance in tumour development. It is generally accepted that tumour cells exhibit a more glycolytic phenotype than normal cells. In this study, we investigate the bioenergetic phenotype of two widely used cancer cell lines, RD and U87MG, by monitoring intracellular oxygen concentrations using phosphorescent Pt-porphyrin based intracellular probes. Our study demonstrates that cancer cell lines do not always exhibit an exclusively glycolytic phenotype. RD demonstrates a reliance on oxidative phosphorylation whilst U87MG display a more glycolytic phenotype. Using the intracellular oxygen sensing probe we generate an immediate readout of intracellular oxygen levels, with the glycolytic lines reflecting the oxygen concentration of the environment, and cells with an oxidative phenotype having significantly lower levels of intracellular oxygen. Inhibition of oxygen consumption in lines with high oxygen consumption increases intracellular oxygen levels towards environmental levels. We conclude that the use of intracellular oxygen probes provides a quantitative assessment of intracellular oxygen levels, allowing the manipulation of cellular bioenergetics to be studied in real time.
1 Introduction

Oxygen has an important role to play in mammalian cell homeostasis, providing ATP for energy, assisting in enzymatic reactions (oxidation and hydroxylation) and generating reactive oxygen species (ROS), which are all key parameters in metabolism [1]. There are many physiological (and pathophysiological) implications for oxygen dynamics, especially in cancer and the adaptive response to hypoxia. Cells and tissues have different levels of intracellular and extracellular oxygen, with oxygen gradients existing across tissues. Cells and tissues are exposed to different levels of oxygen: most tissues have a partial pressure of oxygen (pO$_2$) of between 20-40mmHg, whereas a large tumour can be severely hypoxic or anoxic with pO$_2$ levels <0.1mmHg [2]. A frequent occurrence in most solid tumours is exposure of cells to levels of hypoxia equivalent to oxygen dissolved at less than 1% in solution [3]. This hypoxia results in key adaptive responses, altering cell metabolism and making the cells resistant to chemotherapy and radiotherapy.

With the development of new 3D culture systems, the ability to determine intracellular oxygen levels and model the in vivo situation will be of fundamental importance when investigating the effects of novel therapeutics to treat cancer or reduce ischaemic injury.

A recently-developed in vitro tool was utilised in this study to investigate the level of monolayer oxygenation. Monolayer oxygenation is a result of the balance between oxygen entering the cells by diffusion and oxygen consumed by physiological process. In most cells oxidative phosphorylation will be the major consumer of oxygen within the cell. MitoXpress®-Intra is an easy-to-use cell-penetrating phosphorescence-based oxygen sensing probe that allows the real-time quantitation of oxygen within the cell monolayer using a plate reader platform [4, 5]. We employed this new tool to examine the metabolism of two established cancer cell lines, RD (rhabdomyosarcoma) and U87MG (glioblastoma multiforme) under different applied oxygen concentrations.

2 Methods

RD, U87MG and 786-O cells (ATCC) were seeded at 50,000 cells/well in black, clear bottomed 96 well plates (Nunc) in 150µl Dulbecco’s modified eagles medium (Invitrogen) containing 25mM glucose and supplemented with 1mM pyruvate, 2mM glutamine, 10% FBS and left overnight to adhere. Media was then removed and replaced with 150µl fresh culture media containing 10µg/ml MitoXpress-Intra probe. The cells were then returned to the incubator overnight to allow for probe internalisation. Before assaying, the media was removed and the cells were carefully washed thrice in PBS before 150µl fresh assay media was added. The plate was read on a BMG Omega FluoStar plate reader with Ex 340 ± 50nm and Em
650 ± 50nm using dual delay time resolved measurements. The delay times were 30 and 70μs. An atmospheric control unit (ACU) maintained the temperature at 37°C and allowed us to control ambient oxygen levels. The raw data are expressed as TR-F intensity, requiring a conversion into phosphorescence lifetime values. Lifetime is calculated using the following equation: lifetime τ = (t₂-t₁)/ln(D₂/D₁) [t₁=delay times, D₁=measured intensity values], with each specific lifetime value corresponding to a specific oxygen concentration.

3 Results

To generate absolute values of probe fluorescence at different biological oxygen concentrations, a calibration curve for the MitoXpress-Intra was constructed using probe-containing antimycin-A treated cells. Prior to measurement, cells were treated with 1µM antimycin-A in order to inhibit any mitochondrial respiration which would interfere with baseline probe signal. Atmospheric oxygen was set to 18% and once the level of probe fluorescence reached a steady state, the oxygen in the reader was reduced in a stepwise manner as depicted in Figure 1. Complete de-oxygenation was achieved by the addition of glucose oxidase to adherent cells in media. The average lifetime values of the non-respiring cell monolayer at each steady state were then plotted against their corresponding oxygen concentrations to produce the calibration curve depicted in Figure 2. This calibration process facilitated the conversion of measured lifetime values into absolute percentage intracellular oxygen concentrations using the equation [% O₂] = A exp(-Bτ), where the numerical values of the parameters A ≈ 370 and B = 0.1 μs⁻¹ are derived from least-squares fitting the expression to the combined U87MG and RD data. We also computed 95% confidence intervals on this fit using bootstrapping with the percentile method.

RD and U87MG cells, loaded with MitoXpress intra probe, were cultured in high glucose media and measured at different oxygen concentrations. The results show that, at 18% ambient oxygen, RD cells contained intracellular O₂ levels of 15.2% ± 0.7% (Figure 3). The difference between ambient oxygen and intracellular oxygen concentrations is greater when ambient oxygen levels are dropped to more physiological levels. At 10% and 5% ambient oxygen these metabolically proficient cells show a 50% reduction in the level of intracellular oxygen (Figure 3). In contrast U87MG cells, known for their glycolytic phenotype, were found to contain intracellular oxygen levels of 17.1% ± 0.8% at 18% ambient oxygen levels, more representative of ambient concentration (Figure 3). In Figure 4 we give an example of how the oxygen probes can be used to monitor drug effects in real time. 786-0 renal cancer cells pre-loaded with MitoXpress –Intra were incubated with different concentrations of the drug phenformin under an ambient concentration of 5% oxygen. The cells show an initial drop in oxygen levels (increase in lifetime) as ambient oxygen levels change from 20% to 5% followed by a com-
plete inhibition of oxygen consumption in the phenformin treated samples (Figure 4).

4 Discussion

A substantial body of evidence indicates that aerobic glycolysis, commonly known as the Warburg effect, is a hallmark of cancer [6]. Here we show that RD cells consume oxygen on high glucose media and do not rely on glycolysis for ATP production to the same extent as U87MG cells. This is also a feature of a large number of cancer cell lines (data not shown) indicating that not all cancer cells display this aerobic glycolysis 'hallmark' as previously suggested.

Our data also suggest that significant differences can exist between the oxygen concentration at the cell monolayer and the applied ambient oxygen concentration. This difference is not accounted for in standard in vitro analysis, where it is assumed the intracellular oxygen concentration reflects environmental levels (for example, assuming that an ambient oxygen level of 18% implies an intracellular level of 18%), consonant with previous reports that the rate of oxygen diffusion into the cells is faster than the oxygen consumption rate [7]. However, we observed that the aerobic RD cells consume and deplete intracellular oxygen to a dramatically greater extent than the glycolytic U87MG cells. The aerobically proficient RD cells, at ambient oxygen concentrations of 18, 10 and 5%, have intracellular levels of 13.2, 5.4 and 2.1%, respectively. In contrast to this are the glycolytic U87MG cells that displayed oxygen levels of 17.1, 9.3 and 4.2%, more similar to ambient levels. The greater differences observed between the intracellular oxygen levels in the RD line compared to ambient are likely due to a lower level of back diffusion from the environment as ambient oxygen levels are reduced. If mitochondrial respiration becomes limiting at lower levels of oxygen this difference may decrease. Interestingly our recent study in primary human bone cells showed very high rates of mitochondrial respiration even when ambient oxygen levels were reduced to 2% [8].

The higher than expected value for the U87MG cells at 18% oxygen with an error of 4% is a property of the probe when measuring high non-physiological concentrations of oxygen. The probe is much more robust when working with oxygen levels in the physiological range <8% O\textsubscript{2} with much smaller errors. Interestingly when the ambient oxygen levels were 2%, the aerobic RD cells were almost anoxic (data not shown). Oxygen levels fluctuate and will drop significantly when glucose is used up during normal growth (as cells utilise mitochondrial substrates to generate ATP, thus consuming higher levels of oxygen). The intracellular oxygen monitoring system described here allows changes in oxygen levels to be monitored in real time. This monitoring can be either rapid (over minutes or hours, as with the mitochondrial inhibitor phenformin), or over several days (if the assays are run in a plate reader with an atmospheric control unit). The MitoXpress intra
probe thus allows an insight into cellular oxygenation that has not previously been attainable. This new probe could be extremely useful as a tool to identify compounds or gene knockouts that modulate mitochondrial respiration in a high throughput format.

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References


Figure legends

**Figure 1:** Kinetic trace of the intracellular probe showing the response of a non-respiring cell monolayer (antimycin-A treated) to decreasing atmospheric oxygen concentrations (labelled). Coloured traces show three technical replicates

**Figure 2:** Calibration curve, with 95% confidence intervals, relating lifetime values to percentage ambient oxygen. Values represent the mean and standard devia-
tion of 6 replicates. The equation gives an expression quantitatively linking lifetime measurements with $O_2$ levels, derived from fitting a model to these data points.

**Figure 3:** At 18% ambient oxygen RD cells were found to contain intracellular oxygen levels of 13.2% ± 0.7%, thus suggesting that they are metabolically proficient cells. In contrast, U87MG cells, known for their glycolytic phenotype, had intracellular oxygen levels of 17.1% ± 0.8% at 18% ambient oxygen. ** - p < 0.01, *** – p < 0.001 using an unpaired t-test (n=3).

**Figure 4:** Real-time measurement of the effect of phenformin treatment on intracellular oxygen levels on 786-0 renal carcinoma cells maintained in a BMG Omega FluoStar reader at 5% $O_2$ and 5% $CO_2$ (a) Lifetime measured over 18 hours (b) Estimated intracellular oxygen levels from lifetime values., using a calibration curve derived from HEPG2 cell data.
Figure 2

Model fit:
\[ \% O_2 = 370 \exp(-0.10 \tau) \]
Figure 3

The bar graph shows the percentage of intracellular oxygen for different levels of ambient oxygen. The x-axis represents the percentage of ambient oxygen (18%, 10%, and 5%), and the y-axis represents the percentage of intracellular oxygen. The graph compares two conditions: Old U87MG and RD.

- At 18% ambient oxygen, the percentage of intracellular oxygen is significantly higher for Old U87MG compared to RD, with a p-value of 0.003.
- At 10% ambient oxygen, the percentage of intracellular oxygen is still higher for Old U87MG, with a p-value of less than 0.0001.
- At 5% ambient oxygen, the percentage of intracellular oxygen is lower for both conditions, but Old U87MG remains significantly higher than RD, with a p-value of 0.0009.

The error bars indicate the standard error of the mean for each condition.