ON-LINE QUANTITATIVE IN VITRO MONITORING OF CELLULAR GLUCOSE AND LACTATE METABOLISM: TECHNIQUE AND APPLICATIONS

ABSTRACT

Current biosensor technology is mostly used for discrete off-line analysis. We describe a novel on-line in vitro monitoring technique, with applications for monitoring cellular glucose metabolism. Using continuous perfusion and biosensor technology, we can measure glucose and lactate metabolism at a minute-to-minute time resolution for periods up to several days. The application of our perfusion-detection technique for in vitro monitoring is demonstrated in a wide variety of cells, including primary neuronal and astroglial cultures, yeast cells and human lymphocytes. The method shows that variations in oxygen delivery or exposure to a non-competitive pseudo-substrate (here 2-deoxyglucose, (2DG)) affects normal glucose metabolism. An innovative advantage of this system is that metabolism can be calculated per cell. The potential of in vitro on-line monitoring is discussed for application in studying normal and abnormal metabolism, toxic and non-toxic drug effects, and human tissue biopsies.
INTRODUCTION

Monitoring cellular physiology in vitro does not only provide information on normal metabolic functions, but can also be used to test the effects of drugs or to characterize the possible pathophysiological nature of cells. The main energy substrate for most cells is glucose, whereas cellular efflux of lactate reflects anaerobic metabolism and is often considered an index of insufficient oxygenation of tissue (Schurr and Rigor, 1998; Valtysson et al., 1998). In well-oxygenized tissue, lactate is also used as an (alternative) energy substrate (Cater et al., 2001; Pellerin et al., 1998). Glucose uptake and lactate release can inform about energy use and adequate oxygen delivery, as increased cellular activity has a direct influence on energy use (Magistretti and Pellerin, 1999; Pellerin et al., 1998). Therefore, not only the condition of the cell, but also its response to stimuli, such as drugs, can be monitored by measuring glucose consumption and lactate efflux.

Current biosensor technology is mostly used for discrete off-line analysis. Currently used in vitro test systems are based on fluorescence detection of free calcium, resonance energy transfer or electrophysiological alterations (e.g. of single or multiple unit firing activity or membrane impedance) (for example, Bers, 2003; Wakefield et al., 2002).

In the present report a novel in vitro method for on-line monitoring of cellular glucose metabolism is described, that is particularly useful in testing cell cultures. Several other flow-through systems have been described (Gramsbergen et al., 2003; Cucullo et al., 2002; Wiley and Beeson, 2002; Gloeckner and Lemke, 2001; Noll et al., 2000; Noll and Biselli, 1998; Arthur et al., 1998), however, these flow-through systems are not used for on-line monitoring (Cucullo et al., 2002; Wiley and Beeson, 2002; Gloeckner and Lemke, 2001), or are rather large (Noll et al., 2000; Noll and Biselli, 1998; Arthur et al., 1998). The here used submicroliter flow-through detection system is a slightly modified version of that used previously by us to monitor metabolism in vivo (Rhemrev-Boom et al., 2002; Savenije et al., 2002; Tiessen et al., 1999, 2001, 2002; Leegsma-Vogt et al., 2001; Kaptein et al., 1997, 1998), and more recently, organotypic brain cultures (Gramsbergen et al., 2003). Submicroliter flow-through on-line monitoring systems have, however, never been described for cell preparations. Our detection method, based on flow injection analysis (FIA) and biosensors, measures changes in glucose and lactate metabolism at a minute-to-minute time resolution. By applying continuous perfusion, we can monitor cell metabolism under constant conditions for periods up to several days. The chamber containing the viable cells has small dead volumes, allowing perfusion rates under 2 µL per minute. Low rates are essential to achieve minimal dilution of analytes, but low flow rates may also limit oxygen delivery. Here we demonstrate the potential of our submicroliter flow-through perfusion-detection technique for in vitro monitoring of a variety of cell cultures, including primary neuronal and astroglial cells, yeast cells and human lymphocytes. The technique demonstrates that both glucose metabolism and the aerobic ratio (the percentage aerobic metabolism by the cell culture), can be manipulated by changing flow rate and thus oxygen delivery, and that exposure of these cells to a non-competitive pseudo-substrate (here 2-deoxyglucose, 2DG, which is taken up by cells but cannot be metabolized), affects normal metabolism.
EXPERIMENTAL SECTION

Cell cultures and cell chamber

The cell types studied were: freshly isolated cells, primary cell cultures and cell-lines. All cell types were grown (primary cells and cell-lines) or pipetted (freshly isolated cell suspension) on a semiporous membrane of a culture plate insert (Millicell-CM, 12 mm, pore size 0.4 µm, Millipore Corporation, Bedford, USA). The culture inserts allowed us to transfer the cells directly from the incubation chamber to the monitoring unit, by placing the culture insert tightly into the specially developed cell chamber (figure 2-1). To investigate the applicability of our cell chamber for free-floating, thus non-adhered cells, yeast (Saccharomices cerevisiae) and freshly isolated human lymphocytes were monitored. Dry yeast (Bruggeaman, Gent, Belgium) was dissolved in Dulbecco’s modified eagle medium (DMEM without glucose, Gibco, Invitrogen Corporation, Breda, The Netherlands) supplemented with 5 mM glucose (Merck, Darmstadt, Germany), of which 50 µl cell suspension was pipetted onto the insert (between 1.1x10^8 and 3.5x10^8 cells/ml). For the isolation of lymphocytes, 10 ml blood was mixed with EDTA and AKE solution (155 mM NH₄CL, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.4). This suspension was kept at 0 °C for 20 minutes and centrifuged. The pellet was resuspended in AKE, and again centrifuged. Cells were counted, and 25 or 50 µl cell suspension was pipetted onto the insert (between 1x10^8 and 1.95x10^8 cells/ml).

Four cell cultures were investigated: an astroglial cell culture, a neuronal cell culture, the neuronal cell line NG108, and a human embryonic kidney (HEK) cell line. Primary cells were derived from mouse cortex (embryonic day 16-18). The cortex was excised and dissociated by trypsinization (0.25% trypsin at 37 °C for 20 minutes, followed by trypsin inhibition medium at room temperature for 4 minutes). Cells were triturated, filtered, and centrifuged (10 minutes, 800 rpm). The pellet was resuspended in 1 ml Neurobasal medium (NB medium, supplemented with penicilline (100 U/ml)/streptomycine (100 µg/ml), 1 mM Na-pyruvate, 0.5 mM glutamine, B27 (1x) (All Gibco, Invitrogen Corporation, Auckland, New Zealand), and counted (between 2.8x10^6 and 3.2x10^7 cells/ml). 0.3x10^6 cells were plated on coated (poly-D-lysine, Sigma Chemical Co, St. Louis, MO) cell culture inserts, 6-12 inserts were plated per animal sacrifice. The inserts were placed in 6-wells culture plates (Costar, Corning Incorporated, New York, USA). Neuronal cultures (primary cells and NG108 cells) were grown in supplemented Neurobasal medium (see above) with either high glucose (25mM) or low glucose (5mM). Astrocytic primary culture and HEK cells were cultured in DMEM with 10% fetal calf serum (FCS) (DMEM high (25 mM) glucose (astrocytes: or low (5 mM) glucose), penicilline (100 U/ml)/streptomycine (100 µg/ml), 1 mM Na-pyruvate, 2 mM glutamine). Cell cultures were incubated at 37 °C and 95% O₂, 5% CO₂. Metabolism of the cultures was monitored between 1 to 10 days after incubation, except astrocytes, which, due to maturation time, were used 7-20 days after incubation. On the day of the monitoring experiment, the culture insert was placed inside the cell chamber (figure 2-1) and connected to the Flow Injection Analysis (FIA) system. The specially designed cell chamber (Delrin, Central Instrumental Service, University of Groningen, The Netherlands) consists of a three-piece holder, which did (primary neuronal and astrocytic culture) or did not (cell lines, yeast, lymphocytes) have two metal spacers. The assembled cell chamber has outer dimensions of 3.5 cm by 2.2 cm, with an inner diameter of 7.6 mm. The cell chamber is screwed tightly together to minimize dead space,
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with approximately 10 µL volume area on both sides of the insert membrane. Trapped air bubbles, which can be created by the placement of the insert, are guided outward by the slightly convex shape (3%) of the inner pieces of the cell chamber (closest to the insert membrane). Also, remaining air bubbles are removed by gently pulling medium through the assembled chamber before connecting the cell chamber to the FIA.

Flow injection analysis (FIA) system and biosensors

The FIA system and the biosensors used in these experiments are essentially the same as described earlier (Leegsma-Vogt et al., 2001, 2003). In short, the cell chamber is connected to the analytical part of the set-up by an intercalated valve with a 20 nl internal loop (Vici-Valco Instruments, Houston, USA). The valve injects a cell chamber sample every minute. By applying underpressure, oxygenized (95% O₂, 5% CO₂) DMEM with 5 mM glucose is pulled through the cell chamber towards the FIA with a Harvard 22-syringe pump (Harvard Apparatus, South Natick, MA, USA).

Glucose and lactate are measured using glucose oxidase and lactate oxidase respectively, placed in separate enzyme reactors together with horseradish peroxidase (all enzymes from Roche, Mannheim, Germany). The cell chamber and the enzyme reactors are kept at 37°C. The resulting current is measured using wall-jet-type electrochemical flowcells (VT-03, Antec Leyden B.V., Zoetermeer, The Netherlands) and two potentiostates: the Decade (Antec Leyden B.V., Zoetermeer, The Netherlands), and an Amor amperometric detector (Spark Holland, Emmen, The Netherlands). A schematic representation of the experimental set-up is shown in figure 2-2.
Before the experiment, an in vitro calibration curve is run on the FIA (glucose: 0, 2.5, 5, 10, 20 mM; lactate: 0, 1.25, 2.5, 5 and 10 mM). During the experiment, the glucose and lactate concentration is measured every minute and the currents (in nano-amperes) are recorded on a double-pens recorder (BD 112, Kipp en Zonen, Zoetermeer, The Netherlands) and by a data acquisition program (Chromeleon, Dionex Corporation, Sunnyville, CA, USA).

**Experimental procedure and calculations**

Most experiments lasted two days, in which monitoring was continued during the intermediate night. We were able to monitor cellular metabolism continuously for at least 4 days.

To analyze the potential of our monitoring system we performed several manipulations. The “standard” medium consisted of oxygenized DMEM supplemented with 5 mM glucose. Various flow rates were applied to study oxygen delivery. The ability of the cultures to use lactate as an energy source was studied with lactate as the sole substrate (5 mM in DMEM). Also the effect of a noncompetitive pseudo-substrate, the glycolysis inhibitor 2-deoxyglucose (5 mM 2DG in DMEM with 5 mM glucose), was studied on glucose use and lactate output. The ability of yeast to metabolize glucose under anaerobic conditions was studied by bubbling helium through the 5 mM glucose DMEM, thereby removing all oxygen.

Glucose use, lactate use, lactate output and the aerobic ratio, a possible indicator of aerobic glucose metabolism, were calculated as follows (Gramsbergen et al, 2003):

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\text{Glucose used (nmoles/min) = flow (µl/min)} \times (\text{glucose in medium before perfusion (mM)} - \text{glucose detected after perfusion (mM)}).
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Lactate used (nmoles/min) = flow (µl/min) * (lactate in medium (without glucose) before perfusion (mM) – lactate detected after perfusion (mM)).

Lactate output (nmoles/min) = flow (µl/min) * lactate detected (after perfusion with glucose) (mM).

Lactate converted to glucose equivalents = lactate output/2

The aerobic ratio is a measure of the percentage aerobic glucose metabolism, and is defined as the portion of glucose which is not transformed and released as lactate:

Aerobic ratio (%) = 100% * (glucose used – lactate output/2)/glucose used.

RESULTS AND DISCUSSION

Measurements were taken simultaneously for glucose and lactate every minute, resulting in almost 1,000 measurements in an 8-hour experiment.

Flow rates between 0.03-1.5 µl/min were used. Figure 2-3 is an example of an experiment in which oxygen delivery was studied by applying different flow rates. Glucose use increases with flowrate (figure 2-3), from 0.14 nmoles/min at 0.05 µl/min to 1.08 nmoles/min at 1.5 µl/min, or, as glucose use per cell can be calculated: from 0.4 to 3.3 femtomoles/min/cell. Lactate output shows no apparent relation with flowrate, and ranges between 0.24 nmoles/min at 0.05 µl/min to 0.86 nmoles/min at 1 µl/min (0.8 to 2.9 femtomoles/min/cell). The percentage aerobic metabolism was calculated by adjusting the glucose uptake for the amount of lactate produced. In our closed cell chamber system, oxygen is delivered through the medium, and is thereby flow rate dependent. The aerobic ratio is higher at higher flowrates, ranging from 15% at 0.05 µl/min to 60% at 1.5 µl/min in this experiment. In all of our experiments, the calculated aerobic ratio was highest at the highest flowrates used (1-1.5 µl/min), and reached up to 90%. The calculated aerobic ratio is in the upper range of estimated aerobic ratios in cell cultures (10-75%, calculated from (Cucullo et al., 2002; Gloeckner et al., 2001)). In an open cell chamber system, as used by Gramsbergen et al (2003), oxygen delivery is not dependent on flow rate, and a stable aerobic ratio of approximately 50-60% is reached at 0.5 and 1 µl/min (Gramsbergen et al., 2003).

In about 50% of our experiments with cultures grown in high glucose levels (25 mM), we measured a negative aerobic ratio caused by a high release of lactate or glucose (example in figure 2-4D). This occurred in 15% of experiments with cultures grown on low glucose levels (5 mM). As glycogen levels are high in normal (high glucose) culture (Dringen et al., 1993), glycogen can be broken down to glucose and lactate (Dringen et al., 1993), and (astroglial) glycogen stores contribute to lactate efflux (Gramsbergen et al., 2003), the presence of glycogen, and the release of (mainly) lactate from these glycogen stores in our cell cultures is suggested. Possibly the incubation in low glucose concentrations decreases the amount of glycogen in the cells.

The aerobic ratio was calculated under assumption that almost all of the glucose taken up by the cells is metabolized via glycolysis and subsequently metabolized aerobically via the Krebs cycle or converted anaerobically to lactate (Gramsbergen et al., 2003). However, glucose may be metabolized via other pathways (e.g. pentose phosphate pathway (Sanchez-Abarca et al., 2001; Bonarius et al., 2001), amino acid synthesis (Attwell and Laughlin,
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2001), which would decrease the aerobic ratio. On the other hand, as we have found evidence that lactate may be (partly) derived from glycogen instead of glucose, the calculated aerobic ratio may be an underestimation of the real value.

Figure 2-4 shows four examples of experiments, with lymphocytes (2-4A), yeast (2-4B), a primary astrocytic culture on glucose or lactate medium (2-4C), and a neuronal cell line, with the effects of 2DG (2-4D). Lymphocytes show decreased glucose use at lower flowrates (figure 2-4A). The aerobic ratio in lymphocytes was very high: 85.5% at 0.03 µl/min, 91.1% at 0.1 µl/min, and 91.5% at 0.2 µl/min.

There was no lactate efflux in any of the yeast-experiments (example in figure 2-4B); the aerobic ratio could thus not be calculated. All other cell types released lactate in the glucose containing medium. Yeast (S. cerevisiae) does not produce lactate, as yeast avidly transforms glucose to ethanol (van Dijken et al., 1993). Glucose use is higher at 0.5 µl/min compared to 0.1 µl/min, in both aerobic and anaerobic conditions (0.5 µl/min aerobic: 0.76 nmoles/min, 0.5 µl/min, anaerobic: 0.97 nmoles/min (=8.4 x10exp-2 femtomoles/min/cell), 0.1 µl/min, aerobic: 0.27 nmoles/min, 0.1 µl/min, anaerobic: 0.17 nmoles/min).

In vitro lactate can serve as an energy source in primary cell cultures (Sanchez-Abarca et al., 2001;Tabernero et al., 1996), and hippocampal slices (Gramsbergen et al., 2003;Schurr et al., 1997). In our experiments, lactate consumption was detected in primary neurons and astrocytes (example in figure 2-4C, glucose use: 3 femtomoles/min/cell, lactate use: 5.5 femtomoles/min/cell or 2.75 femtomoles/min/cell in glucose equivalents), but we did not observe lactate consumption in any of our cell line experiments (n=4). Experiments varied between 1.5 and 18 hours. The absence of lactate consumption in cell lines could be partly due to the use of the cell chamber without spacers.
We also investigated the effect of 2DG, a glycolysis inhibitor, on glucose and lactate metabolism (n=3, example in fig. 2-4D). Cultured cells exposed to 2DG exhibited glucose release (in one instance there was glucose release before 2DG was introduced), and either an increased lactate output (n=2, NG108 cells, HEK cells) or a decreased lactate output (example in figure 2-4D, NG108 cells) compared to 5 mM glucose medium. Decreased lactate output and diminished glucose use (although no glucose release) were also found by Gramsbergen et al (2003). When cells were supplied with glucose medium after the 2DG experiment (n=2), little change in glucose or lactate release was seen when altering flowrate, suggesting impaired glucose metabolism after 2DG (results not shown). The 2DG experiments, all incubated in high glucose levels, imply substantial glycogen stores, as both glucose and lactate are released from the cells during 2DG challenge.

Figure 2-4 A,B,C,D: Examples of monitoring experiments. Figure 2-4A shows the effect of various flow rates on the glucose use and lactate output in lymphocytes, figure 2-4B shows the same for yeast cells under both aerobic and anaerobic conditions. Yeast (S. cerevisiae) does not produce lactate. There is both glucose and lactate use in primary astrocytes (figure 2-4C, total experiment 0.5 µl/min, during lactate medium glucose is zero as it is not present in the medium). The effect of the glycolysis inhibitor 2DG is shown in figure 2-4D. Both before and during 2DG measurement, glucose is released into the medium. During 2DG challenge lactate output decreases.
CONCLUDING REMARKS

The present study has shown the capability of the on-line monitoring of cellular glucose and lactate metabolism. We demonstrated its potential in various cell types. The on-line monitoring system has due to the small-volume tubing, low dead volumes of the connections, and the on-line monitoring, low lag-times (approximately 5 minutes) and high temporal resolution. With the miniaturization of biosensors (Rhemrev-Boom et al., 2001 a,b, 2003), the lag-time may become even lower. An innovative advantage of this system is that metabolism can be calculated per cell. Our (preliminary) data suggest that neurons and astrocytes metabolize glucose in the same order of magnitude, whereas yeast cells consume a hundred times less glucose per cell. By miniaturizing the system, metabolism can be monitored in very low cell numbers (approximately 75,000 cells), increasing the time resolution of energy usage profiles. Application of in vitro monitoring may ultimately decrease the use of laboratory animals and optimize patient care. Other metabolites (e.g. glutamate, uric acid, ethanol) can be measured by simply changing the enzymes used for detection. The on-line measurement of glucose and lactate metabolism allows to study effects of drugs and other stimuli on cells, for example, to study individual metabolism separately in normally cooperating cells (e.g. neurons and glia), to study ischemic resilience in cell types, for the development of drugs for new therapeutic interventions (e.g. heart cells), and to study (human) biopsy samples, including chemotherapeutic agents on cancer cells.

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REFERENCES


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