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Quantitative On-Line Monitoring of Cellular Glucose and Lactate Metabolism in Vitro with Slow Perfusion

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An on-line in vitro perfusion technique is described that allows the continuous quantification of cellular glucose metabolism in vitro. Using biosensor technology, we 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 astroglia cultures, yeast cells, and human lymphocytes. The method shows that variations in oxygen delivery or exposure to a noncompetitive pseudosubstrate (here 2-deoxyglucose) affects normal glucose metabolism. The innovative advantage of the present system is that, in contrast to other devices including a recently described system, metabolism per cell can be quantified. The potential of in vitro on-line monitoring is discussed for application in studying normal and abnormal metabolism, toxic and nontoxic drug effects, and human tissue biopsies.

Monitoring cellular physiology in vitro not only provides 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. Accordingly, on-line monitoring of glucose and lactate metabolism allows the assessment of the effects of drugs and other stimuli on cells. So such a technique may facilitate studies on individual metabolism in normally cooperating cells (e.g., neurons versus glia), on ischemic resilience in various cell types, and on the development of new therapeutic drugs and metabolic and pharmacological studies with (human) biopsy samples, including cancer 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.[1,2] In well-oxygenized tissue, lactate may also be used as an (alternative) energy substrate, as shown in vitro as well as in vivo.[3−5] 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.[6,7] Therefore, the measurement of glucose consumption and lactate efflux can be used to monitor not only the condition of the cell but also its response to stimuli, such as drugs. Biosensor technology has most often been used for 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).[8−10]

Here we report a novel in vitro method for on-line monitoring of cellular glucose metabolism that is particularly useful in testing cell cultures. Several other flow-through systems have been described;[11−18] however, these flow-through systems have not been used for on-line monitoring[12−14,18] or are relatively large.[15−17] During the publication phase of the present study, a novel system allowing the measurement of extracellular oxygen, glucose, pH, and lactate on a discontinuous basis (stop-flow) appeared,[19] also emphasizing the need for in vitro monitoring systems. The here

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used submicroliter flow-through detection system is a slightly modified version of that used previously by us to monitor metabolism in vivo[20−25] and, more recently, organotypic brain cultures.[11] By applying continuous perfusion, we can monitor cell metabolism under constant conditions for periods up to several days. The chamber containing the viable cells should have a small dead volume, allowing perfusion rates lower than 2 μL/min. Low rates are essential to achieve minimal dilution of analytes, although low flow rates may 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. By changing flow rate (and consequently oxygen delivery), glucose metabolism and the aerobic ratio (the percentage aerobic metabolism by the cell culture) were optimized. The present technique allows the demonstration of the toxic effects of drugs (e.g., exposure to a noncompetitive pseudosubstrate, 2-deoxyglucose, 2DG, which is taken up by cells but cannot be metabolized). Finally, we illustrate the potential of the approach to quantify metabolism of individual cells. The potential of the present continuous monitoring system will be discussed in view of a related and recently published discontinuous monitoring system.[19]

EXPERIMENTAL SECTION

Cell Cultures and Cell Chamber. The cell types studied include 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 Corp., Bedford, MA). The culture inserts were transferred directly from the incubation chamber to the monitoring unit, by placing the culture insert tightly into the specially developed cell chamber (Figure 1). To investigate the applicability of our cell chamber for free-floating, thus nonadhered cells, yeast (*Saccharomices cerevisiae*) and freshly isolated human lymphocytes were monitored. Dry yeast (Bruggeman, Gent, Belgium) was dissolved in Dulbecco's modified Eagle medium (DMEM without glucose, Gibco, Invitrogen Corp., Breda, The Netherlands) supplemented with 5 mM glucose (Merck, Darmstadt, Germany), of which 50 μL of cell suspension was pipetted onto the insert (between 1.1×10^8 and 3.5×10^8 cells/mL). Lymphocytes were isolated from 10 mL of blood mixed with EDTA and AKE solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4). This suspension was kept at 0 °C for 20 min and centrifuged. The pellet was resuspended in AKE and again centrifuged. Cells were counted, and 25 or 50 μL of cell suspension was pipetted onto the insert (between 1×10^8 and 1.95×10^8 cells/mL).

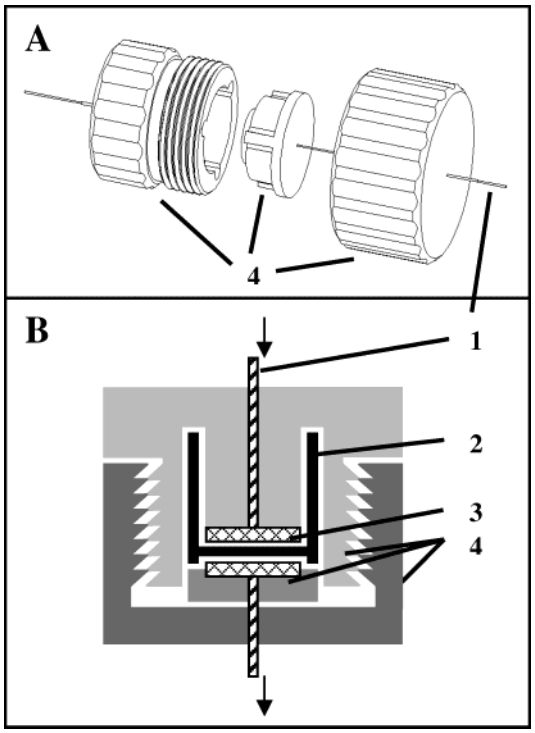

Figure 1. A solid drawing (A) and a schematic diagram (B) of the cell chamber. The central part of the cell chamber is the insert (2), on which the cells are grown (cell cultures) or pipetted (free floating cells). Oxygenized medium is transported through fused-silica tubing (1) and passes the insert. Arrows indicate flow direction. The insert is placed inside a holder (4) with or without two spacers (3). The assembled cell chamber has outer dimensions of 3.5 cm by 2.2 cm, with an inner diameter of 7.6 mm. The inner pieces of the cell chamber are slightly convex shaped (3%).

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 min, followed by trypsin inhibition medium at room temperature for 4 min). Cells were triturized, filtered, and centrifuged (10 min, 800 rpm). The pellet was resuspended in 1 mL of neurobasal medium (NB medium, supplemented with penicillin (100 units/mL)/streptomycin (100 μg/mL), 1 mM sodium pyruvate, 0.5 mM glutamine, B27 (1×) (All Gibco, Invitrogen Corp., Auckland, New Zealand), and counted (between 2.8×10^6 and 3.2×10^7 cells/mL). Cells (0.3×10^6) 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-well culture plates (Costar, Corning Inc., New York). Neuronal cultures (primary cells and NG108 cells) were grown in supplemented NB medium (see above) with either high glucose (25 mM) or low glucose (5 mM). Astrocytic primary culture and HEK cells were cultured in DMEM with 10% fetal calf serum (DMEM high (25 mM) glucose (astrocytes: or low (5 mM) glucose), penicillin (100 units/mL)/streptomycin (100 μg/mL), 1 mM sodium pyruvate, 2 mM glutamine). Cell cultures were incubated at 37 °C and 95% O_2, 5% CO_2. Metabolism of the cultures was monitored between 1 and 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,

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the culture insert was placed inside the cell chamber (Figure 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 (Figure 1), 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, with ~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 flow injection analysis (FIA) system.

Flow Injection Analysis System and Biosensors. The FIA system and the biosensors used in these experiments are essentially the same as described earlier.[22,25] In short, the cell chamber is connected to the analytical part of the setup by an intercalated valve with a 20-nL internal loop (Vici-Valco Instruments, Houston, TX). The valve injects a cell chamber sample every minute. By applying underpressure, oxygenized (95% O_2, 5% CO_2) DMEM with 5 mM glucose is pulled through the cell chamber toward the FIA with a Harvard 22-syringe pump (Harvard Apparatus, South Natick, MA). 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 flow cells (VT-03) and two potentiostats: the Decade (both from Antec Leyden B. V., Zoetermeer, The Netherlands) and an Amor amperometric detector (Spark Holland, Emmen, The Netherlands). 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, 10 mM). During the experiment, the glucose and lactate concentration is measured every minute and the currents (in nanoamperes) are recorded on a double-pen recorder (BD 112, Kipp en Zonen, Zoetermeer, The Netherlands) and by a data acquisition program (Chromeleon, Dionex Corp., Sunnyville, CA).

Experimental Procedure and Calculations. Most experiments lasted 2 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 oxygenated 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 pseudosubstrate, the glycolysis inhibitor 2DG (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. As will be discussed below, even in well-oxygenated medium, oxygen delivery to cells is diffusion dependent.

Use of glucose and lactate, lactate output, and the aerobic ratio, a possible indicator of aerobic glucose metabolism, were calculated, essentially as described.[10] The consumption of the substrates is calculated as the difference between the amounts of glucose or lactate that enter and leave the cell chamber per time unit, respectively. Accordingly, glucose used (mol/min) = flow (L/min) × (glucose in medium before perfusion (M) − glucose detected after perfusion (M)). Lactate used (mol/min) = flow (L/min) × (lactate in medium (without glucose) before perfusion (M) − lactate detected after perfusion (M)). Lactate output (mol/min) = flow (L/min) × lactate detected (after perfusion with glucose) (M). For convenience we expressed the consumptiondata in nanomoles per minute and femtomoles per minute (10^{-9} and 10^{-15} mol/min, respectively).

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 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 ~1000 measurements in an 8-h experiment. Most of the reported experiments lasted between 1.5 and 18 h. Flow rates between 0.03 and 1.5 μL/min were used. Figure 2 shows examples of experiments with lymphocytes (A), yeast (B), a primary astrocytic culture on glucose or lactate medium (C), and a neuronal cell line, with the effects of 2DG (D). Lymphocytes show decreased glucose use at lower flow rates (Figure 2A). 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 2B), so the aerobic ratio could not be calculated. Indeed, yeast (*S. cerevisiae*) does not produce lactate, as yeast avidly transforms glucose to ethanol.[26] The consumption of glucose is higher at 0.5 μL/min as compared to 0.1 μL/min in both aerobic and anaerobic conditions. The ratios were as follows: aerobic, at 0.1 μL/min 0.76 nmol/min and at 0.5 μL/min; anaerobic, 0.97 nmol/min, corresponding to 8.4×10^{-2} fmol/min per cell); 0.1 μL/min, aerobic, 0.27 nmol/min, and anaerobic, 0.17 nmol/min. All the other cell types released lactate in the glucose-containing medium. In about half 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 2D). This occurred in 15% of experiments with cultures grown on low glucose levels (5 mM). The levels of glycogen are high in normal (high glucose) culture, and glycogen can be broken down to glucose and lactate.[27] Accordingly we attribute the observed release of lactate from our cell cultures to the presence of glycogen.

We used the preparation of the astroglia cells for further quantitative estimations. Glucose use increases with flow rate from 0.14 nmol/min at 0.05 μL/min to 1.08 nmol/min at 1.5 μL/min, corresponding to a range of 0.4−3.3 fmol of glucose use per minute per cell. Lactate output was independent from the flow rate and ranges between 0.24 nmol/min at 0.05 μL/min to 0.86

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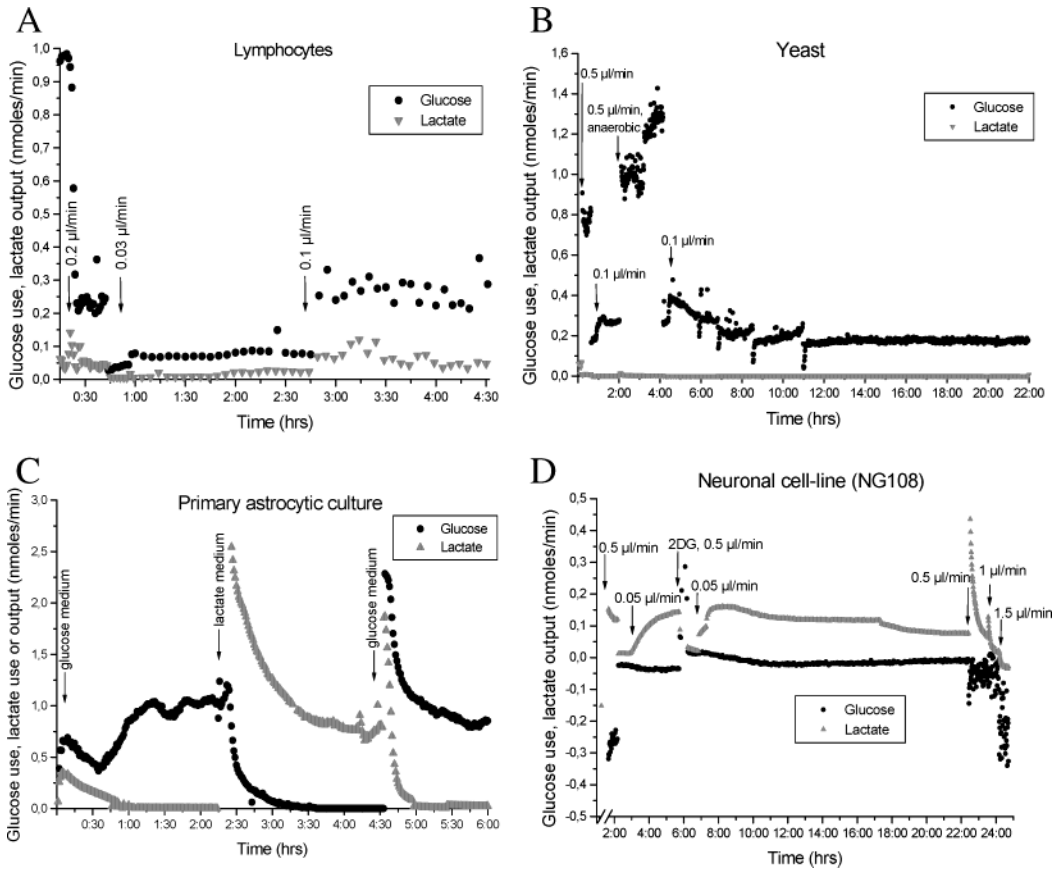

Figure 2. Examples of monitoring experiments. (A) shows the effect of various flow rates on the glucose use and lactate output in lymphocytes; (B) shows the same for yeast cells under both aerobic and anaerobic conditions. Yeast (*S. cerevisiae*) does not produce lactate. There is both glucose and lactate use in primary astrocytes (C, 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 (D). Both before and during 2DG measurement, glucose is released into the medium. During the 2DG challenge, the output of lactate decreases.

nmol/min at 1 μL/min (0.8−2.9 fmol of lactate output/min per cell). The percentage aerobic metabolism was calculated by adjusting the glucose uptake for the amount of lactate produced. The aerobic ratio is higher at higher flow rates, ranging from 15% at 0.05 μL/min to 60% at 1.5 μL/min in this experiment. The calculated aerobic ratio was highest at the highest flow rates 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 refs 1, 3, and 18). In an open cell chamber system,[11] 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. The aerobic ratio was calculated under the 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. However, glucose may also be metabolized via other pathways (e.g., pentose phosphate pathway, amino acid synthesis), which would decrease the aerobic ratio.[25,28,29] In addition, lactate may be (partly) derived from glycogen instead of glucose. Consequently, the calculated aerobic ratio may be an underestimation of the real values. In vitro lactate may serve as an additional energy source in primary cell cultures[6,7] and hippocampal slices.[11] Net lactate consumption was detected in primary neurons and astrocytes (example in Figure 2C: glucose use, 3 fmol/min per cell; lactate use, 5.5 or 2.75 fmol/min per cell in glucose equivalents), but not in any of our cell line experiments ($n = 4$). We also investigated the effect of 2DG, a glycolysis inhibitor, on glucose and lactate metabolism ($n = 3$). 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 2D, NG108 cells) compared to 5 mM glucose medium. We observed, that the consumption of the glucose and lactate remained stable over the whole experimental period, indicating that the cells remained vital, although we cannot exclude the possibility that some cells have been lost before the observation period. Accordingly, the order of magnitude of the rates of cellular consumption is a realistic estimation.[30]

CONCLUDING REMARKS

A setup for on-line continuous monitoring of cellular glucose and lactate metabolism of a variety of cell types is described. Due to the small-volume tubing, low dead volumes of the connections, and the on-line monitoring, the on-line monitoring system has a low lag time (~5 min) and a high temporal resolution. In a recently

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described related approach, based on stopped-flow perfusion technique and biosensor technology,[19] glucose consumption, lactate efflux, pH changes, and oxygen consumption were followed during intervals of 30 s, over periods of at least $2^1/_2$ h. Both this and our approaches emphasize that metabolic monitoring of 100 000 cells or less has now become a realistic option and that such technologies allow the investigation of drug effects of heterogeneous and homogeneous cell populations. The technology of the on/off perfusion approach of Eklund and co-workers[19] raises, however, some doubts about optimal deliverance of glucose and oxygen to the cells. It should be realized that during the stop condition substrate molecules near the cells are rapidly consumed and exhausted because of limited diffusion through the perfusion medium (and the filters containing the cell preparation). Accordingly, glucose will become more exhausted than oxygen, because of the large difference in diffusion rates ($\sim 30\times$), so the cells may become hypoglycemic. Consequently, the (apparent) cellular uptake of glucose and oxygen will rapidly become dependent at least in part on diffusion, rather than on the consumption of energy substrates. Support for this conclusion can easily be deduced from the graphs shown in the paper of Eklund and co-workers[19] as already within 10 s or less following the arrest of the perfusion, the rates of consumption of glucose and oxygen and the production rate of lactate decline. Therefore, we conclude that, compared to other thus far described systems, an innovative advantage of the present system is that it allows us to quantify cellular energy metabolism in vitro.

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