Long-term microfluidic glucose and lactate monitoring in hepatic cell culture

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Monitoring cellular bioenergetic pathways provides the basis for a detailed understanding of the physiological state of a cell culture. Therefore, it is widely used as a tool amongst others in the field of *in vitro* toxicology. The resulting metabolic information allows for performing *in vitro* toxicology assays for assessing drug-induced toxicity. In this study, we demonstrate the value of a microsystem for the fully automated detection of drug-induced changes in cellular viability by continuous monitoring of the metabolic activity over several days. To this end, glucose consumption and lactate secretion of a hepatic tumor cell line were continuously measured using microfluidically addressed electrochemical sensors. Adapting enzyme-based electrochemical flat-plate sensors, originally designed for human whole-blood samples, to their use with cell culture medium supersedes the common manual and laborious colorimetric assays and off-line operated external measurement systems. The cells were exposed to different concentrations of the mitochondrial inhibitor rotenone and the cellular response was analyzed by detecting changes in the rates of the glucose and lactate metabolism. Thus, the system provides real-time information on drug-induced liver injury *in vitro*. © 2014 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution 3.0 Unported License. [<http://dx.doi.org/10.1063/1.4876639>]

INTRODUCTION

Assessing the metabolic activity of a cell culture is a vital step in different applications like the investigation of metabolic pathways, evaluation of cell viability and differentiation, culture condition management in macroscopic bioreactors, or drug safety testing. The classical approach towards hazard identification in the field of *in vitro* toxicology comprises exposure to a chemical with subsequent pathological and histological assessment of organ or cell damage, respectively. A major drawback of such conventional end-point methods is that they do not reveal the pathways leading to the observed harm. Knowing the characteristic cytotoxic mechanism of a compound, however, offers the possibility of predicting its toxicity in target organs by means of *in silico* modeling and *in vitro* assays, thus reducing the need for animal testing. In the liver, the main characteristic types of injury include hepatitis, steatosis, phospholipidosis, and apoptosis.^{1,2} This paper, in particular, focuses on the drug-induced mitochondrial apoptotic pathway. One key method for investigating such toxicological pathways is monitoring the cells' metabolic state which is highly dynamic. This implies that the full picture can only be recognized by constant monitoring of the substrates and their metabolites, but not by taking a snapshot at a single point of time. An important part of this picture is to assess the fundamental glucose and lactate metabolism. Precise monitoring of these analytes allows for detecting chemically induced metabolic aberrations which can help to reveal the compound's full mode of action on the cell or organism level.

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In common cell culture and large-scale bioreactors, the glucose and lactate concentrations of the cell culture medium are usually measured employing colorimetric assays or bulky electrochemical detection systems. This inherently necessitates a manual and tedious sample preparation which poses obstacles to a continuous monitoring over extended periods of time. In contrast, monitoring the glucose and lactate metabolism with an automated set-up as presented here provides continuous information on the state of the investigated cells. Furthermore, it is applicable to all eukaryotic cell types, as glycolysis is, together with oxidative phosphorylation (OXPHOS), a highly conserved mechanism of adenosine triphosphate (ATP) generation.

Here, we probe the mitochondrial apoptotic pathway by exposing the cells to the complex I inhibitor rotenone. This strongly reduces OXPHOS which in turn induces compensation by upregulating other catabolic mechanisms, like anaerobic glycolysis. This is what our set-up then detects. In the cytoplasm of normal mammalian cells, glucose is converted to pyruvate which, in an aerobic environment, connects glycolysis to the citric acid cycle by oxidative conversion to acetyl-CoA. Downstream of this system, the electron donors NADH and FADH₂ are used for ATP generation by means of OXPHOS. In an environment of low oxygen tension or even absence of oxygen, fermentation occurs by conversion of pyruvate into lactic acid in the cytoplasm. This, however, yields much less energy in the form of ATP than OXPHOS.³ Under aerobic conditions, the cells cover their energy requirements almost entirely with ATP generated *via* OXPHOS.⁴ Changes in this energy metabolism are known to be a characteristic of cancer cells.⁵ One example is the Warburg effect, i.e., the increased conversion of glucose to lactic acid even under normoxia. Consequently, the ratio between the fermentation pathway and OXPHOS is shifted in these cases. A near-universal property of primary and metastatic cancers is the upregulation of glycolysis, accompanied by the reduction of pyruvate to lactic acid, even under aerobic conditions.⁶ Most likely, this is an adaptation to hypoxic microenvironments in the pre-malignant stadium of tumor development, when vascularization of the growing tumor tissue has not yet been fully established. Replacing glucose in the culture medium by galactose, i.e., preventing glycolysis, was shown to force cells to rely on OXPHOS, thus strongly sensitizing them to mitochondrial toxicants.⁷ These cytotoxic agents, like rotenone or antimycin A, act by inhibition of the respiratory electron transport chain.^{8,9} In the present study, rotenone was used to induce this effect.

EXPERIMENTAL

Cell culture

The C3A subclone of the human hepatoblastoma cell line HepG2 (CRL-1074; American Type Culture Collection (ATCC), Manassas, VA, US) was been selected for its ability to grow in glucose-deficient medium as well as for its high levels of albumin and alpha-fetoprotein production. High protein secretion was desired in order to demonstrate that the performance of the sensors is not affected by potential biofouling during long-term applications. The cells were maintained in Modified Eagle's Medium (MEM; Biochrom, Berlin, DE) supplemented with 10% fetal calf serum (FCS), 100 U ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin at 37 °C in a cell incubator (Binder, Tuttlingen, DE) at 95% relative humidity and 5% CO₂ supply. During experiments, a concentration of 1% FCS was used to prevent the formation of unintended air bubbles inside the fluidic pathway. Throughout all experiments, the same batch of FCS was used, thus avoiding natural sample-to-sample variations in the FCS composition.

Automated microfluidic measurement set-up

In regular intervals of 2 h, sample aliquots were withdrawn from 25 cm² cell culture flasks in which the cells were cultured. These aliquots were transported first to the sensor module and from there to waste. For that purpose, the flasks had been modified by equipping them with fluorinated ethylene propylene (FEP) tubings (IDEX, Oak Harbor, WA, US). 2 × 10⁶ cells in 7 ml were seeded at the beginning of each experiment run. The automated sampling was performed using a microfluidic set-up shown schematically in Fig. 1 and comprising two peristaltic pumps (Spetec, Erding, DE), five three-way solenoid valves (Buerkner, Ingelfingen, DE), a voltage

FIG. 1. Scheme of the set-up. By means of computer-controlled peristaltic pumps and solenoid valves, PBS buffer (blue), calibration solution (orange), and sample from the customized flask (red) were in turn fed to the sensor module (green), separated from each other by air gaps (light green) in a fully automated manner. Inset: Image of the microfluidic flow cell (PMMA).

source (PS 303 Pro; Voltcraft, CH), FEP tubing (IDEX, Oak Harbor, WA, US), and a computer-controlled relay board (24 V/DC7A; Conrad Electronic SE, DE) for switching of the pumps and valves. Except for the pumps, the complete fluidic part of the set-up was placed inside a cell incubator (TECO 10; Binder, Tuttlingen, DE) with settings identical to those used during the cell culture (see above). The electrochemical sensor module contained a potentiostat (EmStat 2; PalmSens, Utrecht, NL) and two amperometric thick-film sensor chips for the detection of glucose and lactate (sensor 560/660; BST, Berlin, DE) in a modular microfluidic flow cell milled from PMMA (cf. Fig. 1, inset). The fluidic connection was accomplished by flangeless fittings (nut P-226, ferrule P-200; IDEX, Oak Harbor, WA, US) in order to minimize the dead volume of the system. The total inner volume of the microfluidic flow cell was $26\ \mu\text{L}$, viz., $10.35\ \mu\text{L}$ per sensor module (i.e., glucose and lactate) and $2.5\ \mu\text{L}$ for the interconnection between them. The actual measurement cavity of each sensor module had a volume of $2\ \mu\text{L}$. For electrically connecting the sensor chips, spring contacts were integrated in the modules. The sensor chips were based on the enzymatic reactions of glucose oxidase (GOD) and lactate oxidase (LOD), respectively (see Fig. 2). Both yield H_2O_2 in amounts proportional to the analyte concentration. The hydrogen peroxide was then detected using platinum electrodes under polarized condition ($+0.45\ \text{V}$ vs. Ag/AgCl). At the given conditions, the measured current reached its plateau level within about 10 s (Fig. 2). Both the enzymatic generation and the anodic oxidation of H_2O_2 are fast as compared to the diffusion of glucose into the enzyme matrix and of H_2O_2 to the platinum electrode. The kinetics of the former reactions can, thus, be neglected with respect to the latter transport processes. Calibrations were carried out with each individual sensor (exemplarily in Figs. 2(b) and 2(c)). In the case of glucose, these calibrations yielded a linear current signal in the concentration range between 0.5 mM and 15 mM. Above this, a slight deviation from linearity was observed (Fig. 2(b)). However, this was irrelevant for the concentrations of interest here, i.e., below 5 mM. Equivalent calibrations of the lactate sensors showed a linear range from 0.5 mM to 10 mM (Fig. 2(c)). In the continuous experiments, the calibrations were performed using fresh cell culture medium (see below). For the sensor exemplified in Fig. 2(c), the fresh medium yielded a current signal of $(36 \pm 2)\ \text{nA}$ (red and dashed lines in the inset), corresponding to a lactate concentration of $(1.5 \pm 0.1)\ \text{mM}$. This amount was introduced through supplementing the medium with FCS. For measuring, both sensor chambers were filled simultaneously (delay between the sensors below 1 s), the flow was stopped by means of a valve (Fig. 1, valve 5) and the signal was recorded with the fluid stationary. No crosstalk between both sensors in their common flow cell was observed. This was verified in sensor characterization tests using different standard solutions containing both analytes glucose and lactate, either separately or together. Two reservoirs filled with phosphate-buffered saline (PBS; Biochrom, Berlin, DE) and fresh cell culture medium served for flushing and recalibration,

FIG. 2. (a) Exemplary time course of the glucose sensor signal (black curve). Background colors as in Fig. 1. The measurement of the sample from the cell culture (red background) was preceded by the calibration (orange background). The plateau values detected in both aliquots are derived by fitting a gaussian step function (calibration red line, measurement yellow line) to the data points. For details, see text. (b) Exemplary glucose calibration (blue points) with corresponding fits to the steps in current (red). The plateau values are depicted in the inset, also including a weighted fit line (correlation: 99.4%). (c) As before, but for one lactate sensor (correlation: 99.99%).

respectively. Mixing of the three different fluids inside the tubings and valves was prevented by introducing air gaps between them in the sequence indicated in Fig. 2(a). In appropriate interaction with the valves, the first peristaltic pump withdrew all components (fluids, air) through the system, while the second pump pushed back residual cell culture medium from the tubing into the flask by air pressure after completion of a measurement. This was necessary in order to

provide the sensor module with a fresh sample from the cell culture upon the next measurement. Thus, the presented system allows for analysis without pretreatment of the aliquot. The first data point was taken after cell attachment. Subsequently, the following fully automated routine was carried out in intervals of 2 h: first, a calibration with fresh medium was performed prior to each measurement of a sample from the flask. This served to correct for systematic changes of the set-up, i.e., decreasing performance of the sensor chip or slight temperature variations. Cell culture medium was used for calibrating instead of pure solutions, in order to compensate for potentially electrochemically interfering agents in the complex medium. After a subsequent cleaning with PBS, an aliquot from the flask was withdrawn and measured, followed by another buffer step.

Chemicals

Rotenone (Sigma, Schnelldorf, Germany) was dissolved in dimethyl sulfoxide (DMSO) to stock concentrations of 50 mM and 50 μ M, respectively. The compound was added to the culture medium after a significant glucose consumption and lactate synthesis had stably been established, i.e., after 24 h to 36 h. These stock solutions of two different concentrations were used, so that adding them to the culture medium in order to reach final concentrations of 200 nM and 200 μ M, respectively, led to equal DMSO concentrations of 0.4% v/v in all experiments. This concentration of DMSO had no detectable effect on cell viability or the rate of glucose consumption and lactate secretion when compared to controls without DMSO (data not shown). For characterizing the glucose and lactate sensors, a commercial calibration solution (CAL 4+M, Eschweiler, Kiel, Germany) containing 5 mM glucose and 5 mM lactate was used as well as solutions containing various concentrations of each of these analytes in PBS buffer separately (Figs. 2(b) and 2(c)).

Data processing

For processing the time course of the recorded sensor current, the calibration plateau and measurement plateau were analyzed separately but in an identical way (Fig. 2(a)): first, each data point was assigned a weight depending on how strongly it varied from its two neighboring points, in order to favor the plateaus over the rise and noise. Subsequently, periods comprising the baseline, the rise, and the plateau were fitted using a gaussian step, i.e., the error function, to obtain the step height in units of current (Mathematica, Wolfram Research, Long Hanborough, UK). We are aware that this function is symmetrical while the graph of the current is clearly not (cf. Fig. 2(a)); however, it yielded considerably more robust results than analogous routines based on assuming asymmetric (logistic) functions instead. In order to exclude any drift in the sensor signal over the experiment time of several days, each measurement taken was immediately preceded by its own calibration which was used for converting the current value into the concentration. The linear ranges of the sensors (see above) were confirmed by separate tests (data not shown). Glucose consumption and lactate production rates were finally obtained from the slope of the time course of the corresponding concentration (see Figs. 3(b) and 3(d)). Since the concentration of lactate was non-linear in time (cf. Fig. 3(d)), its production rate (slope) was time-dependent.

RESULTS AND DISCUSSION

We developed a measurement set-up that allows for continual monitoring of glucose consumption and lactate secretion by means of a microfluidic sampling of culture medium from a microbioreactor and subsequent evaluation (Fig. 1). The focus was not on optimizing the reactor, but rather on attaining a fully automated sensor system which functions with high reliability over days while consuming only minute sample aliquots as they are available from a microbioreactor. In our case, the minimum aliquot required per simultaneous measurement of both analytes was 50 μ l. In contrast, conventional measurement systems for macroscopic long-term cultivation reactors require a sample volume of 50 μ l–500 μ l per analyte,¹⁰ e.g., Bioprofile

FIG. 3. (a) and (c): Superimposed raw data of glucose (a) and lactate (c) measurements from one exemplary experiment (0.2 μM rotenone). Over the time course of the experiment, the glucose signal obtained from the sample aliquot drawn from the cells decreased while the lactate signal increased. (b) and (d): Changes of the glucose (b) and lactate (d) concentration in culture medium supernatant after the application (arrows) of 0.2 μM rotenone (green) or 200 μM rotenone (red) and in a control without rotenone (blue). In (b), two continuous lines were fitted to the data points of each set, with the zero slope after the bend being fixed, i.e., leaving three free parameters. The solid lines in (d) represent the curves of monoexponential functions fitted to the data points.

100plus, Nova Biomedical, Waltham, MA, US or YSI 7100, YSI Life Sciences, Yellow Springs, OH, US. In general, however, these detection systems only operate off-line, thus necessitating a cumbersome manual sample preparation. The same disadvantage holds true for colorimetric assays which need less volume (2 μl –12 μl).

Measurements were taken in intervals of 2 h. If a higher density of data points was desired, this could be further reduced—up to the time required for one complete measurement run as described above, i.e., about 5 min. In the set-up presented here, each aliquot of medium drawn from the cells was discarded after measurement. An advanced set-up has also been realized that feeds back the sample, thus preserving the reactor volume. This is possible since the measurement does not change the sample composition.

The C3A cells robustly exhibited glucose consumption as well as lactate synthesis within 2 h to 6 h after seeding, when they were fully attached to the culture flask and regained their normal metabolic activity (Fig. 4(a)). Although the emphasis in developing the measurement set-up was on comparing relative metabolic rates observed in cells with and without toxins applied, our system also provides absolute rate values. During the experiments, the cells were cultured in medium containing only 1% FCS which almost entirely halted the proliferation, thus keeping the cell number nearly constant. Our data show that the metabolic pathway of anaerobic glycolysis is not affected by this reduced FCS concentration, since unchallenged control cells showed glucose consumption rates of $(43 \pm 1) \mu\text{M h}^{-1}$ per 10^6 cells, well in line with literary data.^{11,12} Avoiding bubble formation in the microfluidics was not the only reason for choosing a low FCS concentration. It also reduces non-specific protein binding of the test compounds. Additionally, low-serum or even serum-free conditions is often required for primary or iPS cells which is in the focus of developing bioreactors for toxicological assays. Slight deviations in the metabolic rates between different experiments occurred and can safely be ascribed to small variations in the total cell number used during initial cell seeding. These variations were introduced by the manual cell counting and did in no way affect the operation of the

FIG. 4. Confirmation by light microscopy of the cell damage already deduced from the electrochemical sensor signals. HepG2/C3A cells in the microbioreactor (a) before and (b) after application of 200 μM rotenone. The cells in (a) were viable and exhibited their normal shape. The cells in (b) had detached from the substrate and disintegrated; with aggregates of cell debris floating in the cell culture medium. Size bar is 200 μm .

detection set-up. The run-to-run variation was negligible as compared to the huge effect caused by the drug-induced metabolic changes (see Fig. 3). The initial glucose consumption rate of cells exposed to 0.2 μM and 200 μM rotenone were $(49.4 \pm 0.8) \mu\text{M h}^{-1}$ per 10^6 cells and $(42 \pm 3) \mu\text{M h}^{-1}$ per 10^6 cells, respectively, which agrees well with the consumption rate of the control named above. Being able to precisely measure metabolic rates is an added benefit of the presented system and is due to its highly controllable experimental conditions.

Untreated cells consume the glucose in the culture medium completely within 2–3 days, while the lactate concentration reaches a plateau (Figs. 3(b) and 3(d), blue curves). Challenging the cells with chemical compounds that act on the metabolism changes the kinetics of the pathways detailed in the introduction. As a model compound for altering the glucose consumption and lactate secretion, the mitochondrial inhibitor rotenone was employed. Rotenone inhibits the electron transport system in the mitochondrial respiratory chain through binding to the complex I. The cells were exposed to two different rotenone concentrations: at a sub-threshold concentration of 0.2 μM , only minor toxic effects on the cells are to be expected, while a super-threshold concentration of 200 μM causes immediate cell death. Both events were observed by the changes they induced in the glucose consumption and lactate synthesis: after addition of 0.2 μM rotenone, both metabolites were not affected significantly (Figs. 3(b) and 3(d), green curves). In normal cells, hypoxia or an inhibition of the mitochondrial function, like the one introduced through rotenone, induces a shift in ATP synthesis mechanisms towards glycolysis, also known as the Pasteur effect.^{13,14} This is mediated by several steps that are generally based on the adenosine monophosphate (AMP) to ATP ratio in the cell, including activation of phosphofructokinase and the AMP-activated protein kinase (AMPK) pathway.^{15–17} By this, the level of free ATP in the cell is maintained. Wu *et al.* reported this effect of upregulated glycolysis upon mitochondrial inhibition in epithelial cancer cells.¹⁸ Our data do not indicate an enhanced glucose consumption after exposure to a sub-lethal concentration of rotenone. Most likely, this behavior is related to the abnormally high glycolysis rate of the HepG2 cancer cell line.¹⁹ This masks the additional activation of glycolytic pathways. The fact that the glycolysis does not increase may, furthermore, reflect the comparatively very high metabolic activity of hepatocytes. For further elucidating this, analogous measurements using primary human hepatocytes (Mediatec, Heidelberg, DE) or HepaRG cells in mono-culture and co-culture with human hepatic stellate cells are currently underway.

In order to demonstrate the opposite effect, namely, the monitoring of an attenuated or even completely abolished metabolism, the cells were exposed to a super-threshold rotenone concentration of 200 μM . As a result, cell viability dropped drastically within 2 h to 6 h, leading to accordingly reduced metabolic rates (Figs. 3(b) and 3(d), red curves). The inhibition of complex I by exposure to rotenone induces several processes that finally lead to the activation of the apoptotic mitochondrial pathway. Amongst these are the increased production of reactive oxygen species (ROS), the release of cytochrome c, and the reduction of the mitochondrial

transmembrane potential.^{20,21} Exposure to such a high concentration of rotenone renders the aforementioned compensating processes of ATP production negligible, while the whole metabolism of the cells is directed towards apoptosis and necrosis. In consequence, a sudden and strong drop in the number of viable cells were observed (Fig. 4), thus superseding any quantification, e.g., through cell counting. Accordingly, the decrease in the glucose concentration and the increase of the lactate concentration in the medium practically came to a halt after exposure to 200 μM rotenone.

As mentioned, our control measurement showed a constant glucose consumption which depleted the glucose reservoir in the bioreactor after 60 h (Fig. 3(b)). Adding rotenone at a low concentration of 0.2 μM did not change this rate appreciably, resulting in a glucose depletion after 50 h. In contrast, the higher concentration of 200 μM led to a pronounced cytotoxic effect: 9 h after addition of the rotenone, the glucose consumption virtually stopped and its concentration leveled out at (2.86 ± 0.07) mM, i.e., long before the glucose reservoir was exhausted.

Concerning the lactate secretion, again the control and the lower rotenone concentration measurement did not differ significantly from each other, coherent with the glucose measurements (Fig. 3(d)): in the control measurement, the lactate production rate dropped from its initial value of (50 ± 10) $\mu\text{M h}^{-1}$ per 10^6 cells to (17 ± 2) $\mu\text{M h}^{-1}$ per 10^6 cells at the end of the experiment after 62 h. In the measurement using the rotenone concentration of 0.2 μM , this rate decreased from (47 ± 6) $\mu\text{M h}^{-1}$ per 10^6 cells to (13.8 ± 0.9) $\mu\text{M h}^{-1}$ per 10^6 cells (see Fig. 3(d)).

In contrast, monitoring the lactate concentration after the application of 200 μM rotenone confirmed the striking effect of this high effector concentration on the metabolism of the cells. It strongly inhibited the lactate secretion: the initial lactate production rate of (38 ± 3) $\mu\text{M h}^{-1}$ per 10^6 cells fell to (3.4 ± 0.3) $\mu\text{M h}^{-1}$ per 10^6 cells. Presumably, active lactate secretion by the cells stopped together with their glucose consumption. The continued slight rise in the lactate concentration detected in the cell culture medium may be attributed to lactate released from the cytosol of disintegrating, apoptotic cells.

CONCLUSIONS

In this study, we demonstrated the continuous monitoring of glucose and lactate concentrations over several days using minimal sample volumes. This allows for using high-throughput microbioreactors as they are required for toxicity screening of chemical, pharmaceutical, and cosmetic compounds. Our system is automated and sufficiently sensitive to detect changes in the basal metabolism in real-time, without necessarily having to wait for an all-dead plateau as was done here purely for demonstration. The sensor module constitutes a main building block of any bioreactor system with a feedback control for adjusting the medium conditions. Until now, such systems were only available for large-scale bioreactors. Their miniaturization, enabled by equally miniaturized sensor systems as shown here, provides major benefits that allow for analyses not possible before, in that they (a) reduce the necessary amount of cells, thus making it possible to work with rare cells, like iPS cells, (b) enhance the efficiency by means of multiplexed screening, and (c) provide the ability to maintain optimum culture conditions as required for long-term studies, e.g., the chronic exposure to chemicals or repeated-dose testing.

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