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Dose-response in a high density three-dimensional liver device with real-time bioenergetic and metabolic flux quantification

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ABSTRACT

Real-time dose-response curves for fructose have been non-invasively determined in primary rat hepatocyte alginate spheroids cultured in a NMR-compatible fluidized-bed bioreactor. Using ^{13}C–labeled glucose and glycine culture medium, fructose dose was compared to glucose uptake and glycogen synthesis rate using ^{13}C NMR spectroscopy, and to ATP and fructose-1-phosphate concentration using ^{31}P NMR spectroscopy. A highly efficient multicoaxial perfusion system maintains high density 3-D hepatocyte cultures, permitting ^{13}C and ^{31}P NMR spectral time courses with 1 min time points. The perfusion system was turned off to demonstrate its efficiency and effect on the metabolites. Within 16 min, glycogen plummeted, lactate became the largest ^{13}C–glucose metabolite via anaerobic glycolysis, while glutathione was the largest ^{13}C–glycine metabolite. ATP depletion and fructose-1-phosphate formation demonstrated a dose response with a 3 h EC50 of 19 mM ± 8.9 mM and 17.4 mM ± 3.7 mM, respectively. Computational modeling of mass transfer corroborated experimental results and helped determine the optimal bioreactor loading densities, oxygen concentration, and perfusion rates to maintain physiologically-relevant nutrient levels. The total bioreactor plus perfusion loop has a dead volume of 2 ml, and contains 5 million hepatocytes. Due to the non-invasive measurements, there is a reduction of animal tissue by an order-of-magnitude, depending on the number of time points in an experiment. This dynamic flux approach may have generic utility for dose-response studies monitoring multiple metabolic reactions in other primary mammalian cells, such as human, that have strict oxygen demands.

1. Introduction

The first dose-response studies using 2D hepatocyte cultures or suspensions detected LDH leakage as a viability measure in response to toxicity. The first sub-lethal response measurements were the synthetic rates of primary metabolic pathways, specifically glycogen (Wagle et al., 1976; Nyfeler and Walter, 1979; Krack et al., 1980; Rifkin et al., 1983; Rognstad, 1984; Goethals et al., 1984). Glycogen is the major hepatic storage polymer for energy and its rate of synthesis is an early biomarker of toxicity (Goethals et al., 1984). The newest "omics" of systems toxicology that quantifies rates of synthesis or degradation is fluxomics (Cascante and Marin, 2008). We have established a 5 mm NMR-compatible bioreactor that obtains both bioenergetics via ^{31}P NMR spectroscopy and metabolic flux via ^{13}C NMR spectroscopy. We demonstrate the application of this device to fluxomics and toxicology with determining the effect of fructose dose on glucose uptake and glycogen synthetic rate. We also demonstrate the effect of hypoxia on glycogen degradation, anaerobic glycolysis via lactate production, and glutathione (GSH) dynamics. Because NMR is non-invasive, there is a savings in animal tissue using this device which is related to the number of time points in the dynamic experiment. For example, in the 20 time points comprising the time courses presented in this study, there is a savings of 19 time points, which is a function of the total time points in an experiment minus one.

Despite decades of development of NMR-compatible bioreactors for primary hepatocytes, ^{13}C flux measurements have not been attained in bioreactors small enough to fit in a standard 5 mm NMR probe (Lee et al., 2017). Recently, electrostatic alginate encapsulated rat hepatocytes were maintained for 30 h in a 10 mm NMR-compatible fluidized-bed bioreactor with coaxial oxygenation (Jeffries et al., 2013). We

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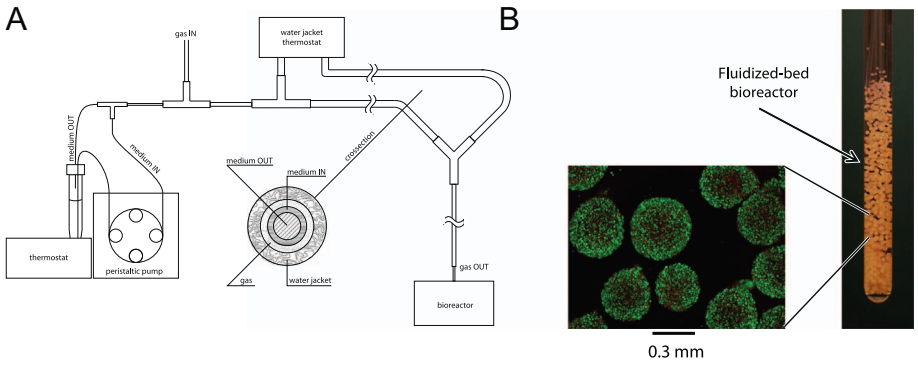

Fig. 1. A schematic of the multicoaxial perfusion line and its integration into the fluidized-bed bioreactor (A). The multicoaxial perfusion line oxygenates the fluidized-bed of alginate-encapsulated hepatocytes of about 500 μm diameter (B). Fluorescent image shows the live (green) alginate-encapsulated hepatocytes using AO/PI live/dead (green/red) stain (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

extend this technology by developing a highly efficient multicoaxial perfusion system for a 5 mm NMR-compatible fluidized-bed bioreactor to accommodate the more commonly available 5 mm NMR probes. The perfusion system maintains efficient oxygen delivery, while maximizing cell density within the sensitive volume of the NMR probe to maximize the NMR signal-to-noise ratio (SNR).

2. Methods

2.1. Multicoaxial perfusion loop with 5 mm fluidized-bed bioreactor

We have extended the multicoaxial bioreactor design (Wolfe et al., 2002) through the entire length of the perfusion lines, comprised of four coaxial tubes, whereby the outer water-jacketed compartment insulates the gas line, which oxygenates the in-going medium compartment (Fig. 1A). The out-going medium compartment is in the center tube, and this medium is heated to 37 °C in the NMR magnet and further heats the in-going medium in the second compartment from center (inset schematic, Fig. 1A). Oxygenating the annular compartment rather than the inner tube compartment increases the surface-area-to-volume ratio for gas exchange by a factor of two. There is also a bubble trap at the top of the 5 mm glass tube, as bubbles will destroy NMR spectral quality. The gas is pre-heated in a heat exchanger prior to entering the perfusion loop, while the media is also preheated (Fig. 1A). There are about 4000 hepatocytes per 500 μm diameter alginate spheroid, and about 1300–1500 spheroids loaded into the bottom of a 5 mm NMR tube resulting in about 5–6 million hepatocytes (Fig. 1B). The spheres are stained with acridine orange/propidium iodine (AO/PI) stain with green and red color representing live and dead cells, respectively.

2.2. Hepatocyte isolation

Freshly isolated primary rat (Wistar) hepatocytes were purchased from Triangle Research Laboratory (Research Triangle Park, NC). The cell suspension was spun at 50 × *g* for 5 min. The supernatant was discarded, cells were resuspended in high glucose DMEM with 10% fetal bovine serum, and viability was assessed using trypan blue exclusion. Experimental criteria required that viability must be > 90%.

2.3. Hepatocyte encapsulation, bioreactor operation, and tissue extraction

A solution of (2:1) rat hepatocytes: 2% alginate making between 4 and 5 × 10^7 cells/ml was electrostatically encapsulated as previously described for the 10 mm bioreactor (Jeffries et al., 2013). The 5 mm NMR tubes were filled to 11 mm with spheroids and perfused with DMEM media with 10% FBS at a rate of 0.6 ml/min, and oxygenated with gas containing 50% O_2 as previously described (Jeffries et al., 2013). At the end of the study the cells were released from spheroids with 150 mM citrate, rinsed three times with phosphate buffered saline, and extracted with 60% acetonitrile. Medium samples were obtained before and after each dosing experiment as previously described (Jeffries et al., 2013).

2.4. NMR spectroscopy and analysis

A Varian INOVA 600 MHz NMR spectrometer with a 5 mm switchable broadband probe was used. A one-pulse ^{31}P NMR spectral parameters were previously described (Jeffries et al., 2013). A one-pulse ^{13}C NMR sequence using a 90° flip angle and 2 s recycle time with WALTZ decoupling during acquisition gave the best SNR for glycogen. The spin-spin relaxation (T_1) of glycogen in rat liver was found to be 0.2 s and 0.3 s at 2 Tesla and 6 Tesla (Roden et al., 2001), respectively, and therefore, polarization transfer pulse sequences such as INEPT or DEPT did not enhance SNR. Nuclear Overhauser effect was not used because the unequal contribution to spectral peaks confounds quantitative analysis, and to avoid probe overheating. Parameters for tissue extracts and medium ^{1}H NMR analysis are previously described (Jeffries et al., 2013). The ^{31}P and ^{13}C NMR spectra were baseline corrected, processed with a 25 Hz and 30 Hz exponential, respectively, and group integrated using ACD software.

2.5. Rate and concentration determination

The ^{13}C NMR peak areas were plotted from 20 to 70 min from the start of the addition of fructose doses and fit with a linear least square function using MatLab. The peak area of the α/β C1 peak of glucose (96/92 ppm) represents 14.22 mM, and subsequent peaks in the time course of glucose, glycogen, and lactate were calibrated to this peak at the 10 min time point. The ^{31}P NMR peak areas were expressed relative to phosphate in the medium, which is 0.92 mM, as previously described (Jeffries et al., 2013).

The percolating spheroids increase to 33 mm axially from initial packed spheroids of 11 mm, or about two-thirds of the initial spheroid density. Randomly packed spheres will fill 64% of total volume, and this total volume filled by the spheres is reduced by two-thirds when spheroids are percolating resulting in 21% of the sensitive volume of the NMR coil filled with spheres. Therefore, the rest of the volume (79%) is filled with media containing 18 mM glucose, resulting in an effective glucose concentration of 14.22 mM within the sensitive volume of the NMR coil. The rates are expressed as the percent of control as previous described by Susanna Baque et al. (1994).

2.6. Computational modeling

COMSOL Multiphysics 4.3 (The COMSOL Group, Stockholm Sweden) with the 2D and 3D computation fluid dynamic (CFD) module was used to simulate oxygen profiles at different oxygen concentration and perfusion rates in medium using Michaelis-Menton oxygen consumption kinetics from Table 1. The geometry of the bioreactor and spheroids are shown in Fig. 4 and other details of the simulation are described in Pedersen et al. (2016).