Differential Incorporation of Glucose into Biomass during Warburg Metabolism

Ying-Jr Chen,[§] Xiaojing Huang,[ǂ] Nathaniel G Mahieu,[§] Kevin Cho,[§] Jacob Schaefer,[§] and Gary J Patti*[,§,¶,ǂ]

Author Affiliations: Department of [§]Chemistry, [¶]Genetics and [ǂ]Medicine, Washington University, St. Louis, MO 63130, United States

Supplementary Methods

Materials

Solvents and chemicals for liquid chromatography were obtained from Sigma-Aldrich (St. Louis, MO). Stable isotope-labeled compounds were obtained from Cambridge Isotope Laboratories (Tewksbury, MA). Cell-culture media and reagents were purchased from Life Technology (Grand Island, NY). HeLa cells were obtained from the Tissue Culture Support Center at Washington University in St Louis.

Cell culture

HeLa cells were grown in high-glucose DMEM (4.5 g/L glucose) containing 10% FBS (Atlanta Biologicals, Flowery Branch, GA) and 1% penicillin/streptomycin at 37 °C with 5% CO_2. Isotope labeling was achieved by culturing cells in media supplemented with ^{13}C-labeled glucose as specified.

Metabolite isolation and liquid chromatography/mass spectrometry (LC/MS)

Approximately $1X10^6$ cells were plated in a 100-mm culture dish containing either natural-abundance glucose or 50% U-^{13}C glucose / 50% natural-abundance glucose. All media had a 22.2 mM concentration of glucose. Cells were cultured for 48 hours. Cells were then washed with phosphate-buffer saline (PBS) and high-performance liquid

chromatography (HPLC) grade water, quenched with 1 mL cold HPLC-grade methanol, scraped from the plate, and pelleted. Pellets were dried on a SpeedVac and subsequently lyophilized. Dried samples were weighed out and extracted by using the previously described protocol (detailed below).[1]

Metabolites were isolated from lyophilized cell pellets by using methanol/acetonitrile/water (2:2:1) adjusted to maintain a ratio of 1 mL of solvent per 1 mg of dried cellular material. Following the previously described protocol, extracts were dried with a speedVac and then reconstituted in acetonitrile/water (1:1). The final volume of reconstitution solvent was adjusted to 100 µL per 1 mg of starting dried cellular material.

Eight µL of each extracted sample was injected onto a Luna Aminopropyl column (3 µm, 150 mm × 1.0 mm I.D., Phenomenex, Torrance, CA) coupled to an Agilent 1260 capillary HPLC system (Santa Clara, CA) with a flow rate of 50 µL/min. The column was used in hydrophilic liquid interaction chromatography (HILIC) mode with the following buffers and gradients: A = 95% water, 5% acetonitrile (ACN), 10 mM ammonium hydroxide, 10 mM ammonium acetate, pH 9.5; B = 95 % ACN, 5 % water; 100 % B from 0-3 min, 100 % to 0 % B from 3-37 min and 0 % B from 37-40 min. For the analyses performed with reversed-phase (RP) chromatography, 5 µL of each extracted sample was injected onto an Agilent ZORBAX SB-C18 column (5 µm, 150X0.5 mm) coupled to an Agilent 1260 capillary HPLC system with a flow rate of 20 µL/min. The solvent system for liquid chromatography comprised of water with 0.1% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B). The solvent gradient was 2% to 10% B from 0-10min, 10% to 20% B from 10-15 min, 20% to 95% B from 15-50 min,

95% to 98% B from 50-60 min. Mass spectrometry (MS) detection was carried out on an Agilent 6540 Q-TOF in negative ESI (electrospray ionization) mode with a mass range of 30-1500 m/z. These data were processed by using X^{13}CMS.[2]

Extracted samples were also analyzed using a Thermo Q Exactive Plus mass spectrometer in negative mode at 70,000 resolving power interfaced with a Dionex UltiMate 3000 UHPLC system to perform HILIC separation according to the same method and flow rate detailed above.

Data processing

All raw data files were converted into mzXML files using msconvert.[3] The data analysis was performed by using the XCMS package implemented in R with the following parameters: centWave algorithm for feature detection (peakwidth = 20-200 s and ppm = 5) and obiwarp algorithm for retention-time correction. The output xcmsSet() object containing labeled and unlabeled data was then forwarded to X^{13}CMS for the isotopic labeling analysis.[3] The peaks corresponding to the isotoplogues of each base peak were identified based on the following inputs: (i) mass difference between ^{13}C and ^{12}C=1.003355 Da, (ii) mass of ^{12}C=12.000000 Da, (iii) ppm = 20, (iv) RTwin = 10 s, and (v) baseline noise = 10,000.

Fate of U-^{13}C-glucose in HeLa cells by solid-state ^{13}C NMR

Approximately 4X10^6 cells were cultured in a T-175 flask containing either natural-abundance glucose or 5% U-^{13}C glucose / 95% natural-abundance glucose. NMR experiments were also performed using 50% U-^{13}C glucose / 50% natural-abundance glucose. All media had a 22.2 mM concentration of glucose. The results from each experiment were consistent; however, data from the 5% U-^{13}C glucose experiment have

reduced spectral overlap and are therefore shown in Figure 2. Cells were cultured for 48 hours and media were collected. Cells were then washed with PBS three times, scraped from the plate, combined, pelleted, and snap-frozen in liquid nitrogen (LN_2). Frozen cell pellets and media were lyophilized prior to NMR analysis.

Solid-state NMR spectrometer and pulse sequences

Experiments were performed at 12 Tesla with a transmission-line probe having a 12-mm long, 6-mm inner-diameter analytical coil, and a Chemagnetics/Varian ceramic spinning module. Samples were spun using a thin-wall Chemagnetics/Varian (Fort Collins, CO/Palo Alto, CA) 5-mm outer diameter-zirconia rotor at 7143 Hz, with the speed under active control and maintained to within ±2 Hz. A Tecmag Libra pulse programmer (Houston, TX) controlled the spectrometer. A 2-kW American Microwave Technology (AMT) power amplifier was used to produce radio-frequency pulses for ^{13}C (125 MHz). The ^{1}H (500 MHz) radio-frequency pulses were generated by a 2-kW Creative Electronics tube amplifiers driven by 50-W AMT amplifier. All final-stage amplifiers were under active control.[4] The π-pulse lengths were 9 μs for both ^{13}C and ^{1}H. Proton-carbon-matched cross-polarization transfers were made in 1.5 ms at 56 kHz. Proton dipolar decoupling was 100 kHz during data acquisition. Quantitation for Table 1 was based on the following ^{13}C NMR resonance frequencies: secreted lactate, 22 ppm (lyophilized media); insoluble proteins, 174 ppm (after extraction); soluble peptides, 174 ppm (lyophilized supernatant); sugars/glycerol, 70 ppm; lipids, 30 ppm; and purines, 150 ppm. The methylene carbon peak at 30 ppm is unique to lipids and was therefore chosen for quantitation of lipids. Error is estimated to be 10% of stated percentage based on signal-to-noise ratios (e.g., the percent of glucose-derived carbons in lipids is 4 ± 0.4%).

Statistical Analysis

All experiments were performed in pairs, with parallel cultures being identical other than replacement of U-^{12}C glucose with U-^{13}C glucose. Each NMR spectrum shown resulted from averaging 20k scans. The NMR was performed on glucose-enriched whole cells (n=5) in addition to associated natural-abundance controls (n=5) as well as fresh/spent cell-culture media. NMR spectra for replicate experiments showed no significant differences. Samples analyzed by LC/MS included cells enriched with U-^{13}C glucose (50%) for 48 hours (n=3) in addition to associated natural-abundance controls (n=3). Isotopologue patterns shown represent group averages for all samples analyzed. Isotopologue patterns from natural-abundance control samples (grown in parallel) are also shown as averages within the same plots.

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