INTEGRATION OF FLUX MEASUREMENTS TO RESOLVE CHANGES IN ANABOLIC AND CATABOLIC METABOLISM IN CARDIAC MYOCYTES

*Andrew A. Gibb\textsuperscript{1,2,3}, *Pawel K. Lorkiewicz\textsuperscript{1,2}, Yu-Ting Zheng\textsuperscript{1,2}, Xiang Zhang\textsuperscript{4,5,6}, Aruni Bhatnagar\textsuperscript{1,2}, Steven P. Jones\textsuperscript{1,2}, and Bradford G. Hill\textsuperscript{1,2}

\textsuperscript{1}Institute of Molecular Cardiology, \textsuperscript{2}Diabetes and Obesity Center, \textsuperscript{3}Department of Physiology, \textsuperscript{4}Department of Chemistry, \textsuperscript{5}Department of Pharmacology and Toxicology, \textsuperscript{6}Center for Regulatory and Environmental Analytical Metabolomics, University of Louisville, Louisville, KY 40202

*Indicates equal contribution

Running Head: Integrated flux measurements in cardiomyocytes

Corresponding Author: Bradford G. Hill, Ph.D., Institute of Molecular Cardiology, Diabetes and Obesity Center, Department of Medicine, University of Louisville, 580 S. Preston St., Rm 321E, Louisville, KY, 40202; Tel: (502) 852-1015, Fax: (502) 852-3663, E-mail: bradford.hill@louisville.edu
ABSTRACT

Although ancillary pathways of glucose metabolism are critical for synthesizing cellular building blocks and modulating stress responses, how they are regulated remains unclear. In this study, we used radiometric glycolysis assays, $[^{13}\text{C}_6]$-glucose isotope tracing, and extracellular flux analysis to understand how phosphofructokinase (PFK)-mediated changes in glycolysis regulate glucose carbon partitioning into catabolic and anabolic pathways. Expression of kinase-deficient or phosphatase-deficient 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in rat neonatal cardiomyocytes coordinately regulated glycolytic rate and lactate production. Nevertheless, in all groups, >40% of glucose consumed by the cells was unaccounted for via catabolism to pyruvate, which suggests entry of glucose carbons into ancillary pathways branching from metabolites formed in the preparatory phase of glycolysis. Analysis of $^{13}\text{C}$ fractional enrichment patterns suggests that PFK activity regulates glucose carbon incorporation directly into the ribose and the glycerol moieties of purines and phospholipids, respectively. Pyrimidines, UDP-N-acetyl-hexosamine, and the fatty acyl chains of phosphatidylinositol and triglycerides showed lower $^{13}\text{C}$ incorporation under conditions of high PFK activity; the isotopologue $^{13}\text{C}$ enrichment pattern of each metabolite indicated limitations in mitochondria-engendered oxaloacetate, acetyl CoA, and fatty acids. Consistent with this notion, high glycolytic rate diminished mitochondrial activity and the coupling of glycolysis to glucose oxidation. These findings suggest that a major portion of intracellular glucose in cardiac myocytes is apportioned for ancillary biosynthetic reactions and that PFK coordinates the activities of the pentose phosphate, hexosamine biosynthetic, and glycerolipid synthesis pathways by directly modulating glycolytic intermediate entry into auxiliary glucose metabolism pathways and indirectly regulating mitochondrial cataplerosis.
Keywords: glycolysis, mitochondria, metabolomics, stable isotope, heart failure, cardiac hypertrophy

ABBREVIATION LIST

DHAP, dihydroxyacetone phosphate; ECAR, extracellular acidification rate; F6P, fructose-6-phosphate; FA, fatty acids; G6P, glucose-6-phosphate; GAP, glyceraldehyde-3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GLP, glycerolipid synthesis pathway; HBP, hexosamine biosynthetic pathway; kd-PFK2, kinase-deficient PFK2; LDH, lactate dehydrogenase; NRCM, neonatal rat cardiomyocyte; OCR, oxygen consumption rate; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PG, phosphoglycerol; PS, phosphatidylserine; pd-PFK2, phosphatase-deficient PFK2; PFK1, phosphofructokinase 1; PFK2, phosphofructokinase 2 (also denoted 6-phosphofructo-2-kinase/fructo-2,6-bisphosphatase); PFKFB1, 6-phosphofructo-2-kinase/fructo-2,6-bisphosphatase isoform 1; PFKFB2, 6-phosphofructo-2-kinase/fructo-2,6-bisphosphatase isoform 2; PFKFB3, 6-phosphofructo-2-kinase/fructo-2,6-bisphosphatase isoform 3; PPP, pentose phosphate pathway; TAG, triacylglycerol; UDP-HexNAc, UDP-N-acetyl hexosamine
INTRODUCTION

Glycolysis is the central pathway of intermediary carbon metabolism. Not only does catabolism of glucose via glycolysis provide useable energy for the cell, but it contributes glycolytic intermediates that serve as branch-point metabolites for the entry of glucose-derived carbon into ancillary pathways of glucose metabolism. These pathways are required for de novo synthesis of cellular components. For example, the activity of the pentose phosphate pathway (PPP) is required for nucleotide biosynthesis and the maintenance of cellular redox state, whereas the hexosamine biosynthetic pathway (HBP) regulates posttranslational protein modifications, protein processing, and stress responses. The synthesis of glycero(phospho)lipids and serine are also dependent on the production of glycolytic intermediates (1). In the heart, the PPP is activated during hypertrophy (2-4) and heart failure (5), and modulating the activity of this pathway regulates the severity of cardiac pathology (6-11). Similarly, O-linked β-N-acetylglucosamine (O-GlcNAc)-modified proteins, which require synthesis of the sugar donor UDP-GlcNAc via the HBP (12, 13), are elevated in hypertrophied and failing hearts (14-17). Although less is known about the glycerolipid synthesis pathway (GLP), increased activity of this pathway may underlie pathologic cardiac hypertrophy (18). In addition, numerous studies have identified aberrant ancillary pathway behavior in the context of diabetes [e.g., as reviewed in (19, 20)].

The activities of ancillary pathways of glucose metabolism are regulated in part through expression of their rate-limiting or committed-step enzymes, cofactor availability, allosterism, and/or post-translational modifications [e.g., (21-27)]. Flux through these pathways may be regulated by other means as well. Specific enzymatic reactions in the glycolytic pathway, especially the phosphofructokinase 1 (PFK1) reaction, can coordinate the activity of ancillary pathways in several cell types (28-31); however, the effects of PFK activity and glycolytic rate
on biosynthetic pathway flux in the heart remain unclear. Understanding how PFK regulates ancillary biosynthetic pathway activity is important because its activity is modulated by increased work ex vivo (32, 33), and catecholamines (34) and pressure overload in vivo (35).

In this study, we examined how PFK activity regulates biosynthetic pathways in cardiomyocytes. For this, we altered PFK1 activity by expressing mutant forms of phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2) in primary rat neonatal cardiomyocytes. This method of regulating glycolysis leverages the strong allosteric regulation of PFK1 by F-2,6-P_2 (36-39) and is relevant to mechanisms regulating cardiovascular homeostasis. For example, kinases known to be important for cardiac responses to stress (40, 41) are strong regulators of PFK2 activity (42-45). By integrating radiometric assays, stable isotope tracing, and extracellular flux analyses, we demonstrate that the amount of glucose available for entry into ancillary pathways appears relatively high and that the PFK node of glycolysis exerts varying degrees of control over glucose incorporation in the end products of the PPP, the HBP, and the GLP by not only diverting glucose carbon into these pathways, but by regulating mitochondrial activity and cataplerosis as well.

**EXPERIMENTAL PROCEDURES**

*Materials:* DMEM growth media, containing L-glutamine, D-glucose, and pyruvate, was purchased from US Biological (Swampscott, MA, USA). Insulin, transferrin and selenium (500×) were purchased from Lonza (Walkersville, MD, USA). Humulin R was from Eli Lilly (Indianapolis, IN, USA). All other reagents were from Sigma-Aldrich Corp. (St. Louis, MO, USA), unless indicated otherwise.

*Rodent models:* All procedures were approved by the University of Louisville Institutional Animal Care and Use Committee and were in accordance with NIH guidelines. The euthanasia
procedures were consistent with the AVMA *Guidelines for the Euthanasia of Animals*. Neonatal rat cardiomyocytes (NRCMs) were isolated from 1- to 2-day-old Sprague-Dawley rats as previously described (46-48). Culture media was changed to serum-free DMEM media 24 h prior to each experiment.

*Gene transfer and expression analysis:* Replication-deficient adenoviral vectors were constructed and purified by Vector BioLabs (Malvern, PA, USA) using cDNA for mutated forms of rat liver 6-phosphofructo-2-kinase/Fru-2,6-P₂ bisphosphatase (PFKFB1 isoform of PFK2). The enzyme was engineered to have single amino acid point mutations to foster a kinase deficient- (S32D and T55V; Glyco<sub>lo</sub>) or phosphatase deficient- (S32A and H258A; Glyco<sub>hi</sub>) form of PFK2 (37, 38, 49-51). A 1.4 kb BamHI/NheI fragment of a pLenti6-3×FLAG-pd-PFK2 plasmid was subcloned into a Dual-CCM(+) shuttle vector, which has dual CMV promoters to drive both green fluorescent protein (GFP) and the insert. The backbone of the adenoviral vector is type 5 (dE1/E3). An Ad-GFP control virus, in which GFP is driven by the CMV promoter, was also purchased from Vector BioLabs.

Cardiac myocytes were transduced with adenovirus for 4 h in fresh media, followed by PBS wash and addition of fresh media. For all viral treatments, a multiplicity of infection (MOI) of 50 was used. Functional expression of the target protein was confirmed by immunoblotting and eGFP fluorescence. For measuring protein abundance, cells were lysed in a common lysis buffer containing 20 mM HEPES, 110 mM KCl, 1 mM EDTA, 1% NP-40, 0.1% SDS, and phosphatase and protease inhibitors. Cell lysates were then centrifuged at 13,000g for 20 min and the supernatant was collected. Protein was measured using the standard Lowry Assay (Bio-Rad Laboratories, Hercules, CA, USA) and separated by SDS-PAGE, electroblotted to PVDF membranes, and probed for PFKFB1 (Novus Biologicals, Littleton, CO, USA) according to manufacturer’s protocol. A horseradish peroxidase-linked secondary antibody (Cell Signaling
Technology, Danvers, MA, USA) was used to detect and visualize the protein band with a Typhoon 9400 variable mode imager (GE Healthcare, Chicago, IL, USA).

Radiolabeled glycolysis assay: The rate of glucose utilization was determined using [5-\textsuperscript{3}H]-glucose as previously described (52-54). Briefly, after 48 h of transduction, the media was replaced with 2 ml of fresh, serum-free media containing 5.5 or 25 mM glucose with the addition of 2 µCi/ml [5-\textsuperscript{3}H]-glucose (Moravek Biochemicals, Brea, CA, USA). Following incubation for 3 h, 100 µl of media was collected and added to 100 µl of 0.2 M HCl in a microcentrifuge tube. This tube, with the tube cap removed, was placed in a scintillation vial containing 500 µl of dH\textsubscript{2}O to allow for evaporation diffusion of [\textsuperscript{3}H]2O in the microcentrifuge tubes into the scintillation vials. To account for incomplete equilibration of [\textsuperscript{3}H]2O and background, known amounts (µCi) of [5-\textsuperscript{3}H]-glucose and [\textsuperscript{3}H]2O (Moravek Biochemicals) were placed into microcentrifuge tubes, and these were placed into separate scintillation vials containing 500 µl dH2O. After incubation at 37ºC for 72 h, the microcentrifuge tube was removed from the vial, 10 ml of scintillation fluid was added, and scintillation counting was performed using a Tri-Carb 2900TR Liquid Scintillation Analyzer (Packard Bioscience Company, Meriden, CT, USA). Glucose utilization was then calculated as reported by Ashcroft et al. (52), with considerations for the specific activity of [5-\textsuperscript{3}H]-glucose, incomplete equilibration and background, dilution of [5-\textsuperscript{3}H]- to unlabeled-glucose, and scintillation counter efficiency. To normalize glucose utilization to total protein, the cells were first washed with PBS (to remove any adherent radioactivity) and then lysed in common lysis buffer. After centrifugation, protein concentration was measured using the Lowry assay kit (Bio-Rad Laboratories).

Stable Isotope Tracing: NRCMs were incubated in 6-well plates for 18 h in glucose-free DMEM media containing 1 mM pyruvate and 4 mM glutamine and supplemented with 25 mM [\textsuperscript{13}C\textsubscript{6}]-glucose. Cell reactions were then quenched in cold acetonitrile, and extracted in
acetonitrile:water:chloroform (v/v/v, 2:1.5:1), as described previously (39, 55-57), to obtain the polar, nonpolar, and insoluble proteinaceous fractions. The nonpolar (lipid) layer was collected, dried under a stream of nitrogen gas, and reconstituted in 0.1 ml of chloroform:methanol:butylated hydroxytoluene (2:1 + 1 mM) mixture. The extract was diluted 10× with 1 mM butylated hydroxytoluene solution in methanol and used for Fourier transform ion cyclotron resonance-mass spectrometry (FTICR-MS) analysis.

For stable isotope nucleotide analysis, the samples were prepared using a previously published protocol (39, 57), with slight modifications. Briefly, lyophilized polar extracts were reconstituted in 50 μl of 5 mM aqueous hexylamine, adjusted to pH 6.3 with acetic acid (solvent A). Samples were then loaded onto a 100-μl capacity C18 tip (Thermo Fisher Scientific, Waltham, MA, USA) followed by washing twice with 50 μl of solvent A. The metabolites were eluted with 70% solvent A and 30% 1 mM ammonium acetate in 90% methanol, pH 8.5 (solvent B). The resulting eluates were diluted 3× with methanol and analyzed via FTICR-MS.

[^13]C\textsubscript{6}-glucose and[^13]C\textsubscript{3}-lactate measurements in the media: For quantification of[^13]C\textsubscript{6}-glucose and[^13]C\textsubscript{3}-lactate in cell media, 10 μL of media were mixed with 9990 μL of 5 mM NH\textsubscript{4}OH in methanol containing 5 μM [D\textsubscript{2}]-glucose and 5 μM [D\textsubscript{2}]-lactate internal standard and used without any additional pre-treatment for FTICR-MS analysis and quantification. Blank incubations without cells were run in parallel. The concentrations of[^13]C-labeled glucose and lactate were assessed using seven-point calibration curves (0.3125 μM to 20 μM).

FTICR-MS Analysis: Lipid and nucleotide spectra and[^13]C-glucose/lactate spectra were acquired using a hybrid linear ion trap-FT-ICR mass spectrometer (Finnigan LTQ FT; Thermo Electron, Bremen, Germany), equipped with a TriVersa NanoMate ion source (Advion Bio-Sciences, Ithaca, NY, USA) with an A electrospray chip (nozzle inner diameter, 5.5 μm). The TriVersa NanoMate was operated by applying 1.5 kV with 0.5 psi head pressure in positive ion
mode and 1.6 kV and 0.7 psi in the negative mode. High mass accuracy data were collected using the FT-ICR analyzer over a mass range from 150 to 1600 Da (lipids; + and – mode) for 15 min at the target mass resolution of 400,000 at 400 \( m/z \). For quantification of \(^{13}\)C\(_6\)-glucose and \(^{13}\)C\(_3\)-lactate in cell media, data were collected in the negative ion mode for 2 min over a mass range from 50 to 250 Da at the target mass resolution of 100,000 at 400 \( m/z \). Prior to acquisition, the LTQ-FT was tuned and calibrated according to the manufacturer’s default recommendations to achieve mass accuracy of 2 ppm or less.

**Stable Isotope Data Analyses:** The FT-ICR MS data were exported as exact mass lists into a spreadsheet file using Qual-Browser 2.0 (Thermo Electron). Isotopologue peak deconvolution, assignment, natural isotope abundance stripping, and quantification were performed using MetSign software, as described previously by us (39, 58).

**Extracellular flux analysis of cellular energetics:** To measure cellular energetics in intact cardiomyocytes, the Seahorse Bioscience XF24 extracellular flux analyzer was used as previously described (59). For this, 75,000 cells were plated into each well. An hour before each experiment, the media was replaced with 675 µl of assay media, i.e., unbuffered DMEM containing glucose (5.5 mM), glutamine (4 mM), and pyruvate (1 mM), pH 7.4. Following 1 h in a 37ºC, CO\(_2\)-free incubator, the cells were placed in the instrument for analysis. Basal oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured using a programmed protocol: 3 cycles of 2 min mix, 2 min wait, and 3 min measure. To interrogate the effects of each treatment on cardiomyocyte bioenergetics, the following compounds were injected into each well with 2 cycles of 2 min mix, 2 min wait, 3 min measure following each injection: Port A, oligomycin (Oligo; 1 µM); Port B, carbonyl cyanide-\( p\)-(trifluoromethoxy)phenylhydrazone (FCCP; 1 µM); and Port C, antimycin A and rotenone.
(AA/Rot; 10 µM / 1 µM). The OCR and ECAR values were normalized to the total amount of protein in each well.

Statistical Analysis: All values are mean ± S.E.M. All comparisons were made to the relevant control group and performed using one-way analysis of variance (ANOVA) with Dunnett’s correction for multiple comparisons. The null hypothesis was rejected when $p > 0.05$.

RESULTS

*PFK regulates glycolysis and the distribution of glucose.* To understand the role of PFK activity in cardiac myocyte metabolism, we expressed kinase-deficient (Glyco\textsuperscript{Lo}) or phosphatase-deficient (Glyco\textsuperscript{Hi}) PFK2 mutant constructs in NRCMs, which are conducive to well-controlled and detailed metabolic flux analyses. The mutant genes were driven by a CMV promoter (Fig. 1A), and the bicistronic vector also contained GFP, which was used to determine relative transduction efficiency. As shown in Fig. 1B, the vast majority of NRCMs were transduced with virus. These PFK2 mutants were intentionally derived from the liver-specific isoform of PFK2 (i.e., PFKFB1) so that expression of the proteins could be assessed by immunoblotting, without obfuscation by other isoforms (e.g., PFKFB2/PFKFB3). Myocytes transduced with the Glyco\textsuperscript{Lo} or the Glyco\textsuperscript{Hi} constructs showed strong bands at the predicted molecular mass for the mutant forms of PFK2, whereas cells transduced with Ad-GFP showed no expression of PFKFB1 (Fig. 1C). Although Glyco\textsuperscript{Hi}-expressing cells routinely displayed a higher level of mutant protein expression relative to Glyco\textsuperscript{Lo}-expressing cells, the strength of the relative effect of each PFK2 mutant on cellular glycolytic rate was equal, with a 2.1-fold increase and 2.2-fold decrease in glycolytic rate for the Glyco\textsuperscript{Hi} and Glyco\textsuperscript{Lo} constructs, respectively (Fig. 1D). This effect on glycolytic rate was independent of glucose concentration, as rates were similar for groups incubated with either 5.5 or 25 mM glucose.
To examine the effect of each mutant PFK2 enzyme on aerobic glycolysis, we replaced the normal growth media with media containing $[^{13}C_6]$-glucose and, after 18 h, measured the relative levels of $[^{13}C_6]$-glucose and $[^{13}C_3]$-lactate in the media. As shown in Fig. 1E, the levels of $[^{13}C_6]$-glucose consumed by the cell was 1.8-fold higher in Glyco$^\text{Hi}$-expressing cells compared with Ad-GFP, with no difference in Glyco$^\text{Lo}$-expressing cells. Levels of $[^{13}C_3]$-lactate extruded into the media was 2.5-fold higher in Glyco$^\text{Hi}$ cells compared with GFP control cells and >10-fold higher compared with Glyco$^\text{Lo}$ virus transduced cells (Fig. 1F). As shown in Fig. 1G, 29% of $[^{13}C_6]$-glucose was metabolized to $[^{13}C_3]$-lactate in GFP control cells, whereas in Glyco$^\text{Lo}$-expressing cells, 6% of labeled glucose was metabolized to lactate; in Glyco$^\text{Hi}$-expressing cells, 44% of labeled glucose was converted to lactate.

Assessment of $[^{13}C_6]$-glucose uptake, $[^{13}C_3]$-lactate production, and [5-$^3$H]-glucose utilization provides estimates of not only how much glucose entered the cell and was later extruded as lactate, but also how much made it through the enolase step of glycolysis, where the tritiated label is eliminated as $^3$H$_2$O. The mean values of glucose uptake and lactate production (provided by stable isotope tracing) and of radiometric measurements were used to calculate the amount of glucose unaccounted for by catabolism to pyruvate. In GFP control cardiomyocytes, only 44% of glucose was catabolized past the enolase step in glycolysis; of this fraction, the majority (65%) was converted to lactate (Fig. 2A,B). These data show that, under control conditions, a substantial amount of glucose taken up by NRCMs is not catabolized to pyruvate and that the majority of glucose that is catabolized to pyruvate is fated for lactate production.

As shown in Fig. 1E, glucose uptake was not different between GFP and Glyco$^\text{Lo}$ cells, yet Glyco$^\text{Lo}$ myocytes demonstrated 2-fold lower glucose utilization through the enolase step of glycolysis and 5-fold lower lactate production. In these cells, 78% of glucose-derived carbon was partitioned upstream of the enolase reaction, leaving 22% available for pathways downstream of enolase (Fig. 2B). Although lactate production was markedly suppressed in
Glyco\textsuperscript{Lo} cells, the absolute amount of glucose-derived carbon available for either mitochondrial oxidation, transamination to alanine, or carboxylation to oxaloacetate was identical to that of control cells. Interestingly, while Glyco\textsuperscript{Hi} cells showed 80% higher levels of glucose uptake compared with GFP control cells (see Fig. 1E), 44% of glucose (by mass) was not catabolized via glycolysis. Nevertheless, a portion of glucose equivalent to that apportioned upstream of enolase (0.67 nmol/h/µg protein) taken into the cell could be accounted for as lactate (0.66 nmol/h/µg protein), and 21% of glucose carbon that was catabolized past the enolase reaction was available for fates other than lactate production (Fig. 2B).

\textit{PFK activity regulates glucose carbon partitioning into the PPP in NRCMs.} To determine how the activity of PFK regulates critical anabolic reactions derived from glycolysis, we modulated glycolytic rate by expressing the Glyco\textsuperscript{Lo} or Glyco\textsuperscript{Hi} PFK2 mutants in cardiomyocytes and then assessed carbon incorporation into end products of ancillary biosynthetic pathways using stable isotope-resolved metabolomics (60). Expression of the Glyco\textsuperscript{Hi} mutant modestly diminished incorporation of glucose-derived carbon into ATP. Lower fractional enrichment of \textsuperscript{13}C in Glyco\textsuperscript{Hi}-expressing cells is attributable to decreased carbon allocation into the phosphoribosyl ring of the nucleotide (\textit{m}+5 isotopologue) (Fig. 3A, 3B), suggesting diminished flux of \textsuperscript{13}C-labeled glucose-6-phosphate (G6P) through the oxidative PPP (oxPPP). The higher level of the \textit{m}+0 isotopologue of ATP (i.e., the unlabeled ATP pool) in Glyco\textsuperscript{Hi} cells further indicates that the flux of \textsuperscript{13}C\textsubscript{6}-G6P through the oxPPP was lower in Glyco\textsuperscript{Hi} cells. Similar changes were observed in other purines such as GTP (Fig. 3C). Myocytes expressing the Glyco\textsuperscript{Lo} mutant showed \textsuperscript{13}C fractional enrichment values into ATP that were similar to GFP-transduced control cells (Fig. 3A, 3B), suggesting that low PFK activity does not strongly affect purine synthesis in these cells.

For the pyrimidines CTP and UTP, we found a higher \textit{m}+0 isotopologue value in Glyco\textsuperscript{Hi} cells and a lower \textit{m}+0 isotopologue value in Glyco\textsuperscript{Lo} cells compared with GFP controls. These
data suggest that high rates of glycolysis diminish incorporation, and that low rates of glycolysis increase incorporation, of glucose-derived carbon into pyrimidines (Fig. 3D, 3E). Interestingly, both the GlycoLo and the GlycoHi NRCMs showed increased levels of CTP and UTP having only the ribose ring labeled ($m+5$ isotopologue). These data could indicate that either decreasing or increasing glycolysis augments oxPPP flux. This interpretation would be consistent with the observation that both GlycoLo and GlycoHi cells show more glucose available for entry into ancillary biosynthetic pathways (see Fig. 2); however, it is also possible that higher levels of $m+5$ labeling could be due to an accumulation of these pyrimidine isotopologues, where the contribution of $^{13}$C-glucose to de novo synthesis of the uridine ring is diminished. Indeed, examination of the $m+7$ and $m+8$ isotopologues of UTP, which likely correspond to addition of carbons from $^{13}$C-labeled aspartate to the molecule (see Fig. 3D), show that both the GlycoLo and GlycoHi cells have less incorporation of $^{13}$C, which is known to derive from Krebs cycle-($m+7$) or pyruvate carboxylase-engendered ($m+8$) oxaloacetate. The $m+6$ isotopologue likely corresponds to complete $^{13}$C$_5$-labeling of the ribose ring and $^{13}$C$_1$ labeling of aspartate, occurring as a result of scrambling during the 2$^{nd}$ turn of the Krebs cycle, as suggested previously (61). Overall, these data suggest that high PFK activity modestly diminishes oxPPP flux and $^{13}$C incorporation into purines. Pyrimidine synthesis appears to be more robustly affected by PFK activity, with high rates of glycolysis diminishing incorporation of glucose-derived carbon into UTP and CTP molecules.

*High PFK activity diminishes glucose-derived carbon incorporation into UDP-HexNAc.* The molecular synthesis of UDP-N-acetyl hexosamines requires end products or intermediates of several metabolic pathways, including those from the Krebs cycle, the pyrimidine biosynthetic pathway, glutamine, and glycolysis; hence, these pathways collectively contribute to UDP-HexNAc synthesis (Fig. 4A). To determine how the glycolytic activity affects glucose-derived carbon allocation into the HBP, we measured fractional enrichment of $^{13}$C from [$^{13}$C$_6$]-glucose
into UDP-HexNAc in NRCMs transduced with Glyco\textsuperscript{Lo} or Glyco\textsuperscript{Hi} virus. After 18 h, >90% of the UDP-HexNAc pool integrated $^{13}$C-glucose-derived carbon (Fig. 4B), which suggests that UDP-HexNAc has a faster rate of glucose carbon incorporation relative to purines and pyrimidines. That the $m+0$ isotopologue was higher in Glyco\textsuperscript{Hi} cells indicates that high rates of glycolysis diminish glucose carbon incorporation into UDP-HexNAc. The lower $^{13}$C enrichment of many isotopologues (e.g., $m+7$, $m+14$, $m+15$, $m+16$) appeared to be due to diminished input of metabolites that require mitochondrial transformation of $^{13}$C-glucose-derived carbon; however, the $m+5$, $m+6$, and $m+11$ isotopologues (which are the forms of UDP-HexNAc with the ribose, hexose, and ribose+hexose moieties constituted with $^{13}$C, respectively) demonstrate significantly higher fractional enrichment in Glyco\textsuperscript{Hi} cells. Given that both the Glyco\textsuperscript{Lo} and the Glyco\textsuperscript{Hi} cells had similar levels of glucose available for reactions upstream of enolase (see Fig. 2), the simplest explanation for this result is that the mitochondrial cataplerotic metabolites (deriving from the $^{13}$C-glucose-derived carbon source) are limiting for the synthesis of UDP-HexNAc; as a result, the $m+5$ and $m+6$ isotopologues of the metabolite accumulate as $^{13}$C incorporation from cataplerotic metabolites slows down. This would be consistent with findings in pyrimidines, which suggest that cataplerotic generation of aspartate is limiting for the incorporation of glucose-derived carbon into CTP and UTP.

Although low rates of glycolysis did not affect the overall level of incorporation of glucose-derived carbon into UDP-HexNAc, that the $m+11$ isotopologue in Glyco\textsuperscript{Lo} cells was higher than that in control cells suggests that low PFK activity promotes channeling of F6P not only into the HBP (contributing six $^{13}$C), but, because the ribose ring is included (contributing five $^{13}$C), the PPP as well (Fig. 4A, 4B). Changes in the $m+8$ isotopologue are difficult to interpret because this isotopologue could be comprised of $^{13}$C-labeled N-acetyl hexosamine as well as stably labeled UDP. Altogether, these results suggest that high PFK activity decreases glucose carbon allocation into UDP-HexNAc primarily via limiting the mitochondria-derived and cataplerotic intermediates (e.g., acetyl CoA, aspartate) derived initially from $^{13}$C-glucose.
PFK regulates glycerolipid synthesis in cardiomyocytes. In cardiac myocytes, glycerolipid synthesis requires free fatty acids (FFAs) and glycerol-3-phosphate (G3P). While FFAs can be derived from transporter-mediated uptake across the plasma membrane (62), myocytes have a relatively low capacity for free glycerol uptake; therefore, there is little contribution of glycerol kinase to G3P synthesis (63, 64). However, G3P can be generated from DHAP by glycerol-3-phosphate dehydrogenase (Fig. 5A). Nevertheless, it is unclear whether or how the rate of glycolysis affects glycerolipid synthesis. We found that in comparison with cells transduced with GFP virus, the GlycoLo cells showed diminished incorporation of $^{13}$C from $^{13}$C$_6$-glucose into the glycerol (m+3) moiety of phosphatidylcholine (PC; Fig. 5B), phosphatidylinositol (PI; Fig. 5C), phosphatidylethanolamine (PE; Fig. 5D), and phosphatidylycerine (PS; Fig. 5E). Incorporation of $^{13}$C$_6$-glucose-derived carbon into fatty acids in PC, PI, PE, and PS was not different in GlycoLo cells compared with control cells, suggesting that only the synthesis of the glycerol backbone portion of these phospholipids is affected by low rates of glycolysis. High glycolytic rates affected $^{13}$C incorporation into phospholipids to a lesser extent. Although PE synthesis showed a slight increase in $^{13}$C fractional enrichment in glycerol (and a concomitant decrease in fractional enrichment of the unlabeled pool) (Fig. 5D), PI showed diminished labeling characterized by lower incorporation of $^{13}$C into fatty acids. Glycerol $^{13}$C labeling in PI from GlycoHi cells was identical to that of control cells. This dichotomy in labeling patterns in different phospholipids could suggest the presence of unique enzyme complexes that participate in metabolic channeling for the synthesis of distinct glycerolipids. Consistent with this idea, triacylglycerol (TAG) labeling was remarkably different from that of other glycerolipids. As shown in Fig. 5F, TAG species collectively showed little $^{13}$C in the glycerol moiety. Furthermore, low rates of glycolysis had little effect on glucose carbon incorporation into TAGs; however, GlycoHi cells showed lower $^{13}$C incorporation into fatty acids. Collectively, these results suggest that, in isolated NRCMs, low rates of glycolysis diminish phospholipid synthesis by the
DHAP→G3P pathway and that high rates of glycolysis decrease incorporation of glucose-derived carbon into the fatty acyl chains of PI and TAGs.

*PFK activity regulates mitochondrial activity in NRCMs:* Stable isotope labeling patterns of several end products of the PPP, HBP, and GLP suggest that high PFK activity diminishes the relative abundance of those $^{13}$C isotopologues that require metabolites derived from mitochondria. This suggests that cataplerosis (65) is diminished under conditions of high PFK activity. Because cataplerotic reactions depend upon mitochondrial activity, we next measured respiration in cells expressing either the control construct (GFP) or the Glyco$^{\text{Hi}}$ or Glyco$^{\text{Lo}}$ constructs. Glyco$^{\text{Hi}}$-expressing cells showed 50% lower basal oxygen consumption, whereas Glyco$^{\text{Lo}}$-expressing cells showed significantly higher basal OCR values (**Fig. 6A, 6C**). These PFK-mediated changes in mitochondrial OCR are likely due to the Crabtree effect, which is characterized by decreases in mitochondrial activity under conditions of increased glucose availability or glycolytic activity (66-69). Although addition of oligomycin showed marginal effects on ATP-linked OCR, proton leak was significantly diminished in Glyco$^{\text{Hi}}$ cells. Upon addition of a mitochondrial uncoupler, Glyco$^{\text{Lo}}$ and Glyco$^{\text{Hi}}$ cells showed relative changes in maximal respiratory rate and in reserve capacity that mirrored the pattern found for basal OCR and proton leak (**Fig. 6A, 6C**). These data are consistent with the notion that high PFK activity diminishes mitochondrial activity, which limits the cataplerotic generation of metabolites required for the synthesis of end products of the PPP, HBP, and GLP.

Although cells transduced with the Glyco$^{\text{Hi}}$ virus had >2-fold elevation in basal extracellular acidification (ECAR) compared with Ad-GFP controls, we observed no differences in ECAR in NRCMs expressing the Glyco$^{\text{Lo}}$ PFK2 mutant (**Fig. 6B**). Addition of oligomycin increased ECAR to similar values in all groups, suggesting that inhibition of mitochondrial ATP production overrides PFK2-mediated changes in glycolysis. Although extracellular acidification is a surrogate measure of lactate extrusion and aerobic glycolysis, protons are also generated
during respiration via pyruvate dehydrogenase-mediated production of \( \text{CO}_2 \) (i.e., \( \text{CO}_2 \rightarrow \text{HCO}_3^- + \text{H}^+ \)) \((70, 71)\); thus, the inconsistency of ECAR with absolute measurements of glycolytic rate and \( ^{13}\text{C}_3\)-lactate in Glyco\(^{1^0}\) cells (see. Fig. 1) is likely due to an increase in mitochondrial activity. Taken together, measurements from radiometric assays, stable isotope tracing, and extracellular flux analyses indicate that PFK regulates ancillary pathways by not only direct partitioning of glucose (e.g., in the PPP and the GLP) but by regulating mitochondrial cataplerotic reactions as well.

**DISCUSSION**

Most studies of cardiac metabolism have focused primarily on the catabolic reactions required for energy provision \((72)\). Considerably less effort has been devoted to understanding the regulation of ancillary pathways of glucose metabolism in the heart and how these pathways integrate with catabolic reactions. In this study, we examined how PFK activity, which immediately precedes the “lysis” step in glycolysis, regulates several 6-carbon- and 3-carbon-initiated anabolic pathways that require glycolytic intermediates. Our results demonstrate that in cardiac myocytes PFK regulates glucose carbon incorporation into ancillary biosynthetic pathways by: (1) directly regulating carbon flow into purines and phospholipids; and (2) indirectly influencing mitochondrial cataplerotic activity required for the generation and subsequent incorporation of glucose-derived carbon into products of the PPP, HBP, and GLP. These studies also illustrate how radiometric measurements, stable isotope tracing, and extracellular flux analyses can be integrated to examine substrate fate and develop detailed understanding of how interventions simultaneously affect anabolic and catabolic metabolism.

We chose to study the integration of anabolism and catabolism in the context of glycolytic regulation at the PFK node. This enzyme controls 65% of the flux through glycolysis in the heart \((73)\), making it the major regulator of glycolytic activity. It is unlikely that, under the
experimental conditions used, glucose entry was a major limiting step for its metabolism because NRCMs express both GLUT1 and GLUT4 (74). Our stable and radiometric isotope tracing measurements show that, under control conditions, <50% of glucose taken into myocytes is catabolized past the enolase reaction. This suggests a majority of the glucose entering the myocyte is not used for energy production, but is likely used by biosynthetic pathways. Although PFK activity was found to be a potent determinant of glycolysis-engendered lactate production, it did not have diametrically coordinate effects on the relative amounts of glucose found upstream of the enolase reaction. Surprisingly, both a decrease and an increase in glycolytic activity led to the accumulation of glucose upstream of the enolase reaction; however, the mechanisms by which low and high PFK activity augment the levels of glucose-derived carbon upstream of the enolase reaction appear to differ: when PFK activity was high, glucose uptake was increased, but glucose utilization was elevated; when PFK activity was low, glucose uptake was the same as that found in control cells, but glucose utilization was remarkably diminished. These results suggest that conditions of low PFK activity promote accumulation of metabolites (likely upstream of the PFK step), and that conditions of high glycolytic activity support levels of glucose-derived carbon upstream of the enolase reaction by augmenting glucose uptake. Additionally, our data suggest that the level of glucose available for glucose oxidation, alanine transamination, or pyruvate carboxylation was relatively stable and independent of PFK activity.

Although studies in some cell types such as adipocytes suggest that the HBP is a relatively minor pathway, utilizing only 2–3% of incoming glucose (75), our stable isotope tracing data suggest that the rate of entry of glucose-derived carbon into this pathway may be higher in cardiac myocytes. The unlabeled pool of UDP-HexNAc was below 10% in all groups, suggesting that sugar donors such as UDP-GlcNAc and UDP-GalNAc are synthesized and utilized at high rates. In contrast, >20% of the pool of nucleotides was unlabeled and $^{13}\text{C}$ enrichment of the glycerol moiety of glycerolipids was below 20%. These findings appear to
indicate that the HBP utilizes more glucose-derived carbon than either the PPP or the GLP. Given that up to 20% of glucose taken up by the heart could enter the PPP (76), our comparisons of $^{13}$C enrichment in end products of these pathways suggests that HBP flux in cardiomyocytes may be much higher than currently appreciated.

Our results are consistent with previous findings in epithelial cells (30), fibroblasts (77, 78), and developing embryos (79) which show that glycolysis regulates glucose entry into the PPP. Our findings are also consistent with computational predictions of the role of PFK in regulating the PPP in the intact heart (73). Similar to findings in neurons (80), in cardiac myocytes high glycolytic rates driven by PFK decreased the incorporation of glucose carbon into the ribose ring of purines. This suggests that high rates of glycolysis decrease the flux of glucose through the oxPPP. Nevertheless, we identified remarkable differences in $^{13}$C labeling in ATP and GTP versus CTP and UTP. Although both classes of nucleotides use the metabolic precursor ribose-5-phosphate to form the ribose ring, purine ring carbon atoms originate from $\text{N}^{10}$-formyltetrahydrofolate, $\text{CO}_2$ and glycine, while pyrimidine ring carbons come from carbamoyl phosphate and glutamine, as well as aspartate generated by cataplerosis (81). In the timeframe examined, purines did not demonstrate incorporation of carbons that derive from metabolic products of the serine biosynthetic pathway and one-carbon metabolism. Hence, it is possible that glycine and $\text{N}^{10}$-formyltetrahydrofolate derived from the serine biosynthetic pathway might be inadequate to participate in purine ring synthesis during the 18 h incubation time used in our experiments. Alternatively, unlabeled serine and folate present in the media could be used by these cells for de novo purine biosynthesis.

The decrease in mitochondrial activity detected by extracellular flux analysis in Glyco$^{13}$ cells, paired with stable isotope tracing results in pyrimidines, suggests low cataplerotic generation of aspartate. Oxaloacetate generated in the Krebs cycle or via pyruvate carboxylation is transaminated to aspartate, which is subsequently used for pyrimidine biosynthesis. That the $m+7$ and $m+8$ isotopologues were lower in Glyco$^{13}$ cells shows that these
pathways were diminished under conditions of high PFK activity. From these data, we inferred that the decrease in \textit{de novo} pyrimidine biosynthesis in the Glyco$^{\text{Hi}}$ cells may be due to lower rates of cataplerosis. Because tricarboxylic acid anions are delicately balanced by the input and output of intermediates involved in mitochondrial substrate oxidation, anaplerosis, and cataplerosis (65), we reasoned that mitochondrial activity measurements could provide convergent evidence of PFK-mediated regulation of auxiliary glucose biosynthetic pathways. Indeed, the decrease in mitochondrial activity in Glyco$^{\text{Hi}}$ cells, paired with the observation that $^{13}$C-aspartate entry into pyrimidines was low, suggests that inhibition of pyrimidine synthesis under conditions of high glycolytic activity is due to low rates of cataplerosis.

Similar reasoning as well as previous modeling, validation, and biochemical interpretation of $^{13}$C isotopologue data for UDP-GlcNAc (82) were used to determine how PFK affects the HBP, which is important given the known role of UDP-hexosamines in modulating N- and O-linked glycosylation (12, 13). The fact that the unlabeled pool of UDP-HexNAc was higher in Glyco$^{\text{Hi}}$ cells suggests that the net effect of high PFK activity is a decrease in HBP flux, and, like that found for pyrimidines, this appears to be in part due to diminished mitochondrial activity. Nevertheless, there are limitations for interpreting current data on HBP flux. Because building UDP-HexNAc requires metabolites from many sources, it is possible that the contribution of carbon from non-labeled pools of metabolites may hinder interpretation. A potential solution to this problem would be to use a pulse-chase approach, where isotopic labeling in the system is first saturated (e.g., with both $^{13}$C and $^{15}$N) and then the stably labeled substrate(s) are replaced with unlabeled substrate(s). The disappearance of the stable label over time in isotopologues of UDP-HexNAc should, with higher confidence, reveal which intersecting metabolic pathways limit HBP activity and UDP-Glc(Gal)NAc synthesis under a given set of conditions.

To date, little evidence exists for whether or how glycolysis regulates GLP activity. Interestingly, G3P formed via glycerol-3-phosphate dehydrogenase 1 has been shown to inhibit
PFK2 and activate fructose bisphosphatase 2 (83), suggesting feedback regulation of the GLP on both glycolytic and gluconeogenic activity. An indication that the glycolytic rate regulates GLP flux and glycerolipid synthesis is provided by studies showing that genetic deletion or overexpression of PFKFB3, a highly active form of PFK2, in adipose tissue coordinately regulates fat deposition and obesity (84, 85), which is consistent with the notion that glycolysis in adipocytes regulates TAG synthesis and storage. We found that low PFK activity directly limits glucose-derived carbon incorporation into the glycerol backbone of several phospholipids including PC, PI, PE, and PS; however, this was not the case with triacylglycerols, where $^{13}$C incorporation into glycerol was not different, but fatty acyl chain labeling was diminished in Glyco$^\text{Hi}$ cells. These results suggest that the fatty acid pool derived from cataplerotic reactions is differentially channeled to route newly synthesized fatty acids for esterification into triacylglycerols rather than phospholipids.

There are several limitations to our study. Although NRCMs are beating cardiac myocytes, they rely on glycolysis for energy to a much greater extent than the adult heart (86). Thus, findings regarding pathways that involve metabolites generated by cataplerosis may not entirely reflect metabolism in the adult heart. Glucose carbon fate in NRCMs appears to differ from the adult heart primarily at the level of coupling between glycolysis and glucose oxidation. In the adult human heart, previous studies show that 26% of extracted glucose undergoes oxidation and 12% is released as lactate (87). In contrast, in NRCMs, we show that <13% of glucose carbon undergoes oxidation and ~24% is released as lactate. Nevertheless, our finding that a large portion of exogenous glucose extracted by cardiac myocytes is apportioned for metabolic pathways other than glycolysis and glucose oxidation is supported by human in vivo isotope tracing studies in the myocardium, which demonstrate that 60% of exogenous glucose extracted by contracting myocytes enters a slow turnover pool amenable to entry into ancillary biosynthetic pathways (87). Another consideration is that the NRCM culture media is devoid of fatty acids, which could influence the coupling between glycolysis and glucose oxidation as well
as biosynthetic pathway activity. Last, it is important to consider that our stable isotope analyses did not include glycogen, glycine, serine, or sorbitol, which limits our understanding of how glycolysis regulates glycogen biosynthesis, serine biosynthesis, and the polyol pathway. Nevertheless, computational models created by Cortassa et al. (73) suggest that the polyol pathway is under strong control by activation or inhibition of PFK. Similarly, previous studies show that PFK activity regulates glycogen abundance in the heart (37, 38). Such a behavior would be consistent with coordinate regulation of ancillary biosynthetic pathway activity by PFK.

In summary, by using several methods to measure metabolic pathway activity, we found that >40% of glucose consumed by NRCMs is not catabolized to pyruvate, and that PFK has strong effects on the coupling between glucose oxidation and glycolysis. Pairing results from stable isotope-resolved metabolomics with extracellular flux analysis demonstrated that PFK activity regulates glucose carbon incorporation directly into the ribose and the glycerol moieties of purines and phospholipids, respectively, and that high PFK, by indirectly limiting mitochondrial activity, diminishes $^{13}$C incorporation into pyrimidines, UDP-N-acetylhexosamine(s), and the fatty acyl chains of phosphatidylinositol and triglycerides. These insights could help elucidate how pathological or physiological changes in glucose metabolism regulate the anabolic behavior and remodeling of the myocardium.

Acknowledgments and Funding Information: This work was supported by grants from the National Institutes of Health [HL122580 (to B.G.H.), HL130174 (to B.G.H.), HL55477 (to A.B.), GM103492 (to A.B.), HL131647 (to S.P.J.), and HL78825], a Predoctoral Fellowship from the American Heart Association [16PRE31010022 (to A.A.G.)], and the American Diabetes Association Pathway to Stop Diabetes Grant [1-16-JDF-041 (to B.G.H)].
Declaration of interests: The authors declare no competing interests.

Author contributions: AAG, financial support, experimental studies, assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; PL, experimental studies, data analysis and interpretation, manuscript writing, final approval of manuscript; YZ, experimental studies, final approval of manuscript; XZ, experimental studies, final approval of manuscript; AB, financial support, final approval of manuscript; SPJ, manuscript writing, final approval of manuscript; and BGH, financial support, concept and design, assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript.

REFERENCES


FIGURE LEGENDS

Figure 1: Phosphofructokinase activity regulates glucose metabolism in isolated cardiomyocytes. Glucose utilization analyses in neonatal rat cardiomyocytes: (A) Constructs of kinase-deficient and phosphatase-deficient PFK2 engineered to decrease (Glyco₄ Lo) or increase (Glyco₄ Hi) glycolytic rate, respectively; (B) GFP expression in cardiomyocytes 48 h after transduction (50 MOI) with adenoviruses expressing GFP only or the Glyco₄ Lo or Glyco₄ Hi adenoviruses; (C) Immunoblot of mutant PFK2 protein expression; (D) Glycolytic rate measured with [5-³H]-glucose under 5.5 or 25 mM glucose culture conditions. n = 3–5 independent isolations per group; (E) ¹³C₆-glucose uptake; (F) ¹³C₃-lactate production, and (G) the percentage of glucose converted to lactate following 18 h incubation with isotopic glucose. Data in Panels E–G represent three replicates per group. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 2. Phosphofructokinase-mediated glucose distribution in isolated cardiomyocytes. Schematics illustrating glucose utilization in myocytes transduced with GFP, Glyco₄ Lo or Glyco₄ Hi adenoviruses. Measurements from radiometric and isotopic glucose utilization assays were used to delineate glucose distribution in neonatal rat cardiomyocytes transduced with each virus. (A) Schematic illustrating glucose distribution in control (GFP) myocytes. Distributions of glucose are drawn to relative scale. (B) Pie chart illustrating distribution of glucose: Numbers (in nmol/h/µg protein) and percentages (%) indicate the absolute and relative distribution of glucose ascribed to the portions of glucose metabolism: upstream of enolase (light red), through enolase (grey shading), converted to lactate (yellow) and available for transamination to alanine, anaplerotic formation of oxaloacetate (OAA), or
glucose oxidation (light blue). Pie charts are drawn to scale. Both schematics consider glucose uptake and lactate production provided by $^{13}$C-glucose tracing and by glucose utilization measured by radiometric assays, respectively.

**Figure 3:** Phosphofructokinase coordinates pentose phosphate pathway activity. Isolated cardiomyocytes were transduced with GFP, Glyco$^{\text{Lo}}$, or Glyco$^{\text{Hi}}$ adenoviruses and incubated with $^{13}$C$_6$-glucose for 18 h. Stable isotope tracing of nucleotides in NRCMs incubated with media containing U-$^{13}$C-glucose: (A) Atom-resolved map illustrating the biological and biochemical history of $^{13}$C incorporation into the purine AMP (phosphates in ATP omitted to conserve space); Fractional enrichment values of $^{13}$C in (B) ATP and (C) GTP after incubation with $^{13}$C-glucose; (D) Atom-resolved map illustrating the biological and biochemical history of $^{13}$C incorporation into the pyrimidine UMP; Fractional enrichment values of $^{13}$C in (E) UTP and (F) CTP after incubation with $^{13}$C-glucose. Colored x-axis labels correlate to the labeled molecule biosynthetic components indicated in the atom-resolved maps. Graph represents three replicates per group from one isolation. *$^5$ p<0.05, **$^5$ p<0.01, ***$^5$ p<0.001, ****$^5$ p<0.0001.

**Figure 4:** High phosphofructokinase activity diminishes glucose carbon incorporation into UDP-HexNAc. Isolated cardiomyocytes were transduced with GFP, Glyco$^{\text{Lo}}$, or Glyco$^{\text{Hi}}$ adenoviruses and incubated with $^{13}$C$_6$-glucose for 18 h. Stable isotope tracing of UDP-HexNAc in NRCMs incubated with media containing $^{13}$C-glucose: (A) Atom-resolved map illustrating the biological and biochemical history of $^{13}$C incorporation into UDP-GlcNAc; (B) Fractional enrichment values of $^{13}$C in UDP-HexNAc (which includes both UDP-GlcNAc and UDP-GalNAc) after incubation with $^{13}$C$_6$-glucose. Colored x-axis labels correlate to the labeled molecule biosynthetic components indicated in the atom-resolved maps. Graph represents three replicates per group from one isolation. *$^5$ p<0.05, **$^5$ p<0.01, ***$^5$ p<0.001, ****$^5$ p<0.0001.

**Figure 5:** Phosphofructokinase regulates glycerolipid biosynthesis. Stable isotope tracing of phospholipids and triacylglycerols in cardiomyocytes incubated with media containing $^{13}$C$_6$-glucose for 18 h: (A) Atom-resolved map illustrating the biological and biochemical history of $^{13}$C incorporation into glycerolipids; Fractional enrichment values of $^{13}$C into: (B) phosphatidylcholine (PC); (C) Phosphatidylinositol (PI); (D) phosphatidylethanolamine (PE); (E) phosphatidylserine (PS); and (F) triacylglycerols (TAG). Graph represents three replicates per group from one isolation. *$^5$ p<0.05, **$^5$ p<0.01, ***$^5$ p<0.001, ****$^5$ p<0.0001.

**Figure 6:** Phosphofructokinase activity regulates mitochondrial activity in isolated cardiomyocytes. Extracellular flux analyses of NRCMs: (A) Oxygen consumption traces (OCR), (B) Extracellular acidification rate (ECAR), and (C) Calculated indices of respiration of cardiomyocytes transduced with GFP, Glyco$^{\text{Lo}}$, or Glyco$^{\text{Hi}}$ adenoviruses. n=3 independent isolations per group. *p < 0.05, **p < 0.01.
Figure 1

A  

Glyco\textsuperscript{Lo}  
kinase-deficient PFK2 construct  
CMV \textcolor{red}{\begin{tabular}{c} Kinase \end{tabular}} \textcolor{blue}{\begin{tabular}{c} Phosphatase \end{tabular}}  
S32D T55V  

Glyco\textsuperscript{Hi}  
phosphatase-deficient PFK2 construct  
CMV \textcolor{red}{\begin{tabular}{c} Kinase \end{tabular}} \textcolor{blue}{\begin{tabular}{c} Phosphatase \end{tabular}}  
S32A H258A  

B  

GFP  
Glyco\textsuperscript{Lo}  
Glyco\textsuperscript{Hi}  

C  

Mutant PFK2  
58 kDa  

D  

Glucose utilization  

E  

Glucose uptake  

F  

Lactate production  

G  

% \textsuperscript{13}C\textsubscript{6}-Glucose to \textsuperscript{13}C\textsubscript{3}-Lactate  

|  | 5.5 mM  | 25 mM  | 5.5 mM  | 25 mM  | 5.5 mM  | 25 mM  | 5.5 mM  | 25 mM  | 5.5 mM  | 25 mM  | 5.5 mM  | 25 mM  |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Glyco\textsuperscript{Lo} | 0.0 | 0.4 | 0.8 | **|
| Glyco\textsuperscript{Hi} | 1.0 | 1.5 | 2.0 | ***|
| Lactate production | 0.5 | 0.5 | 1.0 | ***|
| % \textsuperscript{13}C\textsubscript{6}-Glucose to \textsuperscript{13}C\textsubscript{3}-Lactate | 20 | 30 | 40 | **|

* p < 0.05  
** p < 0.01  
*** p < 0.001
Figure 2

Part A: Metabolic pathway diagram showing glucose metabolism with key enzymes like PFK1 and Enolase.

Part B: Pie charts representing different glycolytic states: GFP, Glyco^Lo, and Glyco^Hi.

- **GFP**:
  - Above Enolase: 0.47 (56%)
  - Through Enolase: 0.37 (44%)
  - Converted to Lactate: 0.13 (35%)

- **Glyco^Lo**:
  - Above Enolase: 0.64 (78%)
  - Through Enolase: 0.18 (22%)
  - Converted to Lactate: 0.13 (72%)
  - Oxidation, carboxylation, or transamination: 0.05 (28%)

- **Glyco^Hi**:
  - Above Enolase: 0.67 (44%)
  - Through Enolase: 0.84 (56%)
  - Converted to Lactate: 0.66 (79%)
  - Oxidation, carboxylation, or transamination: 0.18 (21%)
Figure 3

A) Serine/folate metabolism

B) Fractional Enrichment of Isotopologue ($m+x$) for GFP, GlycoLo, and GlycoHi

C) Fractional Enrichment of Isotopologue ($m+x$) for GTP

D) Pyrimidine Biosynthesis

E) Fractional Enrichment of Isotopologue ($m+x$) for CTP

F) Fractional Enrichment of Isotopologue ($m+x$) for UTP
Figure 5

A

Glycolysis

CAC

{\(^{13}C\_2\)-Pyruvate} \(\rightarrow\) \({^{13}C\_6}\)-Glucose

\(\text{HK}\)

\({^{13}C\_2}\)-Ac-CoA

ACL

ACC

FAS

FA Synthesis

GFP

Glyco\(^{-}\)

Glyco\(^{+}\)

Glyco\(^{Lo}\)

Glyco\(^{Hi}\)

PI Biosynthesis

CCA

\({^{13}C\_2}\)-Pyruvate \(\leftarrow\) \({^{13}C\_2}\)-Ac-CoA

\(\text{HK}\)

\({^{13}C\_2}\)-F6P

PPF

ALDO-LASE

F1,6P\(_2\)

\({^{13}C\_2}\)-G3P \(\leftarrow\) \({^{13}C\_2}\)-DHAP

Triglyceride Biosynthesis

B

PC

C

PI

D

PE

E

PS

F

TAG

Fractional Enrichment

Unlabeled

Glycerol

FA Only

Glyc+FA

Unlabeled

Glycerol

FA Only

Glyc+FA

Unlabeled

Glycerol

FA Only

Glyc+FA

Unlabeled

Glycerol

FA Only

Glyc+FA

Unlabeled

Glycerol

FA Only

Glyc+FA

Unlabeled

Glycerol

FA Only

Glyc+FA

Unlabeled

Glycerol

FA Only

Glyc+FA

Unlabeled

Glycerol

FA Only

Glyc+FA