Enhancing cardiac glycolysis causes an increase in PDK4 content in response to short-term high-fat diet

The healthy heart has a dynamic capacity to respond and adapt to changes in nutrient availability. Metabolic inflexibility, such as occurs with diabetes, increases cardiac reliance on fatty acids to meet energetic demands, and this results in deleterious effects, including mitochondrial dysfunction, that contribute to pathophysiology. Enhancing glucose usage may mitigate metabolic inflexibility and be advantageous under such conditions. Here, we sought to identify how mitochondrial function and cardiac metabolism are affected in a transgenic mouse model of enhanced cardiac glycolysis (GlycoHi) basally and following a short-term (7-day) high-fat diet (HFD). GlycoHi mice constitutively express an active form of phosphofructokinase-2, resulting in elevated levels of the PFK-1 allosteric activator fructose 2,6-bisphosphate. We report that basally GlycoHi mitochondria exhibit augmented pyruvate-supported respiration relative to fatty acids. Nevertheless, both WT and GlycoHi mitochondria had a similar shift toward increased rates of fatty acid–supported respiration following HFD. Metabolic profiling by GC-MS revealed distinct features based on both genotype and diet, with a unique increase in branched-chain amino acids in the GlycoHi HFD group. Targeted quantitative proteomics analysis also supported both genotype- and diet-dependent changes in protein expression and uncovered an enhanced expression of pyruvate dehydrogenase kinase 4 (PDK4) in the GlycoHi HFD group. These results support a newly identified mechanism whereby the levels of fructose 2,6-bisphosphate promote mitochondrial PDK4 levels and identify a secondary adaptive response that prevents excessive mitochondrial pyruvate oxidation when glycolysis is sustained after a high-fat dietary challenge.

The heart has the dynamic capacity to metabolize different substrates to generate chemical energy in the form of ATP. This metabolic flexibility allows the heart to respond to continual changes in energy demands and nutrient availability (1). For example, during fasting, the heart relies prominently on fatty acid oxidation and/or ketone bodies (1, 2). However, being an insulin-sensitive tissue, the heart increases glucose oxidation postprandially (3, 4). This response will also depend upon nutrient composition, as a single meal high in fat is sufficient to further reduce cardiac glucose oxidation (5). This phenomenon, described by the Randle cycle, ensures that fatty acid oxidation supersedes glycolysis and glucose oxidation via feedback mechanisms at discrete regulatory points (6). Thus, whereas the heart is able to utilize a multitude of nutrients, there is also a hierarchy in which fatty acids and ketone bodies are prioritized over glucose (7).

A complex network of interconnected pathways mediates cardiac metabolic flexibility. However, there are specific, dynamic nodes that serve as primary sites of regulation. In regard to glycolysis, phosphofructokinase-1 (PFK-1) catalyzes a committed step that requires hydrolysis of ATP (8). As such, the regulation of PFK-1 is complex and dependent upon signals from multiple pathways. It is inhibited by citrate and ATP and activated by ADP and fructose 2,6-bisphosphate (Fru-2,6-P₂) (9). Fru-2,6-P₂ is the most potent allosteric activator of PFK-1, and it is both produced and degraded by the bifunctional enzyme phosphofructokinase-2 (PFK-2)/fructose bisphosphatase-2 (FBPase-2) (9). The importance of Fru-2,6-P₂ in regulating glycolysis is supported by cardiac-specific PFK-2/FBPase-2 transgenic models that demonstrate that modulating the levels of this metabolite is sufficient to affect glycolytic rates in vivo (10, 11). Furthermore, activation of PFK-2 activity and increasing Fru-2,6-P₂ has a strong enough influence on metabolism that it can increase glucose oxidation even in the presence of fatty acids (12). Our laboratory has previously shown that the protein content of PFK-2/FBPase-2 (referred to for simplicity

This work was supported by National Institutes of Health Grants R01HL125625 (to K. M. H.), P30AG050911 (to M. K.), and P20GM103447 (to M. K.); by Grant HR17-094 (to K. M. H.) from the Oklahoma Center for the Advancement of Science; and by National Science Foundation Graduate Research Fellowship Program Grant 1849507 (to M. F. N.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This article contains Tables S1–S6 and Figs. S1–S4.

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Edited by Jeffrey E. Pessin

The abbreviations used are: PFK-1, phosphofructokinase-1; PFK-2, phosphofructokinase-2; PC, palmitylcoenzyme A; Fru-2,6-P₂, fructose 2,6-bisphosphate; FBPase-2, fructose bisphosphatase-2; PDH, pyruvate dehydrogenase; PDK4, pyruvate dehydrogenase kinase 4; HFD, high-fat diet; LFD, low-fat diet; RCR, respiratory control ratio; PCA, principal component analysis; BCAA, branched-chain amino acid; BCKDH, branched-chain α-ketoacid dehydrogenase; AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; PDP, PDH-specific phosphatase; ANOVA, analysis of variance; PP, PFK, pyrophosphate:fructose-6-phosphate phosphotransferase; Iso buffer, isolation buffer; SRM, selected reaction monitoring; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol.

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as PFK-2 in this work) is positively regulated by insulin and that chronic models of insulin deficiency lead to a persistent decrease of this glycolytic regulatory protein (13). Pyruvate dehydrogenase (PDH), located in the mitochondrial matrix, also plays a primary role in metabolic flexibility by regulating the overall rate of glucose oxidation (14). PDH produces acetyl-CoA from pyruvate, feeding into the Krebs cycle, and its inhibition can decrease the overall rate of glycolysis (15). PDH is therefore highly regulated by multiple mechanisms that include product feedback inhibition and phosphorylation. In the heart, PDH is phosphorylated and inhibited by pyruvate dehydrogenase kinase 4 (PDK4), an enzyme that also fluctuates in both protein content and activity, depending upon the nutrient status (5, 16–18). For example, under conditions where fatty acid oxidation is elevated, such as with fasting or under high-fat diet feeding, PDK4 levels are elevated.

Together, the energy-sensing mechanisms of PFK-1/PFK-2 in the cytoplasm and PDH/PDK4 in the mitochondria are critical for the orchestration of macronutrient metabolism in the heart. There is additional complexity because mitochondria exert direct effects on glycolysis via changes in PDH activity and also by releasing citrate into the cytoplasm, where it can inhibit both PFK-1 and PFK-2 (7). However, it is less clear how glycolytic activity and mitochondrial metabolic intermediates may reciprocally regulate or affect mitochondrial functions and processes (19).

It is critical to understand the underlying mechanisms of metabolic flexibility because its disruption is associated with the occurrence and progression of cardiac pathologies, such as diabetic cardiomyopathy. Thus, therapeutically, it may be beneficial to increase glucose metabolism as a way to mitigate the deleterious effects of sustained reliance on fatty acid oxidation that occurs with diabetes. In this study, we sought to define how enhancing glycolysis affects the capacity of the heart to adapt to a short-term dietary stress. To do so, we evaluated mitochondrial function, metabolomic profile, and metabolic reprogramming via quantitative proteomics in WT and a transgenic mouse model with constitutively increased cardiac glycolysis (GlycoHi mice) either on a normal chow diet or following a short-term high-fat diet (HFD) regimen. We report that GlycoHi mice, which express a constitutively active form of PFK-2 in the heart, have functionally distinct mitochondria but nonetheless are able to respond to a high-fat diet, whereas the metabolomic profile is uniquely affected by both genotype and diet. Our proteomics analysis revealed a previously unreported concerted relationship between sustained PFK-2 activity and enhanced PDK4 protein levels. Our results demonstrate that the two primary regulatory points for glucose metabolism in the cytoplasm and mitochondria are integrally linked.

Results

GlycoHi heart mitochondria have a higher rate of pyruvate-supported respiration

Initial experiments were undertaken to determine how constitutive enhancement of glycolysis affects mitochondrial function in mice fed a standard rodent chow. Heart mitochondria were isolated from WT or GlycoHi mice, and respiration was measured with either pyruvate/malate or palmitoylcarnitine (PC)/malate. A physiological concentration of pyruvate (0.1 mM) was used, as hyperphysiological concentrations can mask effects of endogenous regulatory mechanisms (5, 20). GlycoHi heart mitochondria have an increased rate of pyruvate-supported state 3 respiration relative to WT mice but exhibit no difference in respiratory control ratio (RCR) (Fig. 1, A and B). In contrast, PC-supported state 3 respiration was the same among the two groups (Fig. 1A), but there was a significant decrease in RCR in GlycoHi relative to WT (Fig. 1B). This suggests that there is a decreased efficiency of PC-supported respiration in GlycoHi heart mitochondria.

The rates of pyruvate or PC-supported state 3 respiration can change dynamically based upon nutrient availability at the time of mitochondrial isolation. We therefore compared PC-supported with pyruvate-supported state 3 respiration in each individual mitochondrial preparation to identify which substrate generated higher rates (20). For each mitochondrial preparation, WT mitochondria consistently had greater state 3 respiration rates with PC relative to pyruvate, whereas GlycoHi mitochondria had similar rates with each substrate (Fig. 1C). This was quantified by plotting the PC to pyruvate state 3 respiratory rates as a ratio (1.29 ± 0.08 for WT, 1.0 ± 0.06 for GlycoHi; Fig. 1D). This suggests that GlycoHi mice have a mitochondrial respiratory profile that is adapted to maximize pyruvate oxidation generated from persistent glycolysis.

GlycoHi heart mitochondria respond comparably with WT mitochondria after 7 days on HFD

A high-fat diet increases cardiac reliance on fatty acid oxidation and is therefore a means of testing metabolic flexibility (21). We sought to determine how sustained PFK-2 activity in GlycoHi mice affects this mitochondrial adaptation. WT and GlycoHi mice were either placed on a low-fat diet (LFD) or HFD for 7 days. Blood glucose and insulin levels were not significantly elevated under this time frame, but mice did exhibit weight gain that was independent of genotype (6.7 g in WT and 5.3 g in GlycoHi mice, combined sexes; Fig. S1). We also examined Fru-2,6-P2 levels to determine whether it was elevated in GlycoHi mouse hearts under these experimental conditions. As shown in Fig. 2A, GlycoHi hearts had significantly elevated Fru-2,6-P2 levels regardless of the feeding regime.

We next measured mitochondrial respiration from each experimental group. Table 1 presents the respiratory rate data and demonstrates that 1 week on HFD had minimal effects on any of the individual measured parameters. However, examination of the relative rates of PC- to pyruvate-supported state 3 respiration indicates that mitochondria isolated from both WT and GlycoHi mice on HFD exhibited a significant increase in the ratio of PC- to pyruvate-supported state 3 respiration (Fig. 2B). In male mice, the ratio increased from 1.29 to 1.76 in WT and from 1.09 to 1.52 in GlycoHi mice. A similar increase was also observed in the female mice, and a composite of both sexes is presented in Fig. 2C. The significant increase in this ratio suggests that a week on HFD is sufficient to increase the maximal rate of respiration with fatty acids relative to pyruvate. Furthermore, WT and GlycoHi mitochondria have a similar capacity to adapt to alterations in nutrient availability.
Increased PFK-2 activity alters metabolomic profile in a diet-dependent manner

Having characterized mitochondrial function, we next sought to determine how increased glycolytic activity affected the metabolic profile of WT and Glyco\textsuperscript{Hi} hearts after 7 days on HFD. Using semi-targeted GC-MS analysis, we identified 38 unique compounds belonging to primary metabolic pathways (Table S1). The data were first subjected to a principal component analysis (PCA) to evaluate whether there is a global effect of genotype and diet on cardiac metabolic profile. The results demonstrated a decisively clear separation between all four groups, suggesting a high sensitivity of the metabolic response to both elevated PFK-2 activity and diet (Fig. 3). The strongest factor was clearly mediated by genotype (PC1, 55.6% variance). Interestingly, the diet effect (PC2, 16.0% variance) was also genotype-dependent, as there is a reverse distribution of the shape-
grouped samples across PC2 (Fig. 3). This analysis suggests that global metabolism is differentially affected by short-term HFD in GlycoHi mice.

We next identified the metabolites that were significantly different either by diet or genotype (Table S2), and pathway heat maps were generated (Fig. 4, A and B). Diet (Fig. 4A) caused fewer metabolite changes than genotype (Fig. 4B), and they were scattered across multiple pathways. Consistent with the PCA, this indicates that genotype has a larger effect. GlycoHi hearts had lower levels of glucose 6-phosphate and fructose 6-phosphate (Fig. 4B) on both LFD and HFD, which is consistent with an increased rate of glycolysis that may deplete these early glycolytic intermediates. The pathway analysis, based upon genotype, showed a significant up-regulation of only branched-chain amino acid (BCAA) metabolism in the HFD condition in GlycoHi hearts (Table S3). Indeed, BCAAs (Leu, Ile, and Val), which are metabolized in mitochondria, were all more abundant in GlycoHi hearts relative to WT (Fig. 4B). This appears to be a unique effect, as other amino acids (Asn and Asp) had a decrease in abundance relative to WT. Collectively, the metabolic profiling reveals that GlycoHi hearts have decreased levels of early glycolytic intermediates and a unique increase in BCAAs when challenged with short-term HFD.

The branched-chain α-keto acid dehydrogenase (BCKDH) complex catalyzes the rate-limiting step of branched-chain catabolism (22, 23). To investigate a potential mechanism of increased BCAAs, we examined the abundance and phosphorylation state of the E1α subunit of BCKDH. As shown in Fig. 5A, the content of BCKDH-E1α was significantly decreased in GlycoHi hearts relative to WT (Fig. 4B). This appears to be a unique effect, as other amino acids (Asn and Asp) had a decrease in abundance relative to WT. Collectively, the metabolic profiling reveals that GlycoHi hearts have decreased levels of early glycolytic intermediates and a unique increase in BCAAs when challenged with short-term HFD.

GlycoHi mice have distinct proteomic profiles under different diet conditions

In contrast to metabolites, protein expression levels are relatively more stable and consequently can provide further

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**Table 1**

|                | WT LFD | WT HFD | GlycoHi LFD | GlycoHi HFD |
|----------------|--------|--------|-------------|-------------|
| Pyruvate state 3 | 50.6 ± 9.0 | 48.2 ± 13.2 | 61.2 ± 9.7* | 48.7 ± 15.3 |
| Pyruvate state 4 | 6.1 ± 1.2 | 6.6 ± 2.6 | 7.5 ± 2.6 | 6.3 ± 1.7 |
| Pyruvate RCR    | 8.6 ± 2.3 | 8.7 ± 4.6 | 7.7 ± 1.6 | 7.7 ± 1.4 |
| PC state 3      | 70.4 ± 15.0 | 84.4 ± 25.3 | 68.7 ± 23.3 | 80.0 ± 18.8 |
| PC state 4      | 11.8 ± 1.7 | 12.6 ± 4.3 | 12.3 ± 4.4 | 13.4 ± 1.7 |
| PC RCR          | 6.2 ± 0.9 | 6.8 ± 0.8 | 5.7 ± 0.8 | 6.0 ± 1.4 |

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**Figure 2.** GlycoHi mitochondria remain responsive to a change in nutrient availability with a short-term high-fat diet. WT and GlycoHi mice were fed a 7-day HFD or LFD. A, the metabolite fructose 2,6-bisphosphate was measured in total heart homogenates of WT and GlycoHi mice fed the indicated diets; n = 3–4 biological replicates/group. B, state 3 respiration ratios of isolated mitochondria were measured in both female (left) and male mice (right); n = 4–6 biological replicates/group. C, combined male and female state 3 respiration ratios. Scatterplot data are represented as the mean ± S.D. (error bars). *, p < 0.05; ***, p < 0.001 by two-way ANOVA with Tukey post hoc test or unpaired two-tailed Student’s t test.
insight into sustained changes in global metabolism. Using a targeted proteomics approach, we analyzed total heart homogenate samples (WT and GlycoH) either on a LFD or HFD for 7 days for the abundance of 128 metabolic enzymes (Table S4). To visualize, data were broken down into four unique clusters (Fig. 6A). Clusters 1 and 4 include proteins that had unique changes in their relative protein abundance attributable to a diet or a genotypic effect, respectively. Proteins separated into clusters 2 and 3 had distinct responses due to a specific treatment and a specific genotype. For example, within cluster 2, the WT/LFD group was different from the other three groups, whereas in cluster 3, the GlycoH/HFD group was distinct.

Next, individual proteins of each cluster were separately subjected to pathway analysis. The top 20 metabolic pathways identified were plotted in a Venn diagram (Fig. 6B), allowing the examination of pathways that were either exclusive or shared among clusters. Table S5 provides information on the individual proteins within each cluster (in the same order as in the heat map in Fig. 6A), the top 20 pathways that were found to be enriched with the proteins of each cluster, individual proteins that belonged to each of the presented pathways, and the indicated pathways that were shared between clusters. Because many of the top pathways are very general, we chose to focus on the primary metabolic pathway(s) exclusive to each cluster.
Their identities and their relative magnitudes of change as compared with the other experimental groups are shown in Fig. 6C. In cluster 1, where changes were driven by diet, lipid metabolism was the primary and unique metabolic pathway. Specifically, the HFD-treated groups had a higher relative abundance of lipid metabolism proteins regardless of genotype. Clusters 2 and 3 were enriched in proteins belonging to two major, unique metabolic pathways. Proteins involved in carbohydrate metabolism and redox metabolism were more abundant in WT/low-fat animals (Fig. 6C, cluster 2); tricarboxylic acid cycle–related proteins and a different subset of redox metabolic pathway proteins were more abundant in GlycoHi/ high-fat animals (Fig. 6C, cluster 3). In cluster 4, proteins involved in glucose metabolism had greater abundance in both GlycoHi treatment groups. This is consistent with this transgenic model’s increased PFK-2 and glycolytic activity. Intriguingly, the GlycoHi heart under HFD conditions had a higher abundance of proteins involved in both lipid metabolism and glucose metabolism. In contrast, WT/HFD hearts had an increase in lipid metabolism but a decrease in glucose metabolism. These results are consistent with our mitochondrial functional measurements that demonstrated that both genotypes had increased relative rates of lipid-supported respiration. However, they also suggest that GlycoHi hearts sustain elevated glycolytic enzymes when challenged with short-term HFD.

AMP-activated protein kinase (AMPK) content and activity are unaffected by diet or PFK-2 overexpression

AMPK is considered a master regulator of metabolism. It is activated by phosphorylation in response to nutrient stress to initiate downstream signaling events that ensure that energy demand is met with proper nutrient catabolism (24). Given this central role, we sought to identify whether changes in its content and phosphorylation state were apparent under our experimental conditions. We found that there were no changes in AMPK status between WT and GlycoHi groups (Fig. S2, A and B).
Acetyl-CoA carboxylase (ACC) is a well-established downstream target of AMPK that is inhibited by phosphorylation (24, 25). ACC produces malonyl-CoA, an inhibitor of carnitine palmitoyltransferase 1 (CPT1). Thus, an increase in phosphorylation of ACC may indicate increasing rates of fatty acid oxidation. Western blot analysis revealed an overall increase of total ACC in the GlycoHi mice compared with WT groups. However, no significant change in ACC phosphorylation state was seen across any of the groups (Fig. S2, C and D). Cumulatively, these results suggest that AMPK is not a significant contributor to the metabolic changes identified in the pathway analysis.

Nutrient stress enhances PDK4 protein levels in GlycoHi mice

Inspection of the magnitudes of change of individual proteins in our proteomic data revealed that the most marked change was to pyruvate dehydrogenase 4 (PDK4; denoted with an asterisk on the heat map in Fig. 6A). PDK4 levels increased by 2.5-fold in WT HFD, but this was significantly more pronounced in the GlycoHi group (4.1-fold increase from LFD to HFD conditions; Fig. 7A). PDK4 phosphorylates and inhibits pyruvate dehydrogenase, thereby decreasing the overall rate of glucose oxidation and promoting increased fatty acid oxidation (1). The increase in PDK4 was anticipated, as previous studies have shown that a short-term high-fat diet increases PDK4 protein abundance (5). However, an interaction between PFK-2 activity and PDK4 protein levels has not been previously described.

We next sought to determine whether this enhancement of PDK4 content was specific to HFD treatment or whether it represented a broader adaptation in GlycoHi mice to nutrient stress. It is established that fasting also increases PDK4 protein expression as part of the glucose-sparing effect (16). WT and GlycoHi mice were either fed ad libitum or subjected to a 12-h fast, and PDK4 levels were measured by Western blot analysis. A similar trend in PDK4 protein levels was seen with fasting as compared with HFD conditions (Fig. 7B). PDK4 levels were 1.8-fold higher in GlycoHi fasted hearts as compared with WT fasted hearts. This increase in PDK4 content in GlycoHi mice was also reflected in PDH activity. In the fed state, PDH activity in the GlycoHi mice was significantly higher than in the WT control group (2.3-fold higher; Fig. 7C), consistent with our mitochondrial respiration data that demonstrates that under fed conditions, the GlycoHi have enhanced pyruvate-supported respiration (Fig. 1). Under fasting conditions, however, PDH activity returned to a similar rate as in WT hearts (Fig. 7C). These results demonstrate that enhancing glycolysis can induce an increase in mitochondrial PDK4 to act as a secondary means of limiting glucose oxidation.

Reciprocal to pyruvate dehydrogenase kinases, PDH-specific phosphatases (PDPs) mediate the dephosphorylation and acti-
vation of PDH (26). To determine whether PDP activity is different between WT and GlycoHi, experiments were performed in which PDH activity and its phosphorylation state were measured immediately after isolation of mitochondria (from normally fed animals) and then at 2-h increments. As shown in Fig. 7 (D and E), PDH activity increases over time concurrently with a decrease in protein phosphorylation. Furthermore, PDH dephosphorylation plateaued at 2 h in GlycoHi and 4 h in WT. This supports a more rapid PDP-mediated dephosphorylation of PDH in GlycoHi hearts. It was also observed that PDH activity continued to increase in a time-dependent manner independent of further dephosphorylation, suggesting that other reversible regulatory mechanisms may affect PDH activity.

**PDK4 protein levels in GlycoHi mice are regulated at both posttranscriptional and posttranslational levels**

We next sought to determine the mechanism whereby PDK4 levels were increased. First, we examined whether HFD conditions affected PDK4 at the transcriptional level. In WT hearts, there was a significant increase in PDK4 transcript with 7-day HFD treatment. In contrast, GlycoHi hearts had significantly higher levels of PDK4 mRNA levels under LFD, but they were

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Figure 7. PDK4 levels increase more significantly in GlycoHi hearts upon nutrient stress. A, PDK4 protein abundance in total heart homogenates was measured by selected reaction monitoring MS (n = 3–6/group). B, PDK4 protein content and pyruvate dehydrogenase E2 subunit (dihydrolipoyl transacetylase) were measured by Western blot analysis in isolated cardiac mitochondria (quantification of PDK4 above; representative image below) (n = 4/group). The representative image is taken from a single, cropped and cut blot, and the vertical dashed line was added for presentation. C, PDH activity in isolated cardiac mitochondria was measured by a spectrophotometer-based assay under fed or fasted (12 h) conditions (n = 5–6 biological replicates/group). D, PDH activity was measured in mitochondria, isolated from normally fed WT or GlycoHi mice (n = 3–5 biological replicates/group) in 2-h intervals (data from fed condition in C is represented as time 0 h in D). D, a portion of mitochondria from each time point in D were subjected to Western blot analysis to determine the phosphorylation status of PDH, which is expressed as the ratio of signal of phospho-PDH to total PDH (quantification of PDH above; representative image below; n = 3–6 biological replicates/group). The images were taken from a single, cut and cropped blot, and the vertical dashed lines were added for presentation. Scatterplot data are represented as the mean ± S.D. (error bars). *, p < 0.05; **, p < 0.01; ***, p < 0.001 by two-way ANOVA with Tukey post hoc test or unpaired two-tailed Student t test.
not further increased with HFD (Fig. 8A). FOXO1 and PGC1α are two identified transcription factors that regulate PDK4 mRNA expression in the heart (15, 27, 28). FOXO1 is regulated by phosphorylation such that when it is phosphorylated, its transcriptional activity is decreased and it is translocated from the nucleus to the cytoplasm, where it is then degraded (29). FOXO1 phosphorylation was significantly less in GlycoHi mice compared with WT, as determined by two-way ANOVA (Fig. 8B). Multiple-comparison analysis showed a significant decrease between the LFD-treated groups with no additional decrease in the GlycoHi HFD group. Together, the decrease in FOXO1 phosphorylation seen in GlycoHi groups follows the relative increase in levels of PDK4 transcript. PGC1α is another well-described promoter of fatty acid oxidation genes and is up-regulated under HFD conditions (27, 30). Indeed, PGC1α transcript levels were significantly elevated in both WT and GlycoHi hearts on HFD. However, protein levels of PGC1α were only elevated in WT hearts on HFD (Fig. 8C). These results support a greater role for FOXO1 than PGC1α in the elevated levels of PDK4 transcript in GlycoHi hearts.

To better understand the discrepancy between mRNA levels and protein abundance, we next examined factors, CoA and Lon protease, that may affect PDK4 posttranslationally. PDK4 associates with the PDH complex, and this protects it from degradation (18). Upon an increase in the PDH substrate, CoA, PDK4 dissociates from the complex and is degraded by Lon protease (17). We found that there was no difference in CoA levels between the WT and GlycoHi groups (Fig. S3). However,

Figure 8. PDK4 transcript, but not protein, is constitutively increased in GlycoHi hearts. A, quantitative RT-PCR determination of PDK4 mRNA levels in hearts. B, total heart homogenates from mice given a LFD (−) or HFD (+) were used to measure FOXO1 phosphorylation standardized to total FOXO1 (quantification of FOXO1 above; representative image below). C, PGC1α mRNA level measured by quantitative RT-PCR (left) and PGC1α protein levels (standardized to actin) by Western blot analysis (right; representative image shown below). For all conditions, n = 4–5 biological replicates/group. D, Lon protease was measured by selected reaction monitoring MS (n = 3–6 biological replicates/group). The images were taken from a single, cut and cropped blot, and the vertical dashed lines were added for presentation. Scatterplot data are represented as the mean ± S.D. (error bars). *, p < 0.05; **, p < 0.01 by two-way ANOVA with Tukey post hoc test or unpaired two-tailed Student’s t test.
Effect of increased glycolysis on metabolic flexibility

Lon protease levels were significantly elevated in GlycoHi hearts regardless of diet (Fig. 8D). Together, our results suggest that GlycoHi hearts have constitutively elevated PDK4 transcript, and this may be mediated by increased FOXO1 activity. Furthermore, the dynamic increase of PDK4 may be facilitated by a lack of susceptibility to Lon protease degradation due to the lack of a proportional increase in CoA.

Discussion

In this study, we identified how enhancing cardiac glycolysis affects the metabolic response to a short-term high-fat diet challenge. We found that there are adaptations in both mitochondrial function and global metabolism. GlycoHi mitochondria had enhanced pyruvate-supported respiration basally but were similarly affected by a short-term HFD regimen. Consistent with our functional data, quantitative proteomics experiments revealed that both WT and GlycoHi mice exhibited increases in lipid metabolism. Uniquely, however, GlycoHi hearts had increased glucose metabolism pathway expression even with HFD. This suggests that glycolysis may be sustained under the dietary challenge. However, an exaggerated increase in PDK4 protein may serve as an additional means of limiting glucose oxidation.

Among the animal models of perturbed cardiac metabolism, the GlycoHi mice are unique in specifically targeting glycolysis. The mice were engineered by the Epstein group to express a transgene PFK-2 is not under the control of insulin enzyme that has constitutively decreased FBPase-2 activity (11). Unlike endogenously expressed PFK-2, activation of the transgene PFK-2 is not under the control of insulin or adrenergic activation, and the net result is sustained production and accumulation of Fru-2,6-P₂ and increased glycolysis (11). Recently, the Hill group employed the GlycoHi mice to sustain glycolysis under a regimen of exercise (31). However, the role of enhanced glycolysis under a dietary stress has not been previously explored. We chose a short-term-high-fat diet because previous studies have demonstrated that even a single high-fat meal is sufficient to increase cardiac fatty acid oxidation (5, 21). Indeed, mice already exhibit significant weight gain at 7 days on the diet (Fig. S1F). In addition, our GC-MS metabolic profiling data revealed distinct changes induced by diet (Fig. 3).

Our initial experiments focused on changes in mitochondrial function. This is because of their importance in cardiac health and because relatively little is known regarding how upstream changes in glycolysis may affect their function. We found that in response to increased glycolysis, mitochondria isolated from GlycoHi mice on normal chow had enhanced pyruvate-supported respiration and a decreased RCR with palmitoylcarnitine. We thus anticipated that in response to HFD, GlycoHi mitochondria may sustain this phenotype. However, both WT and GlycoHi mitochondria displayed increases in PC-supported relative to pyruvate-supported respiration. This demonstrated that GlycoHi hearts retain metabolic flexibility at the level of mitochondrial respiratory capacity.

Metabolic profiling was performed to identify metabolic phenotypes. As shown in our PCA (Fig. 3), genotype was clearly the dominant source of variation. Nevertheless, both WT and GlycoHi hearts had similar magnitudes of change in response to HFD. The lack of convergence of variance between the groups on HFD supports a clearly differential response to diet. The results of pathway analysis revealed an increase in BCAA biosynthesis as the most distinguishing difference between WT and GlycoHi hearts in response to HFD. We recently reported that BCAAs are also elevated in GlycoHi hearts with fasting (32), suggesting that their increase may be a common response to nutrient stress in this model. BCAAs are of interest because of their bioactivity and potential role in cardiac pathologies (33). In addition, BCAAs can directly inhibit PDH, suggesting another potential layer of regulation that could minimize pyruvate oxidation (34). BCAAs also have a well-described capacity to increase the activity of the master nutrient and energy sensor, mTORc1 (35). As such, we examined whether the short-term HFD and associated increase in BCAAs affected mTORc1 signaling by measuring the phosphorylation status of 4E-BP1, a well-described substrate of mTORc1 kinase activity (36, 37). We found that WT levels of 4E-BP1 decreased in response to a HFD, an effect that has been previously reported to occur under these conditions (38). However, GlycoHi heart 4E-BP1 levels did not exhibit this known diet-dependent decrease (Fig. S4A). Similarly, phosphorylation patterns of 4E-BP1 closely resembled the changes seen in total 4E-BP1. The magnitude and direction of change of phosphorylated 4E-BP1 correlated closely with the total levels (Fig. S4B). Two-way ANOVA revealed a significant interaction between the genotypic and diet effect on total 4E-BP1 levels. This suggests that mTORc1 signaling may be elevated in GlycoHi hearts, but the relationship with elevated BCAAs will be pursued in future studies. Finally, these findings are also provocative given that an increase in 4E-BP1 expression has been found to be protective against diet-induced obesity (38, 39).

Quantitative proteomic analysis further revealed unique genotype-dependent responses to HFD between WT and GlycoHi hearts. Cluster and pathway analysis supported an increase in glucose utilization in GlycoHi hearts. Additionally, short-term HFD increased enzymes involved in fatty acid metabolism, regardless of phenotype. Thus, like the mitochondrial functional data, these results show that enhancing glycolysis affects global metabolism, yet regulatory mechanisms are largely intact to respond to an increase in circulating fatty acid levels.

One such adaptive response we identified is that GlycoHi hearts have an accentuated increase in PDK4 protein levels. Interestingly, PDK4 mRNA levels were elevated even in animals on the control diet, and unlike in WT hearts, expression did not further increase with high-fat diet treatment. We hypothesize that the elevated transcript expression allows for an enhanced and rapid response to changes in nutrient availability. This is supported by the observation that PDK4 protein levels also increased significantly more in GlycoHi hearts, as compared with WT controls, in response to fasting. Reciprocally, elevated Lon protease may promote rapid turnover of PDK4 to facilitate an increase in pyruvate oxidation. Although the regulation of PDK4 activity by metabolites is well-described, our results suggest a newly identified means of regulation of PDK4 content by PFK-2/PFK-1 activity. Future studies will be needed to deter-
mine whether it is Fru-2,6-P$_2$ or other downstream glycolytic metabolites that facilitate the increased PDK4 expression.

Our study demonstrates that sustaining cardiac glycolysis induces compensatory mechanisms when faced with a short-term high-fat diet challenge. This suggests that attempts to intervene with metabolic inflexibility by enhancing cardiac glycolysis may be met by compensatory mechanisms of increased BCAAs and PDK4 content. Future studies will need to determine whether it is specifically the increase in Fru-2,6-P and PFK1 activity that mediated these effects. In addition, further studies with long-term nutrient challenges that induce insulin resistance will be needed to fully investigate the possible beneficial effects of increased glycolysis on disease states like diabetic cardiomyopathy.

**Experimental procedures**

**Experimental animals and diets**

Littermates of transgenic Glyco$^{Ht}$ and WT mice on the FVB/NJ background (30–36 weeks of age) were used in this study. Mice were group-housed and maintained on a 12-h light/dark cycle (light from 06:00 to 18:00). Glyco$^{Ht}$ mice were obtained from the University of Louisville, and the development of these transgenic mice has been described previously (11). Briefly, Glyco$^{Ht}$ mice have cardiac-specific expression of a kinase-deficient PFK-2 bound to the α-myosin heavy-chain promoter. Mice were given a 7-day treatment of either a low-fat diet (10% fat, 70% carbohydrate, and 20% protein, by kilocalories) or a high-fat diet (60% fat, 20% carbohydrate, and 20% protein, by kilocalories) (Research Diets Inc.). For the fasting experiment, food was removed at 21:00, mice were placed in cages with fresh bedding, and then they were sacrificed the following morning at 09:00. Mice were euthanized by cervical dislocation, and hearts were collected. All procedures were approved by the Oklahoma Medical Research Foundation Animal Care and Use Committee.

**Blood and plasma measurements**

Insulin levels were measured in plasma using an insulin ELISA (ALPCO). Food was removed 4 h prior to blood collection. Animals were euthanized by cervical dislocation. Blood was collected immediately following euthanasia by cardiac puncture. Blood glucose was measured via tail snip using a blood glucose monitor (Contour) after euthanasia and before tissue collection.

**Measurement of Fru-2,6-P$_2$**

Fructose 2,6-biphosphate content in the heart was measured as described previously (40). Briefly, Fru-2,6-P$_2$ was extracted from 10–15 mg of pulverized tissue in 200–300 μl of 50 mM NaOH by heating at 80 °C for 20 min. The extract was then cooled and neutralized at 4 °C by the addition of glacial acetic acid in the presence of 20 mM HEPES. After precipitation of insoluble matters by centrifugation at 10,000 × g for 15 min, the supernatant was collected, and 50 μl of the extract from each sample was used for the pyrophosphate:fructose-6-phosphate phosphotransferase (PP$_{1}$-PFK) activity measurement. The assay was performed in 5 mM MgCl$_2$, 50 mM Tris buffer, pH 8.0, supplemented with 0.5 mM PP$_3$ and 1 mM fructose-6-phosphate. PP$_{1}$-PFK activity was measured as the rate of NADH oxidation ($v_{s0} = 6200$ μM·min$^{-1}$·cm$^{-2}$) following the addition of $5 μM$ NADH to the mixture of tissue extract and isolated PP$_{1}$-PFK, in the presence of 2 μg/ml triosephosphate isomerase, 10 μg/ml glycerol-3-phosphate dehydrogenase, and 0.2 units/ml aldolase. All purified enzymes were from Sigma-Aldrich except for PP$_{1}$-PFK, which was enriched from potato tubers as described (40).

**Isolation of cardiac mitochondria**

Heart mitochondria were isolated as reported previously (20, 41). Following euthanasia, the chest cavity was opened, and the heart was perfused with 5 ml of isolation buffer (Iso buffer) containing 210 mM mannitol, 70 mM sucrose, 1.0 mM EDTA, and 5.0 mM MOPS, pH 7.4, via injection into the left ventricle. Hearts were excised and minced in 5 ml of Iso buffer. Next, the minced tissue was passed through a motor-driven Potter–Elvehjem tissue grinder a total of five times. The homogenate was spun at 5000 × g for 5 min at 4 °C, and the supernatant was passed through a cheesecloth into a fresh tube and then spun again at 5000 × g for 10 min. The resulting mitochondrial pellet was resuspended in 60 μl of Iso buffer. Iso buffer was maintained ice-cold at all times, and samples were kept on ice. The protein concentration was determined by the BCA (bicinchoninic acid) method (Thermo Scientific) with BSA as the standard.

**Mitochondrial respiration measurements**

Isolated mitochondria were diluted to 0.25 mg/ml in 210 mM mannitol, 70 mM sucrose, 10 mM MOPS, 5.0 mM KH$_2$PO$_4$, and 0.5 mg/ml BSA, pH 7.4, containing either 0.1 mM pyruvate plus 1.0 mM malate or 30 μM palmitoylcarnitine plus 1.0 mM malate, as indicated. Respiration was measured at 20 °C by using a fiber optic oxygen measurement system (Instech) that utilizes the fluorescence lifetime technique. State 3 respiration was initiated by the addition of ADP (0.5 mM) at 2 min.

**Metabolic profiling and data analysis**

At the end of treatment, mice were euthanized by cervical dislocation, and hearts were excised after perfusion with 5 ml of ice-cold saline solution by injection into the left ventricle. The perfused hearts were quickly blotted dry and snap-frozen. Samples were kept in liquid nitrogen until metabolite extraction and subjected to metabolic profiling by GC-MS. Metabolic profiling was performed based on previously published methods (32, 42). Briefly, snap-frozen whole hearts were pulverized in a Qiagen TissueLyser II bead beater. Next, 35–45 mg of the resulting powder was resuspended in a methanol/chloroform/water (2:1:1) mixture for metabolite extraction, and ribitol (20 mg/ml) was added as internal standard. The samples were sonicated, incubated at 70 °C for 10 min, and centrifuged (16,000 × g). Next, 400 μl of supernatant were dried in a vacuum concentrator for 5 h. Dried residues were derivatized in 40 μl of 20 mg/ml methoxyamine hydrochloride in pyridine for 2 h (37 °C with constant orbital shaking), and then 70 μl of N,O-bis(trimethylsilyl)trifluoroacetamide was added for an additional 30 min. A mixture of alkanes (C10–C24) was used as a retention time standard. The samples were injected into the
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The HPLC column was configured with a splitless capillary column. HPLC system analyzed using selected reaction monitoring with a triple quadrupole instrument. The samples were washed, reduced with DTT, alkylated with iodoacetamide, and digested with trypsin. The hydrophobicity of all peptides was calculated using the resource hs2.proteome.ca/SSRCalc/SSRCalcX.html\(^3\). Peptides with calculated hydrophobicity <10 or >45 were excluded because of early or late elution, respectively, under the LC conditions used. Peptides were also excluded from consideration if they contained methionine (because of oxidation). Peptides seen in other proteins were excluded based on a combination of database searches and consultation with the Peptide Atlas, looking for annotation as multiple gene locations (www.peptideatlas.org). All remaining peptides were tested experimentally by generating an inclusion list and expected retention times for LC-tandem MS analysis on the QEx Plus in a PRM experiment. Data are analyzed using the program Skyline set to find the b- and y-ion series with \(m/z\) tolerances <5 ppm (49). Collision-induced dissociation spectra were recorded for candidate chromatographic peaks were evaluated manually to validate the correct identity of each peptide. The Skyline detection was refined to use the top 10 fragment ions to build an initial transition list for SRM analysis on the triple quadrupole instrument. Based on the SRM data, final choices were made for the two best flyers based on which peptides gave the best chromatographic peak area responses. The final set of product ions to monitor was also refined by eliminating ions that did not contribute significantly to the total response. Peptide and retention time data were entered into our peptide database for use with a relative retention time system known as iRT (52).

These assays are defined as tier 3 using the system created by the targeted quantitative proteomics community (53). This system recognizes the purpose of different experiments, including the highly multiplexed experiments used here.

Proteomics assay development

All assays were developed through experimental testing in our laboratory to find the best flyer peptides (50). Assay development began by calculating the set of peptides formed by tryptic digestion of each protein. The hydrophobicity of all peptides was calculated using the resource hs2.proteome.ca/SSRCalc/SSRCalcX.html\(^3\). Peptides with calculated hydrophobicity <10 or >45 were excluded because of early or late elution, respectively, under the LC conditions used. Peptides were also excluded from consideration if they contained methionine (because of oxidation). Peptides seen in other proteins were excluded based on a combination of database searches and consultation with the Peptide Atlas, looking for annotation as multiple gene locations (www.peptideatlas.org). All remaining peptides were tested experimentally by generating an inclusion list and expected retention times for LC-tandem MS analysis on the QEx Plus in a PRM experiment. Data are analyzed using the program Skyline set to find the b- and y-ion series with \(m/z\) tolerances <5 ppm (49). Collision-induced dissociation spectra were recorded for candidate chromatographic peaks were evaluated manually to validate the correct identity of each peptide. The Skyline detection was refined to use the top 10 fragment ions to build an initial transition list for SRM analysis on the triple quadrupole instrument. Based on the SRM data, final choices were made for the two best flyers based on which peptides gave the best chromatographic peak area responses. The final set of product ions to monitor was also refined by eliminating ions that did not contribute significantly to the total response. Peptide and retention time data were entered into our peptide database for use with a relative retention time system known as iRT (52).

These assays are defined as tier 3 using the system created by the targeted quantitative proteomics community (53). This system recognizes the purpose of different experiments, including the highly multiplexed experiments used here.

Proteomics data processing

The program Skyline was used to evaluate the data (49). The latest version available was used. All chromatograms were inspected manually within the Skyline visualization system to assure correct peak integration. The response for each peptide was calculated by Skyline as the total of all product ions recorded. Those values were used to calculate the total response for each protein as the geometric mean of all peptides measured for that protein, typically two. The amount of each protein in the sample, in pmol, was determined by dividing the total response of the protein by the total response of the BSA internal standard and multiplying by the amount of albumin added. Finally, concentration as pmol/\(\mu\)g total protein is calculated by dividing by the amount of protein taken for analysis. This approach to data analysis incorporates the Best Flyers system described by Aebersold’s laboratory (50). The Best Flyers system recognizes that the relative response for the best flyer peptides for any protein are functionally equivalent, thereby eliminating the need to independently calibrate every pep-
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RNA was extracted from frozen hearts collected in the same manner as the samples frozen for metabolomic analysis. RNA was extracted using Tripure reagent (Roche Applied Science) from heart tissue (~30 mg). cDNA was made using a QuantiTect reverse transcription kit (Qiagen). Quantitative PCRs were performed in duplicate by the CFX96 real-time system (Bio-Rad) as directed by the QuantiTect SYBR Green PCR kit (Qiagen). The data for each target gene were normalized to the gene of three reference genes (Eef1e1, Rpl4, and Tbp) for cardiac-specific gene expression analysis in mouse hearts (58).

Author contributions—M. F. N. and K. M. H. conceptualization; M. F. N., A. B., S. M., Z. T. Y., M. W., N. C. C., L. I. S., and M. K. investigation; M. F. N., A. B., S. M., and M. K. methodology; M. F. N., A. B., and K. M. H. writing-review and editing; M. F. N., A. B., and M. K. data curation; A. B. and K. M. H. fund acquisition.

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PDH activity assay
Pyruvate dehydrogenase activity was measured as described previously (20). Briefly, isolated cardiac mitochondria were diluted to 0.05 mg/ml in a buffer containing 25 mM MOPS and 0.05% Triton X-100 at pH 7.4. Solubilization of mitochondria with 0.05% Triton X-100 inhibits complex I of the respiratory chain, preventing consumption of NADH. PDH activity was measured spectrophotometrically (Agilent, 8452A) as the rate of NAD\(^+\) reduction to NADH (340 nm, \(\epsilon = 6200 \text{ M}^{-1} \text{ cm}^{-1}\)) upon the addition of 2.5 mM pyruvate, 0.1 mM CoASH, 0.2 mM thiamine pyrophosphate, 1.0 mM NAD\(^+\), and 5.0 mM MgCl\(_2\) at pH 7.4.

Statistical analysis
Data were analyzed using GraphPad Prism 7. Data are presented as means. Pairwise comparison between groups was performed using a paired or unpaired (A, B, and D) two-tailed Student’s t test, as specified. Multiple comparison was performed using two-way ANOVA with Tukey post hoc analysis, unless otherwise noted. \(p < 0.05\) was considered statistically significant.

Author contributions—M. F. N. and K. M. H. conceptualization; M. F. N., A. B., S. M., Z. T. Y., M. W., N. C. C., L. I. S., and M. K. investigation; M. F. N., A. B., S. M., and M. K. methodology; M. F. N., A. B., and K. M. H. writing-review and editing; M. F. N., A. B., and M. K. data curation; A. B. and K. M. H. formal analysis; A. B. visualization; M. F. N. and K. M. H. writing-original draft; K. M. H. funding acquisition.
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