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GC-MS metabolic profiling reveals Fructose-2,6-bisphosphate regulates branched chain amino acid metabolism in the heart during fasting

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Abstract

Introduction—As an insulin sensitive tissue, the heart decreases glucose usage during fasting. This response is mediated, in part, by decreasing phosphofructokinase-2 (PFK-2) activity and levels of its product fructose-2,6-bisphosphate. However, the importance of fructose-2,6-bisphosphate in the fasting response on other metabolic pathways has not been evaluated.

Objectives—The goal of this study is to determine how sustaining cardiac fructose-2,6-bisphosphate levels during fasting affects the metabolomic profile.

Methods—Control and transgenic mice expressing a constitutively active form of PFK-2 (Glyco^{Hi}) were subjected to either 12-hours fasting or regular feeding. Animals (n=4 per group) were used for whole-heart extraction, followed by GC-MS metabolic profiling and multivariate data analysis.

Results—Principal component analysis displayed differences between Control and Glyco^{Hi} groups under both fasting and fed conditions while a clear response to fasting was observed only for Control animals. However, pathway analysis revealed that these smaller changes in the Glyco^{Hi} group were significantly associated with branched-chain amino acid (BCAA) metabolism (~40% increase in all BCAAs). Correlation network analysis demonstrated clear differences in response

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Author Contribution Statement

AB, SM, MN, TG and KH conceived and designed research. AB, SM, MN, MW and KH conducted experiments. AB, SM, TG and KH analyzed data. AB, TG and KH wrote the manuscript. All authors read and approved the manuscript.

Author Conflict of Interest Statement

The authors declare that they have no conflict of interest.

Compliance with Ethical Standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Data Availability Statement

The metabolomics data collected for this paper are submitted as supplementary file.

to fasting between Control and Glyco^{Hi} groups amongst most parameters. Notably, fasting caused an increase in network density in the Control group from 0.12 to 0.14 while the Glyco^{Hi} group responded oppositely (0.17 to 0.15).

Conclusions—Elevated cardiac PFK-2 activity during fasting selectively increases BCAAs levels and decreases global changes in metabolism.

Keywords

GC-MS metabolomics; heart pathologies; cardiac metabolism; correlation network

1. Introduction

The healthy heart has the dynamic capacity to switch between fatty acid oxidation and glycolysis to meet its incessant energetic demands. The heart, while primarily using fatty acid oxidation, increases glucose uptake and oxidation in response to insulin and sympathetic stimulation (Griffin et al., 2016). The irreversible conversion of fructose-6-phosphate to fructose-1,6-biphosphate by phosphofructokinase-1 (PFK-1) is the key regulatory step of glycolysis, and altering this step can lead to activation or inhibition of the entire pathway (Jenkins et al., 2011). Fructose-2,6-biphosphate is the strongest allosteric activator of PFK-1 (Depre et al., 1998; Kolwicz and Tian, 2011) and its content is regulated by the bifunctional enzyme phosphofructokinase-2 (PFK-2)/fructose biphosphatase-2 (FBPase-2). The cardiac isoform of this enzyme (gene product of *pfkfb2*) is phosphorylated in response to increases in insulin or sympathetic signaling, which in turn enhances PFK-2 activity and promotes the generation of fructose-2,6-biphosphate. We have found that in the absence of insulin signaling, as occurs with fasting, PFK-2 is dephosphorylated and its content decreases (Bockus et al., 2017). Presumably this decrease in PFK-2 activity acts to impede cardiac glycolysis under conditions of low circulating glucose and is integral to how the heart adapts to changes in nutrient availability. However, the significance of decreased PFK-2 activity and fructose-2,6-bisphosphate to the overall cardiac metabolic response to fasting is not known (Depré et al., 1998). This is important to understand because metabolic flexibility is essential for cardiac health and identifying how the heart responds to normal fluctuations in nutrient availability can be used to further understand pathological conditions.

Gas chromatography - mass spectrometry (GC-MS) analysis is a powerful technique that provides detection of the majority of primary metabolic pathway intermediates. This technique has been applied to numerous biological systems, yet challenges remain in the analysis and interpretation of the vast data produced by metabolic profiling (Fernie et al., 2004; Shen et al., 2016; Spicer et al., 2017). To face this challenge, correlation-based network (CN) analysis is increasingly used to provide an overview of multiple metabolic reactions and regulation patterns in the organism (Angelovici et al., 2017; Chen et al., 2009). In a metabolic CN, individual compounds detected by GC-MS are represented as "vertices", and the "edges" between two vertices correspond to the correlation between two compounds (Batushansky et al., 2016). Many factors can affect the biological interpretation of CNs (Steuer, 2006), but multiple studies have demonstrated the power of CN analysis to describe the systematic effect of the experimental conditions (Elo et al., 2007; Fukushima, 2013;

Saito et al., 2008). Indeed, a strength of correlation networks is that they can be remarkably different even if the content of metabolites did not change significantly.

One of the crucial steps of CN analysis is defining the correlation threshold because this value can drastically affect the CN structure and interpretation. One of the widely accepted solutions is to use the so-called “hard thresholding” concept that converts a correlation coefficient into an edge only if it passes predefined *r*- and *p*-values (Horvath and Dong, 2008). Numerous studies employing metabolic and gene correlation networks have demonstrated that the generally accepted $p < 0.05$, or $p < 0.01$ after correction of multiple test (for example, false discovery rate, FDR) provides a good estimation of the *r*-value threshold (Perkins and Langston, 2009; Voy et al., 2006).

In this study, we sought to 1) characterize the cardiac metabolic profile in response to fasting using GC-MS metabolic profiling and CN analysis, and 2) identify how this is altered when PFK-2 activity is maintained during fasting. The Glyco^{Hi} mouse is a cardiac-specific transgenic model that expresses a kinase active, phosphatase null, form of PFK-2 resulting in accentuated cardiac glycolysis (Gibb et al., 2017; Wang et al., 2008). Using Control and Glyco^{Hi} mice under fed and fasted conditions, we applied the CN approach with a hard threshold as a complementary technique to the classical methods to analyze data obtained via GC-MS metabolic profiling. Our results demonstrate a distinct metabolic profile in Glyco^{Hi} mice relative to controls under fasting conditions, including a robust increase in branched chain amino acids. CN analysis, applied independently of traditional analytical approaches, demonstrated more conservative regulation of Glyco^{Hi} metabolism between the fed and fasted states that was unique from the Control response. This work demonstrates the power of the CN approach in identifying unique metabolic characteristics and identifies a previously unknown relationship between fructose-2,6-bisphosphate and branched chain amino acids.

2. Materials and methods

2.1. Animals and tissue collection

All mouse experiments were performed with approval from the Oklahoma Medical Research Foundation (Oklahoma City, OK) Institutional Animal Care and Use Committee. Transgenic mice expressing a phosphatase-deficient form of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2) under the control of the α -myosin heavy chain promoter (Glyco^{Hi} mice) were kindly provided by Drs. Paul Epstein and Bradford Hill (University of Louisville, Louisville, KY) (Gibb et al., 2017; Wang et al., 2008). Glyco^{Hi} mice are on an FVB/NJ background and male 9-month old heterozygotes were paired with male non-transgenic control (Control) littermates. Mice were group housed and maintained on a 12-hour light/dark cycle (light from 06:00 to 18:00). For the fasting group, 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. Animals had access to water *ad libitum* at all times. Mice were euthanized by cervical dislocation and immediately after death the chest cavity was opened and hearts were perfused with saline solution by injection into the left ventricle. The hearts were excised, quickly blot-dried, and weighed before being snap-frozen in liquid nitrogen.

2.2. Glucose, fructose-2,6-biphosphate and glycogen measurements

Blood was collected from a tail-snip immediately after cervical dislocation and glucose was measured using a blood glucose monitor.

Cardiac fructose-2,6-biphosphate levels were determined spectrophotometrically as described previously (Van Schaftingen et al., 1982). Briefly, fructose-2,6-biphosphate was extracted from 10-15 mg of pulverized heart tissue in 200-300 μ L 50 mM NaOH by heating at 80 °C for 20 minutes. The extract was then cooled and neutralized at 4 °C by addition of glacial acetic acid in the presence of 20 mM HEPES. After precipitation of insoluble matters by centrifugation at $10,000 \times g$ for 15 minutes, the supernatant was collected and 50 μ L of the extract from each sample was used for the pyrophosphate:fructose-6-phosphate phosphotransferase (PP_i-PFK) activity measurement. The assay was performed in 5 mM Mg²⁺, 50 mM Tris buffer pH 8.0, supplemented with 0.5 mM PP_i and 1 mM fructose-6-phosphate. PP_i-PFK activity was measured as the rate of NADH oxidation ($\epsilon_{340} = 6200 \text{ M}^{-1} \text{ cm}^{-1}$) following the addition of 150 μ M NADH to a mixture of heart tissue extract, excess PP_i-PFK (enriched from potato tubers), 2 μ M triosephosphate isomerase, 10 μ g/mL glycerol-3-phosphate dehydrogenase, and 0.2 U/mL aldolase. Cardiac glycogen content was measured enzymatically according to the manufacturer's specifications (Sigma-Aldrich #MAK016).

2.3. Western blot analysis

Pulverized heart tissue was resuspended in a buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM MOPS, 1 mM EDTA, and 1X Halt protease/phosphatase inhibitor cocktail (Thermo Fisher) at a ratio of 1:50 (mg/ μ L) and homogenized by 3 passages with a Potter-Elvehjem homogenizer. Homogenates were then centrifuged for 5 min at 500g and supernatants were diluted with 4x SDS-PAGE sample buffer containing 25 mM dithiothreitol and heated at 95°C for 5 min. Following SDS-PAGE (4-12% NuPage Bis-Tris gel, Thermo Fisher), gels were transferred to nitrocellulose membranes and blocked for 30 min with Odyssey TBS blocking buffer (LI-COR). PFK-2 (Origene #TA347548, 1:3000 dilution) or phospho-Ser483 PFK-2 (Cell Signaling Technologies #13064; 1:2000 dilution) antibodies were added overnight at 4°C, subsequently washed the following day, and the secondary antibody (IRDye 800CW, LI-COR; 1:10,000 dilution) was incubated for 1h. Following additional washing, blots were analyzed on an Odyssey CLx imaging system using the Image Studio software (LI-COR).

2.4. Metabolic profiling

Metabolic profiling was performed based on a previously published method (Lisec et al., 2006) with modifications. Briefly, frozen heart tissue was pulverized using a tissue grinder Qiagen TissueLyser II containing pre-chilled metal beads, and followed by methanol:chloroform:water (2:1:1) extraction. Ribitol, as internal standard, was added to each sample at the first step of extraction. After 10 minutes incubation at 70°C, samples were centrifuged at 20000 rpm for 5 minutes, and 400 μ L of supernatant were transferred to the new 2 mL tubes and completely dried in a vacuum concentrator for 4 hours. Dried residues were derivatized in two steps. First, samples were dissolved in 40 μ L of 20 mg/mL methoxyamine hydrochloride in pyridine for two hours at 37°C with constant orbital

shaking. Second, 70 μ L N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) were added, and the samples were mixed at 37°C for 30 minutes. A mixture of alkanes (C10 to C24) was used as a retention time standard. After derivatization, the samples were transferred into glass vials and 1 μ L was injected to the GC-MS system (Agilent 7890B-5977A) in splitless mode. Each sample was analyzed in duplicate. The full scan from 60-600 m/z was performed. In addition to the tissue samples, analytical standards of pyruvate, lactate, glucose, fructose-6-phosphate, hydroxybutyrate, leucine and isoleucine were prepared and analyzed. All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) except pyridine and methoxyamine hydrochloride (Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Data analysis

The obtained chromatograms were processed using Agilent MassHunter Quantitative Data Analysis software with integrated mass-spectrometry library from the National Institute of Standards and Technology (NIST, Gaithersburg, USA). The metabolites were annotated according to the selected analytical standards and NIST library. The relative abundance of metabolites was calculated by peak area and followed by normalization according to the sample weight and internal standard. Principal component analysis (PCA) was performed using *stats*- and *ggplot2*-packages for R-project (Team, 2017; Wickham, 2016). Two-way ANOVA and Student's t-test were performed in R-project (Team, 2017). Both, multivariate and univariate statistical analyses were performed on Log-transformed data. Pathway analysis was performed using MetaboAnalyst web-platform (Chong et al., 2018) on KEGG database background (Kanehisa and Goto, 2000).

2.6. Correlation network analysis

Correlation based network analysis was performed as previously published (Batushansky et al., 2016). Data were Log-transformed and Pearson correlation matrices were calculated using psych-package for R-project (Revelle, 2018). The metabolites whose pair-wised correlation passed the selected threshold ($|r| \geq 0.7$, $q_{FDR} < 0.05$) were transformed to an adjacent list for CN visualization using Cytoscape (Shannon et al., 2003). Graph theory-based network properties were calculated using the *igraph*-package for R-project (Csardi and Nepusz, 2006).

3. Results

Regularly fed and 12-hours fasted Control and Glyco^{Hi} mice (n = 4 per group) were sacrificed for whole heart extraction. Although 12 hours is a long fasting period for mice (Jensen et al., 2013), we found that blood glucose levels measured at the time of sacrifice were not significantly different (Figure 1a). The content of fructose-2,6-bisphosphate extracted from hearts was also measured to determine whether this potent PFK-1 activator was maintained in Glyco^{Hi} hearts under fasting conditions. We confirmed that fructose-2,6-bisphosphate levels were approximately 10-fold elevated in fed Glyco^{Hi} hearts compared to controls and that this was largely sustained with fasting (Figure 1b). In contrast, fructose-2,6-bisphosphate levels in Control mice trended towards a decrease with fasting (p=0.08). To further confirm that this central regulatory point is indeed affected by fasting conditions in control mice, we also examined the phosphorylation status of PFK-2. Western

blot analysis revealed a significant decrease in the phosphorylation of endogenously expressed PFK-2 in both Control and Glyco^{Hi} hearts that was induced by fasting (Figure 1c, Supplementary data 1). Because phosphorylation is essential for PFK-2 kinase activity, it supports the conclusion that this regulatory point is downregulated under fasting conditions.

3.1. The metabolic profile of Glyco^{Hi} mice demonstrates a higher tolerance to fasting

Using semi-targeted GC-MS analysis we identified and annotated 43 compounds belonging to primary biochemical metabolic pathways according to the selected standards and the NIST library (see material and methods for the details) (Supplementary data 2). Each biological replicate was analyzed in duplicate ($n_{\text{total}} = 8$ per group). Interestingly, not all metabolites were identified within each group. For example, fructose-6-phosphate was detected only in Control/Fed mice, gamma-aminobutyric acid (GABA) was only detected in Control/Fasted mice, and alpha-ketobutyrate was only undetected in Glyco^{Hi}/Fasted mice. For the initial analysis, data were subjected to an unsupervised principal component analysis (PCA) to evaluate a global effect of the genotype and feeding regime on the cardiac metabolic profile. Prior to the PCA, the three metabolites listed above were excluded from the data-set due to the high sensitivity of PCA to missing values and the potential artificial effects of these metabolites on the PCA plot. The results demonstrated a clear separation between the different genotypes across the 1st principal component (PC₁), suggesting a clear effect of the PFK-2 transgene on the cardiac metabolic profile (Figure 2). Strikingly, the separation of the samples across the 2nd principal component (PC₂) were different in respect to the fed versus fasted state in the Control group only. In contrast, the Glyco^{Hi} animals demonstrated a mixed effect (Figure 2). Because both components have an almost equal variance (PC₁=26% and PC₂=24%), these results suggest a weaker metabolic response of Glyco^{Hi} hearts to fasting.

We next extracted eigenvectors for the metabolites with the strongest contribution to the separation between the groups (Supplementary data 3). Intriguingly, the separation across PC₁ was mostly defined by members of mixed compound classes, while separation across PC₂ was by N-enriched compounds that extend beyond the main amino acids (for example urea, ornithine, and oxoproline). Also, overlapping metabolites between both components were remarkably related to the non-glycolytic sugars fructose and sorbitol, and the fatty acid oleate (Figure 2, Supplementary data 3). Taken together, the results of PCA indicate a genotype-dependent response of cardiac metabolism to the feeding regime and suggest a higher tolerance of Glyco^{Hi} mice to a 12-hour fast.

PCA gives a general overview of the multidimensional data but does not provide insight on whether the observed changes are statistically significant nor the directionality of these changes. To explore this, we applied two-way ANOVA to determine metabolites that significantly differed under the experimental conditions. The results supported the observations made by PCA. Thus, the level of 15 metabolites were significantly ($q_{\text{FDR}} < 0.05$) affected by genotype while the feeding regime factor significantly affected fructose only. A significant effect of interaction between the two factors was detected for fructose and oleate. In addition to these altered metabolites, fructose-6-phosphate, GABA and alpha-

ketobutyrate are also amongst the discriminative compounds because they were below detectable levels in one or more groups (Supplementary data 2).

To further explore differences in metabolic profiles, we next performed comparative analysis within each genotype separately and identified the metabolites that changed at least 30% in response to fasting. Overall, the results of this analysis demonstrated wider metabolic changes in Control mice (Figure 3) and contrasting differences were observed in different metabolic pathways. Because Glyco^{Hi} mice are characterized by elevated rates of glycolysis, metabolites related to glucose were specifically examined. Glycogen (measured by an enzymatic assay) was differentially affected by fasting (Figure 4). Control mice had a precipitous decrease in glycogen in response to fasting. Glyco^{Hi} mice, on the other hand, had less glycogen than control mice under fed conditions and this was unaffected by fasting. Looking at individual metabolites, fasting of Control mice induced a slight decrease in glucose content (~1.2-times), a precipitous decrease of fructose-6-phosphate to undetectable levels, and a ~1.2-times decrease of glycerate. Glyco^{Hi} mice had no significant changes in these sugars. Lactate and alanine, both of which are readily converted to pyruvate, were affected by both genotype (Supplementary data 4) and feeding regime (Figure 4) and trended towards a decrease upon fasting.

Other non-glycolytic sugars were also affected by fasting in both genotypes but in opposite directions: fructose and sorbitol declined in Control mice (4.5- and 1.6-times, respectively) and increased in Glyco^{Hi} mice (1.4- and 1.8-times, respectively). Interestingly, the content of oleic and palmitic acids decreased in response to fasting only in the control group. This is consistent with an increased fatty acid consumption in Control that is absent in Glyco^{Hi}. The decreased content of glutamate, a central amino acid, in the Control group (~1.3-times) may be indicative of a physiological stress (Newsholme et al., 2003) and related to the remarkable increase in GABA (detected in Control/Fasted group only; Figure 3) (Chouchani et al., 2014). Surprisingly, we did not detect any changes in TCA cycle intermediates for either genotype except for the elevated content of citrate (~1.4-times) in the Control/Fasted group (Figure 3).

While the Control group responded more broadly to fasting, we observed a number of amino acids that exclusively accumulated under fasting in the Glyco^{Hi} group. This includes an approximate 1.4-times increase in aspartate, glycine, leucine, isoleucine and valine (Figure 3). Lastly, the changes in pyruvate-derived metabolites lactate and alanine showed a similar pattern for both genotypes, decreasing in response to fasting (~1.3 and ~1.2-times decrease, respectively; Figure 4).

3.2. Metabolic response of Glyco^{Hi} mice to fasting was weaker but targeted compared to control group

To further investigate the effect of fasting on the metabolic profile, we next performed pathway analysis on the metabolites that showed a trending change (Figure 3) within each genotype using the MetaboAnalyst platform (<http://www.metaboanalyst.ca>). The results demonstrated that despite the broader changes in Control compared to Glyco^{Hi}, the metabolites in Control were scattered among multiple metabolic routes. Indeed, only an association with general amino acid metabolism was identified in Control (Table 1,

Supplementary data 5). In contrast, the Glyco^{Hi} group had a strong statistically significant association with a specific pathway - branched-chain amino acids (BCAA) metabolism (Table 1, Supplementary data 5). Moreover, this specific effect in fasted Glyco^{Hi} mice was also consistent and led to the accumulation of leucine, isoleucine and valine (1.3-1.5 fold; Figure 4). BCAAs occupy central positions according to our current understanding of metabolism when evaluated by the Impact-parameter. This supports the conclusion that there is a highly specific cardiac metabolic response to fasting in Glyco^{Hi} mice.

3.3 Correlation-based network analysis suggests reciprocal response to fasting of Glyco^{Hi} and Control groups

The classical analysis of the metabolite data demonstrates the role of the PFK-2 transgene on alterations of heart metabolism in response to fasting, specifically emphasizing a smaller but targeted effect on BCAA's in Glyco^{Hi} mice. However, multiple alterations of metabolite levels beyond BCAA's were moderate and often not significant under the limited sample size used for this work. Moreover, the reported results do not fully explain how sustained fructose-2,6-bisphosphate during fasting leads to these alterations. In an effort to understand this process better and to emphasize the role of moderate changes we investigated the systematic effect of each condition on metabolite interactions by the construction of four correlation-based networks for each of the studied groups: Control/Fed, Control/Fasted, Glyco^{Hi}/Fed and Glyco^{Hi}/Fasted. The goal was to test the hypotheses that (i) Glyco^{Hi} mice have a higher tolerance to fasting; and that (ii) PFK-2 transgene has a broad systematic effect of cardiac metabolism by comparing the obtained metabolic networks. As an added analysis, a correlation-based network approach is valuable because it can detect systematic changes even when canonical hypothesis tests fail to identify statistical significance (Donovan et al., 2018; Torkamani et al., 2010). We started with a general qualitative comparison of the networks' structures, continued by calculating the global properties, and finished with a concise, but focused collation of specific components of the networks, as described below.

The networks present metabolites linked to other metabolites through positive or negative correlations as indicated. We first compared fed and fasted networks of each genotype for intersecting edges - those that are present in both networks irrespective of their correlation sign. The fed (Figure 5a) and fasted (Figure 5b) networks in Control hearts changed strongly with fasting and only 9 intersecting edges were observed (conserved edges are represented by dashed lines). In contrast, the Glyco^{Hi} networks had 30 intersecting edges (Figures 5c,d), consistent with a higher tolerance of Glyco^{Hi} to fasting. Further support was obtained by applying the main graph theory-based properties. We calculated density - the ratio of the number of existing edges to the number of total potential edges; transitivity - it reflects the probability of networks to form clusters; and diameter - the longest shortest path between any two vertices in a network. The density of the Control network expanded from the fed to the fasted state (0.12 vs. 0.14, Figure 5e) while the Glyco^{Hi} network shrank (density 0.17 vs. 0.15, Figure 5e), reflecting their distinct responses to fasting. Furthermore, the difference in density between the Control and Glyco^{Hi} networks under fed conditions (0.12 vs. 0.17, Figure 5e) underscores a systematic effect of the PFK-2 transgene on global metabolism. The transitivity of the Control/Fed network, while still high, was nevertheless notably

smaller than the other three networks (Figure 5e). Transitivity increased in Control while it was essentially unchanged in Glyco^{Hi} networks (0.45 to 0.65 and 0.62 to 0.64, respectively), supporting a readjustment of cellular metabolism upon fasting. Finally, the diameter of the Control networks, regardless of fed state, were larger than the Glyco^{Hi} networks. Collectively, the three main graph-theory properties support broad systemic effects regardless of the feeding regime. The inversely directed changes in density and transitivity and the more conserved network structure supports resistance of Glyco^{Hi} mice to fasting.

An examination of specifically connected patterns among metabolites reveals more detailed information. There is a higher interconnectivity of N-compounds across all conditions (Figure 5) indicating their maintained central role. In addition the connectivity of non-glycolytic sugars increased similarly under fasting in both genotypes. However there are remarkable changes in connectivity of glucose and BCAAs exclusively in the Glyco^{Hi} group. Eleven negative edges of glucose in Glyco^{Hi}/Fed network completely disappeared in Glyco^{Hi}/Fasted while leucine and isoleucine vertices also lost most of the connections (12 to 4) (Figure 5c,d). Thus, the CN analysis further highlights the specific changes in BCAA metabolism in response to fasting.

4. Discussion

The heart is one of the most energy demanding organs (Wang et al., 2010) and as such it has a broad capacity to generate chemical energy from the available circulating nutrients (Goodpaster and Sparks, 2017). The heart is also an insulin sensitive tissue and it undergoes a coordinated metabolic response in times of fasting to limit glucose uptake and oxidation and to increase fatty acid usage. We became interested in PFK-2 in this metabolic response because of our recent work demonstrating that the content of this regulator is decreased acutely during fasting and chronically with untreated type 1 diabetes (Bockus et al., 2017). We hypothesized that comparing the metabolic profile of hearts from control versus Glyco^{Hi} mice would offer new insights into: 1) how the healthy heart responds to decreased insulin signaling; and 2) identify how sustaining PFK-2 activity during fasting affects this response.

We observed significant cardiac metabolic changes with 12-hours fasting that were genotype specific. Glyco^{Hi} mice express the *pfkfb1* (liver) isoform of PFK-2 that was engineered to have null-phosphatase activity and sustained kinase activity (Wang et al., 2008). The transgene is driven by the cardiac specific myosin heavy chain promoter and as we show here, it sustains elevated levels of fructose-2,6-bisphosphate during 12-hours fasting. Importantly, elevated fructose-2,6-bisphosphate levels in the Glyco^{Hi} mouse model are sufficient to drive enhanced glycolysis (Gibb et al., 2017; Wang et al., 2008). As such, we observed differential responses in Control versus Glyco^{Hi} mice upon fasting. Fed Glyco^{Hi} mice had decreased glycogen content relative to Control mice, which is consistent with work by Gibb and coauthors (Gibb et al., 2017). Previous studies have found mixed results regarding the effect of fasting on cardiac glycogen content, with an increase occurring in rats and female mice (Feng Wang et al., 1999; Kokubun et al., 2009; Kruszynska et al., 1991; Reichelt et al., 2013). Thus, it was somewhat unexpected to find glycogen decreased in Control mice (Figure 4). Differences in the response to glycogen content compared to these previous studies may be due to the duration of the fast, strain-specific effects, or the sex of

mice. There was also a trend towards a decrease in lactate regardless of genotype (Figure 4). In Controls, this may be expected as a reflection of overall decreased glucose usage. In Glyco^{Hi} mice, though, the decrease suggests that sustaining fructose-2,6-bisphosphate does not necessarily lead to lactate accumulation. Glycolytic intermediates may be shuttled to other ancillary pathways or lactate could be excreted into the circulation. Alanine, which can be reversibly converted into pyruvate via transamination, followed a similar pattern as lactate. This suggests that full glycolysis oxidation to pyruvate, which was undetectable under our experimental conditions, may not be occurring in Glyco^{Hi} hearts upon fasting.

In addition to enhanced glycolysis, Glyco^{Hi} mice display broad effects on global metabolism during fasting that extend beyond glycolysis. Thus, PCA demonstrates Control and Glyco^{Hi} mice have unique metabolic profiles under both fed and fasted conditions (Figure 2). In addition, the PCA suggests Glyco^{Hi} mice are less sensitive to fasting as seen by the lack of clear separation between these groups. This is further supported by the comparative analysis that shows fewer metabolites changing in Glyco^{Hi} mice when fasted (Figure 3). However, fasted Glyco^{Hi} had elevated fructose and sorbitol in the polyol pathway, supporting a shift towards a non-glycolytic fate of glucose. In addition we found that the levels of oleic and palmitic acid decreased in control but not Glyco^{Hi} animals with fasting (Figure 3). This is in agreement with a recent report showing that the PFK-1/PFK-2 nexus has effects on the glucose-fatty acid cycle in the heart (Gibb et al., 2017). A caveat of this study is that the extraction method used for the GC-MS analysis was targeted for polar metabolites and only limited fatty acids (those that were miscible in methanol and water, Supplementary data 2) were detected.

The broad systematic effects induced by fasting are also supported by CN analysis, which is becoming a popular approach in the “omics” studies to unravel complex biological mechanisms (Batushansky et al., 2016; Zhang et al., 2014). In an effort to better understand a systematic effect of sustaining PFK-2 activity on heart metabolism, we constructed correlation-based metabolic networks of all tested conditions separately (Figure 5). Our analysis suggested that the obtained networks cannot be classified as small-world graphs. These type of graphs are characterized by short indirect connections between any two vertices in the network via only a few hub-vertices and a large number of edges (Watts and Strogatz, 1998). Consequently we did not focus on the search of hub vertices (Wang et al., 2015) but instead explored the potential differences between the networks. Interestingly, both networks of the Control genotype were smaller in density and looser in diameter (Figure 5e). These parameters are often used to describe the ability of the networks to respond to stress conditions such as the lack of nutrients, extreme abiotic conditions, etc. In contrast to CN applied to other systems (such as gene, proteins and non-biological), the larger diameter of a metabolic network was suggested to indicate enhanced sensitivity in response to external or internal changes (Jeong et al., 2000). In light of this, we can interpret that the Glyco^{Hi} networks (smaller diameter) are more resistant to changes caused by fasting as compared to the Control networks (larger diameter).

More detailed comparison of the networks revealed that glucose in the Glyco^{Hi}/Fed network had exclusively negative correlation with numerous compounds, including lactate and BCAAs. Considering that positive correlations may indicate the existence of common

precursor, while negative correlations may indicate a substrate-product relationship (Wang et al., 2015), the loss of negative connections by glucose in the Glyco^{Hi}/Fasted network suggests a shift from glycolysis to other metabolic pathways under fasting in Glyco^{Hi} animals (Figure 5c,d). Moreover, CN analysis supports unique effects of fasting on BCAAs in Glyco^{Hi} mice. Leucine and isoleucine went from being vertices with one of the highest degrees to losing most of the connections in Glyco^{Hi}/Fasted network (Figure 5c). This includes lost connections with serine which also had a high degree (together with glucose) in the Glyco^{Hi}/Fed network (Figure 5d). Serine is connected to glycolysis because its synthesis is through the intermediate 3-phosphoglycerate. Thus the broken connection between leucine/isoleucine and serine further supports a disruption between BCAA metabolism and glycolysis.

A central conclusion of our metabolic profiling is that Glyco^{Hi} hearts are more resistant to changes due to fasting. However, there is a pronounced effect on the BCAA pathway (Figure 3, Table 1, Supplementary data 5). Growing evidence demonstrates BCAAs have biological importance systemically (Cummings et al., 2018) and with pathophysiological conditions in the heart (Huang et al., 2011). A recent report showed that overexpression of cardiac Glut1 (the insulin insensitive glucose transporter) lead to increased glucose uptake and utilization and concurrently an increase in BCAA's (Shao et al., 2018). This effect was mediated by changes in expression of proteins involved in BCAA catabolism. Additionally, BCAAs can reciprocally decrease glucose utilization downstream via inhibition of pyruvate dehydrogenase (Li et al., 2017). Thus, one possibility is that the increase in BCAAs observed in Glyco^{Hi} mice serves as a feedback mechanism to restrain uncontrolled glucose oxidation during fasting.

A GC-MS metabolic profile combined with data-mining approaches such as CN analysis provides a powerful combination to study metabolic responses in animal models. Here we have identified unique and differential responses to fasting in hearts of control and Glyco^{Hi} mice. Our analysis has identified a previously unidentified connection between sustained fructose-2,6-bisphosphate levels and increases in BCAAs during fasting. As a limitation, it should be noted that it remains unclear why BCAA accumulation was only observed in transgenic mice. Future studies will be required to identify the specific molecular mechanisms whereby BCAAs levels are regulated and affected by PFK-2 activity and identify its significance under physiological and disease conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Blood glucose levels (a), content of fructose-2,6-biphosphate (b), and phosphorylation status of endogenous PFK-2 (c) in the Control and Glyco^{Hi} animals under regular feeding regime and 12-hours fasting. Data are Mean $\pm$ SD, $n=4$, p -values are indicated according to the Student's t -test.

Figure 2. Principal component analysis (PCA) of metabolic profiles of Control and Glyco^{Hi} animals under regular feeding regime and 12-hours fasting (see legend on the plot). First principal component (PC₁) and second principal component (PC₂) are plotted on the axes. Variance explained by each component is indicated on the plot.

Figure 3.

Effect of fasting on the metabolite profile of Control (left square, blue outline) and Glyco^{Hi} (right square, back outline) compared to regular feeding conditions within each genotype (a). The relative content of primary metabolites is represented in the false color heat-map of the fold-changes between fasted and regularly fed conditions. Only 30% changes between the two feeding regimes within each genotype are presented. Data are fold-change of means in Log₁₀-scale. Dashed arrows represent indirect reactions. Crossed box represents metabolites undetected under both feeding regimes within a genotype (see Supplementary data 2). Asterisk (*) represents metabolites that were not detected under one of the feeding regimes within a genotype (see Supplementary data 2).

Figure 4.

Content of branched chain amino acids and glycolysis related compounds: leucine, isoleucine, valine, glucose, lactate, alanine and glycogen in the Control and Glyco^{Hi} animals under regular feeding regime and 12-hours fasting. Data are Mean ± SD, n=4, *p-values* are indicated according to the Student's t-test.

Figure 5. Pearson correlation-based metabolic networks of Control/Fed (a), Control/Fasted (b), Glyco^{Hi}/Fed (c), Glyco^{Hi}/Fasted (d) animals and main graph theory-based properties of these networks (e). Each vertex represents a metabolite; each edge represents a significant correlation between pairs of metabolites (see Materials and methods). The size of vertices represents vertex degree from 1 to 12 (from smallest to largest). The color code of the vertices represents the compound class (see legend on the plot). The color code of edges represents positive (black) or negative (red) correlation. Dashed edges represent commonality between fed and fasted regimes within each genotype.

Table 1.

The results of pathway analysis of the metabolites that significantly changed in response to fasting within Control and Glyco^{Hi} genotypes. The qFDR column represents significance of association with the whole pathway according to Fisher's exact test. The Impact column represents the importance of the affected metabolites within the pathway based on graph theory betweenness. Pathways up to the first insignificant hit are presented. The full results are presented in Supplementary data 5.

Control	Hits/Total	qFDR	Impact	Glyco ^{Hi}	Hits/Total	qFDR	Impact
Aminoacyl-tRNA	6/69	0.0199	0.1	Aminoacyl-tRNA	6/69	0.0011	0.0
Glyoxylate and dicarboxylate	3/18	0.0704	0.4	Valine, leucine and isoleucine	3/11	0.0039	0.9
				Alanine, aspartate and glutamate	2/24	0.3049	0.2