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METABOLIC REGULATION OF MYOCARDIAL ADAPTATION TO EXERCISE

By

Andrew Alan Gibb

B.S., Ohio University, 2009
M.S., University of Louisville, 2011
M.S., University of Louisville, 2015

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In Physiology and Biophysics

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Louisville, Kentucky

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A Dissertation Approved on

September 1, 2017

By the following Dissertation Committee

Aruni Bhatnagar, Ph.D.

Bradford G. Hill, Ph.D.

Irving Joshua, Ph.D.

Dale Schuschke, Ph.D.

Claudio Maldonado, Ph.D.

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ABSTRACT

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ADAPTATION TO EXERCISE

Andrew Alan Gibb

September 1, 2017

While the benefits of exercise affect several organs, a significant adaptive response occurs within the heart. Exercise promotes cardiac growth, increases angiogenesis, and enhances cardiac function and these adaptations are associated with a cardioprotective phenotype. Additionally, extensive evidence shows that exercise dynamically regulates myocardial metabolism. This can be attributed to both changes in hormonal stimulation, increases in bioenergetic demand, and the bioavailability of circulating substrates. However, it is unclear whether these changes in metabolism contribute to physiologic cardiac growth. We reason that exercise-induced changes in metabolism are required to balance the catabolic and anabolic reactions needed for growth. Specifically, we hypothesize that exercise-induced changes in myocardial glycolytic activity are essential for activation of the physiological growth program and the synthesis of metabolites required for growth. Prior to investigating the effects of exercise on cardiac metabolism, we established a model of cardiac adaptation to exercise training in mice (Chapter II). Once established, as discussed in Chapter III, we found that the exercise-adapted heart demonstrates enhanced glycolytic activity; however,

during exercise glycolytic rate in the heart is diminished. To determine whether high or low glycolytic rates may be a stimulus for cardiac growth, we assessed the effect of cardiac-specific overexpression of phosphofructokinase isoforms that decrease or increase myocardial glycolytic rate. We found that constitutive reduction in glycolytic rate was sufficient to structurally and functionally phenocopy the exercise-adapted heart, replete with activation of the physiologic growth program; however, metabolic inflexibility invoked by constitutive changes in glycolysis results in mild mitochondrial dysfunction. To assess how glycolytic activity affects anabolic metabolism, we utilized stable isotope labeling in an *in vitro* model to interrogate flux of carbon into collateral biosynthetic pathways of glucose metabolism. Results from these studies, discussed in Chapter IV, show that PFK1 and glycolytic activity coordinate the allocation of glucose into pathways that would be important for cardiac growth. Collectively, our results indicate that physiological growth of the heart is intricately regulated by exercise-induced changes in intermediary metabolism, and that preservation of metabolic flexibility is essential for mitochondrial health. Importantly, these findings suggest a causal role of metabolism in myocardial responses to stress.

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CHAPTER I

GENERAL INTRODUCTION

Significance of Physical Activity

Physical activity is firmly embedded in human culture. The ability to adapt to periods of increased physical activity likely played a critical role in the evolution of human physiology. For instance, our early ancestors with low “physical fitness,” likely did not fare well at a time when hunting, gathering, shelter building, and evasion from predators were a necessity. This period in time likely contributed to the evolutionary physiologic advancement of the human species. Much later in ancient Greece, Hippocrates was the first to realize the importance of physical activity and stated that “eating alone will not keep a man well; he must also take exercise. For food and exercise...work together to produce health.”¹ Today, physical activity as a way of life is simply an afterthought. With the obvious transition from manual labor jobs to sedentary desk jobs and the technological advancements made over the last century, a sedentary lifestyle has become the new “normal” for human physiology. Unfortunately, this new “normal” could be considered evolutionarily “abnormal.” While exercise has been labeled as providing “health benefits,” it is in fact the lack of exercise that carries health risks.² This deviation from “normal” and change in lifestyle led the World Health

Organization (WHO) to classify physical inactivity as the fourth leading risk factor for global mortality.³

While physical inactivity is a health risk, it is a modifiable one. The first documented study of the beneficial effects of an active lifestyle was in 1953. This study showed that those in more physically active jobs (e.g., train conductors and postmen) in London had a lower incidence of cardiovascular events and a lower early mortality-rate than their sedentary, desk-based, civil servant counterparts.⁴ Along with this observation, the benefits of physical activity have only become more noticeable over the years. Regular exercise has been shown to promote cardiovascular health,⁵⁻⁷ augment skeletal muscle function,⁸ and increase both healthspan⁸⁻¹⁰ and lifespan.^{5, 11-13} An individual's level of physical fitness, commonly measured by their exercise capacity, is a more powerful predictor of mortality among men than other established cardiovascular risk factors such as hypertension, blood lipids, and smoking.¹² The indication that physical fitness is the most powerful predictor of mortality risk highlights the importance of an active lifestyle.

Despite irrefutable evidence that exercise imparts health benefits, or perhaps more accurately prevents sedentary health risks, the mechanisms by which exercise does so remain poorly understood. For instance, epidemiologic evidence suggests that while exercise training significantly lowers traditional cardiovascular risk factors (i.e. hypertension, inflammation, and blood lipids), only half of the reduction in event risk can be explained by these changes.^{6, 7} These

observations suggest that a large portion of the benefits of exercise remain unexplained and warrant further investigation.

Exercise and the Cardiovascular System

The complexity of studying exercise is rooted in the coordinated response of the body's organ systems. For example, even before beginning exercise (e.g. running), the centers in the brain prime the body for an increase in energy expenditure by increasing respiratory and heart rates (refs). At the onset of exercise, skeletal muscle contracts in response to the increased workload, requiring an increase in the utilization of fuel substrates such as glucose, glycogen, and fatty acids to meet the higher energy demand. This increase in muscle metabolism must be met by an equal increase in oxygen delivery, which is accomplished by a further increase in pulmonary respiratory rates and heart rates to increase oxygen delivery. These changes from homeostasis in the muscular, respiratory and circulatory systems are driven in part by changes in both the autonomic tone of the nervous system and the release of circulating hormones and catecholamines from the endocrine system. Interestingly, exercise profoundly affects the cardiovascular system in particular, resulting in significant adaptive responses of the heart. For instance, exercise promotes cardiac growth, increases perfusion, and enhances cardiac function and substrate utilization, all of which contribute to cardioprotection (**Fig. 1**). Understanding the mechanisms that underlie this response could lead to therapeutic strategies to improve cardiac health in disease.

Figure 1: Summary of the cardiac benefits of exercise. SV, stroke volume; CO, cardiac output.

FIGURE 1

Image courtesy of Nicholas Reader

The heart is highly susceptible to changes in workload brought upon by exercise. Simply put, the primary response of the circulatory system is to increase blood flow to active areas (e.g. skeletal muscle) and to decrease it to non-working, less critical ones (e.g. GI tract). This is critical as the working muscle requires an increase in oxygen delivery to match the increase in metabolic demand required for the maintenance of contractile function. Not surprising, the heart is quite efficient at matching oxygen delivery to metabolic demand, resulting in little energetic waste. To accomplish this, the heart increases cardiac output, the product of heart rate (HR) and stroke volume (SV). Several factors determine cardiac performance and are discussed below:

- 1) *Heart rate*: The single greatest factor determining cardiac output is heart rate. Under resting conditions, the human heart beats approximately 70 times per minute (bpm); however, during intense exercise this can increase nearly 3-fold to 200 bpm. Under parasympathetic control via the Vagus nerve, vagal tone is diminished during exercise and sympathetic stimulation predominates, leading to an increased HR. In addition to its significant contributions to cardiac output, heart rate is the most important factor determining myocardial oxygen consumption. This increase in oxygen utilization is essential to match the increase in metabolic demand. For this reason, increases in HR and cardiac output are tightly regulated to be directly proportional to the increase in oxygen requirements of not only the skeletal muscle, but the heart itself. Lastly,

HRs are useful in estimating the myocardial metabolic cost of exercise. For example, the product of HR and systolic blood pressure is the rate-pressure product (RPP), a rough index of the coronary blood flow myocardial oxygen consumption.

- 2) *Preload*: Several factors contribute to SV with preload being one of them. Preload, also known as end diastolic volume (EDV), is the volume of blood that stretches the ventricles to their greatest dimensions prior to contraction. Preload directly contributes to SV by means of the Frank-Starling law. This law describes the positive relationship between SV and EDV and is driven by the length-tension relationship of the cardiomyocyte sarcomeres. As blood fills the ventricles, the myocyte myofibrils are stretched, creating more opportunities for cross-bridge interactions. An increased number of possible cross-bridges promotes greater contractile force, resulting in a larger volume of blood ejected, i.e., SV. Preload is directly related to the venous return of blood to the heart: its increase during exercise is caused by augmented skeletal and respiratory muscle pump function, higher venous tone (i.e. venoconstriction), and elevated cardiac output. Interestingly, it has been suggested that high heart rates may in fact decrease preload due to the loss of filling time; however, this remains controversial.¹⁴

- 3) *Afterload*: Unlike preload, afterload is referred to as a pressure and is negatively correlated to SV and cardiac output. Afterload is defined as the load which the ventricles must overcome to eject blood. The typical afterload (systolic blood pressure) in a healthy adult is 120 mmHg; however, during resistance exercise, such as weight lifting, arterial pressures can reach 480 mmHg and is due to the arterial constriction of contracting skeletal muscle.¹⁵ These pressures, however, are not maintained as weight lifting is typically intermittent and dynamic. While this acute increase in afterload causes an immediately and relatively large decrease in SV, this leads to an increase in preload during the next cardiac cycle, increasing the effect size of Frank-Starling's law and increasing SV on the subsequent beat. While the increase observed with endurance exercise is much less severe (~ 100 mmHg), this increase in afterload is overcome by the heart due to the increases in preload and contractility.
- 4) *Contractility*: Contractility can be defined as the degree of force the heart muscle produces to eject blood and is positively correlated with SV. Unlike skeletal muscle, which is capable of recruiting additional fibers, the myocardium is fully engaged. Therefore, changes in contractility are completely dependent on intracellular Ca^{2+} availability, critical for muscle excitation-contraction coupling. When intracellular Ca^{2+} levels rise, Ca^{2+} binds to troponin-C causing a conformation change allowing actin-

myosin cross-bridge interactions to occur. Utilizing ATP, the actin and myosin filaments slide past each other, shortening the sarcomeres and contracting the cell. Increased sympathetic stimulation and circulating catecholamines released during exercise increase intracellular Ca^{2+} , resulting in enhanced myocardial contractility during exercise.

Similar to the effects on skeletal muscle,⁸ the plasticity of the heart allows for adaptation to repetitive bouts of exercise. This adaptive state following training elicits significant cardioprotection from cardiac insults including myocardial ischemia,^{16, 17} pressure-overload (i.e. TAC),¹⁸ and diabetic cardiomyopathy.¹⁹ Part of this cardioprotection is associated with improvements in cardiac function. The exercise-adapted heart, also known as athlete's heart, is associated with modestly enhanced diastolic function, whereas systolic function is primarily unaffected.²⁰ This improvement in diastolic function can be linked back to those factors affecting preload. Endurance exercise training has been well documented to result in hypervolemia²¹ which contributes to increases in preload and cardiac output. Additionally, exercise reduces not only resting HR, but also the HR achieved at submaximal workloads, providing additional filling time for preload.¹⁴ The chronic effects of exercise on decreasing afterload in healthy individuals are not as large, as systolic blood pressures are only slightly reduced; however, in hypertensive individuals, exercise has a slightly greater effect size.²² At the cellular level, enhanced cardiac contractility contributes significantly to the improvement in cardiac performance. In exercise-trained rodents, fractional shortening improves

40-50% while relaxation rates improve 20-40%.²³ These improvements are, in part, explained by an increase in myofilament sensitivity to Ca^{2+} .²⁴ Additionally, exercise has been shown to increase the activity of SERCA2a and phospholamban, contributing not only to enhanced Ca^{2+} release required for excitation-contraction coupling but also Ca^{2+} reuptake into the sarcoplasmic reticulum for relaxation.^{23, 25}

In addition to improving Ca^{2+} handling, regular exercise promotes cardiac growth, also referred to as physiological hypertrophy. This form of remodeling is characterized by a 10-20% increase in cardiac mass. Endurance exercise selectively induces an eccentric form of hypertrophy, wherein an increase in left ventricular (LV) volume is met with an equal increase in LV and septal wall thicknesses, coordinated by an increase in cardiomyocyte width and length. This is in stark contrast to a pathologic form of hypertrophy, where a much larger increase in LV volume is accompanied by a preferential myocyte lengthening, leading to wall thinning. Concentric hypertrophy, more prominent in pathology due to chronic elevations of afterload, is characterized by wall thickening as cardiomyocytes increase in width more so than length, reducing LV volume. While some strenuous exercise, such as weight training may induce a milder form of concentric hypertrophy, it is not believed to be pathologic.^{26, 27} Also, different from pathological hypertrophy is the observation that following only a few weeks of detraining in college and Olympic athletes, physiological hypertrophy regresses, indicating exercise-induced cardiac growth is a reversible phenotype.^{28, 29}

Other important factors also clearly distinguish these different forms of hypertrophy. For example, physiologic and pathologic hypertrophy differ

significantly in their signaling mediators and transcriptional responses. In particular, while pathological hypertrophy is associated with the induction of the fetal gene program (e.g. ANP, BNP, β -MHC, smooth muscle α -actin), physiological hypertrophy is not.³⁰ Additionally, the molecular signaling events driving physiologic growth of the myocardium following exercise training are distinct. The most well established signaling and transcriptional events governing exercise-induced physiologic growth are discussed below and in **Fig. 2**:

1. IGF1: Insulin-like growth factor 1 (IGF1), and its receptor (IGF1r) is the best described and potentially most important signaling event governing exercise-induced cardiac growth. Acute exercise increases the release of growth hormone (GH) from the anterior pituitary gland which acts distally on the liver to increase IGF1 production.³¹ Additionally, exercise increases local IGF1 production in several target tissues, including the heart, thereby acting in an autocrine and/or paracrine fashion.³² Several transgenic mouse models have demonstrated the critical role of IGF1 in regulating physiologic cardiac growth. Both *Igf1*- and *Igf1r1*-null mice display a growth retardation phenotype with high perinatal mortality³³ while *Igf1* overexpressing mice display a physiologic form of cardiac hypertrophy.³⁴ Furthermore, cardiomyocyte-specific deletion of *Igf1r1* in adult mice prevents exercise-induced cardiac growth.³⁵ These results clearly demonstrate the importance of IGF1 signaling in not only exercise-induced cardiac growth, but perinatal development as well.

Figure 2: Exercise-mediated cardiac adaptive signaling pathways. Signaling pathways involved in the adaptation to exercise training. Physiologic growth is initiated by IGF1 through the IGF1/PI3K/AKT axis. Activation of AKT downregulates *Cebpb* transcription, allowing the upregulation of CITED4. This cascade leads to the upregulation of gene sets specific to promote cardiomyocyte growth. Inactivation of PTEN is also associated with the upregulation of the IGF1/PI3K/AKT signaling cascade. Nrg1, through ErbB, also activates AKT and leads to adaptive signaling events. Mitochondrial biogenesis also occurs through the IGF1/PI3K axis that may or may not involve AKT signaling. However, AKT signaling does upregulate the transcription of *Pgc1 α* , resulting in the upregulation of adaptive metabolic gene sets. Catecholamine surges during exercise activate β -adrenergic receptors, leading to eNOS activation and the production of NO to increase cardiac perfusion. AMPK, an cellular sensor of the energy state of the cell, is also involved in the activation of eNOS as well as AKT, contributing to the overall adaptive response. Abbreviations: IGF1, insulin-like growth factor-1; IGF1r, insulin-like growth factor-1 receptor; Nrg1, neureglin1; ErbB, member of epidermal growth factor receptors; β -AR, beta-adrenergic receptor; PI3K, phosphoinositide-3 kinase; PTEN, phosphatase and tensin homolog; Akt, protein kinase B; C/EBP β , CCAAT/enhancer binding protein β ; Cited4, cbp/p300-interacting transactivator with Glu/Asp-gamma co-activator 1 α ; PGC1 α , peroxisome proliferator-activated receptor gamma coactivator 1-alpha; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; AMPK, AMP-activated protein kinase.

FIGURE 2

- 2) PI3K: IGF1r phosphorylation causes the activation of PI3-kinase, an intracellular family of signal transducing enzymes. Specifically, the activity of the PI3K (110 α) isoform appears to be necessary and sufficient for IGF1-mediated physiologic growth, as expression of a cardiac-specific dominant-negative PI3K (110 α) prevented exercise-induced cardiac growth in mice.³⁶ Conversely, constitutive activation of PI3K (110 α) in the heart promotes a physiologic form of hypertrophy.³⁶
³⁷ In addition to the direct role of PI3Ks in regulating cardiac growth, deletion of *Pten* (phosphatase and tensin homolog), which dephosphorylates PI3Ks, has also been shown to mediate cardiac growth.³⁸ Thus, the signaling events propagated by PI3K, and its regulation by PTEN, are critical for the physiologic growth response.
- 3) AKT: The serine/threonine-specific protein kinase B, also known as AKT, also plays a critical role in physiologic hypertrophy. Functioning downstream of PI3K, exercise training enhances the phosphorylation state of AKT at both the threonine 308 and 473 residues.^{35, 39-42} This activation of AKT is required for the hypertrophic response as mice with genetic deletion are resistant to exercise-induced cardiac growth.⁴³ Interestingly, cardiac-specific overexpression of a constitutively active form of AKT, while initially promoting a physiologic form of hypertrophy, progressed to a pathologic form, suggesting that duration, and likely also intensity, determine the cardiac response to AKT signaling.⁴⁴

4) C/EBP β and CITED4: Recent studies have identified transcriptional events downstream of AKT activation that are essential for exercise-induced physiologic growth. For example, exercise training was shown to downregulate cardiomyocyte expression of the CCAAT/enhancer binding protein- β (C/EBP β), in an AKT-dependent manner. In that study, cardiomyocyte-specific overexpression of C/EBP β resulted in physiological hypertrophy and resistance to the pathologic response to pressure overload.⁴⁵ Additionally, the exercise-induced transcriptional downregulation of *Cebpb* led to the transcriptional upregulation of *Cited4*,⁴⁵ and overexpression of *Cited4* was shown to be sufficient to drive physiologic cardiac growth and promote recovery from ischemic injury.⁴⁶ While C/EBP β and CITED4 are important for mediating the response to exercise, additional mechanisms of activation and downstream targets require further investigation.

5) *Additional effectors of physiologic cardiac growth*: Several other factors have been shown to be involved to some degree in the response to exercise. Nuregulin 1 signaling through the ErbB family of tyrosine kinase receptors produces similar effects to that observed in the exercise-adapted heart, in part, through the activation of AKT.⁴⁷ AMP-activated protein kinase (AMPK), important for its role in cellular energy homeostasis, has been suggested to play a role in the development of both physiologic and pathologic hypertrophy.²⁷ Additionally, signaling

though MAPK (mitogen-activated protein kinase)⁴⁸ and mTOR (mechanistic target of rapamycin)⁴⁴ have also been suggested to play roles in governing the cardiac hypertrophic program.

Recent evidence suggests that cardiomyocytes, once thought incapable of regeneration and proliferation, may in fact do so at a low rate in the adult heart.⁴⁹⁻
⁵¹ Increasing lines of evidence also suggest that exercise not only stimulates cardiomyocyte hypertrophy, but potential hyperplasia as well. Rodents subjected to exercise show elevated makers of proliferation such as PCNA expression, Ki67 positivity, and BrdU incorporation in the heart along with the activation of c-kit⁺ and Sca-1⁺ cells, believed to be cardiac progenitor cells.^{45, 52-54} While these results suggest that exercise-activated cardiomyocyte proliferation may contribute to physiological growth, more detailed studies, such as cell fate-mapping are required before more definitive conclusions can be drawn.

As cardiomyocytes grow in response to exercise, several other adaptive responses, both intrinsic to the cardiomyocyte and within the myocardium as a whole, must occur as well. One such response is an enhancement of cardiac perfusion. This is a critical adaptive response as both increases in workload and a larger myocyte both have a higher energy and oxygen demand. This increase in cardiac perfusion occurs primarily through two modes of action, vasodilation and angiogenesis. First, exercise results in the release of catecholamines which, in addition to their effect on Ca²⁺ dynamics and contractility, increases the phosphorylation of endothelial nitric oxide synthase (eNOS).⁵⁵ Enhanced eNOS

activity increases nitric oxide (NO) bioavailability, a potent vasodilator. This effect is also driven by the exercise-induced activation of AKT and AMPK, both of which promote the phosphorylation of eNOS.^{56, 57} Secondly, chronic exercise training induces an angiogenic response within the myocardium, resulting in *de novo* capillary formation.⁵⁸ This angiogenic response is in part associated with exercise-mediated increases in VEGF secretion⁵⁹ and circulating angiogenic progenitor cells.⁶⁰

The most common metabolic adaptation of the heart to exercise is mitochondrial biogenesis.⁶¹⁻⁶³ While various signaling events likely lead to an increase in mitochondrial mass, exercise-induced increases in peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) play a critical role.⁶⁴ Interestingly, induction of PGC-1 α in response to exercise was prevented in *IGF1r* knockout mice, and it has been shown that signaling through PI3K, but not AKT, is required for this biogenic response.^{35, 65} Accompanying the increase in mitochondrial mass, respiratory function and capacity appears to be enhanced in the physiologically hypertrophied heart. In both isolated permeabilized cardiac muscle fibers⁶⁵ and the isolated perfused heart,^{40, 66} exercise training increased the rate of mitochondrial fatty acid oxidation (FAO). This is in part due to the increase in mitochondrial mass, but also the upregulation of genes associated with FAO.⁶⁷

In addition to FAO rates, others have shown an increase in glucose oxidation (GO) as well.⁶⁶ This enhanced metabolic capacity of the exercised heart is a hallmark characteristic, distinguishing it from pathologic forms of hypertrophy

where it is well accepted that metabolic flexibility and energy homeostasis are compromised. Therefore, studying the metabolic phenotype of the physiologically hypertrophied heart may provide therapeutic avenues to treat or delay the onset of maladaptive pathologic remodeling. In the following section, the acute and chronic effects of exercise on cardiac metabolism will be discussed in detail.

Cardiac Metabolism in Exercise

The heart has an incredibly high energy demand, which requires continuous adenosine triphosphate (ATP) generation to sustain contractile function, ionic homeostasis, metabolic processes, and signaling. While myocardial ATP turnover is high ($\sim 30 \mu\text{mol} \bullet \text{g wet wt}^{-1} \bullet \text{min}^{-1}$), ATP content is relatively low ($\sim 5 \mu\text{mol} \bullet \text{g wet wt}^{-1}$) resulting in complete turnover approximately every 10 s.⁶⁸⁻⁷⁰ The heart fuels this turnover by generating >95% of its ATP from mitochondrial oxidative phosphorylation with the remaining 5% derived primarily from glycolysis.^{70, 71} Although the majority (60-70%) of the ATP generated supports contractile function, 30-40% is necessary to maintain ionic homeostasis through ion pumps, specifically the Ca^{2+} -ATPase in the sarcoplasmic reticulum.^{72, 73}

Basally, it is well accepted that the oxidation of free fatty acids (FFA) contributes 70-90% to oxidative phosphorylation with glucose, glycogen, lactate, ketones, and amino acids contributing the remaining 10-30%. With the ability to utilize a myriad of substrates, the heart is considered a metabolic “omnivore”, utilizing whichever substrates are available at a given time; however, with multiple substrates consistently available for use, competition for utilization exists. The

competition of substrates has long been demonstrated in both *in vivo* arterial-coronary sinus difference studies⁷⁴⁻⁷⁷ and perfused heart preparations *ex vivo*.⁷⁸
⁷⁹ The first observation of substrate competition was observed in the isolated perfused heart by Philip Randle in 1963⁸⁰ In this study, Randle demonstrated that increasing delivery of FFAs to the heart increased FAO and decreased glucose to pyruvate conversion and oxidation. Interestingly, exercise is also associated with the competition of substrates for utilization.

Acute exercise (i.e. an acute increase in workload) has a robust effect on not only cardiac metabolism. For example, it is estimated that TCA cycle flux increases 70- to 100-fold in an effort to match energy production to the large increase in consumption in working muscle.⁸¹ In the heart, exercise increases LV contractile power and myocardial oxygen consumption 4- to 6-fold above resting rates, which requires a significant increase in the generation of NADH and FADH₂, from substrate oxidation.⁸² Several *ex vivo*^{78, 83} and *in vivo*⁸⁴⁻⁸⁷ studies demonstrate that an increase in myocardial workload is accompanied by significant, yet selective, increases in the metabolism of various substrates. This increase in myocardial metabolism is accomplished by increased substrate availability and delivery to the myocardium as well as through extensive intracellular signaling that regulates substrate selection and utilization, coordinating myocardial metabolism. The effects of exercise on individual substrate metabolism is discussed below and in **Fig. 3**:

Fatty Acids: As already mentioned, the oxidation of FFA contributes the most to energy provision in the normal healthy heart. Because of their hydrophobic properties, FFA are bound to albumin for transport in circulation at a concentration of 0.2–0.6 mM;^{88, 89} however, during an acute bout of exercise, these levels can increase to 2.4 mM.⁸⁹ Because the availability of circulating FFAs is a primary determinant of their uptake by the myocardium,⁷⁴ the exercising heart demonstrates enhanced rates of FFA uptake.⁹⁰ This increase in circulating FFAs during exercise is primarily due to elevations in catecholamines, which activate hormone sensitive lipase in adipose and skeletal muscle tissues to de-esterify FFAs and promote their release into circulation.⁹¹ Exercise likely also facilitates the translocation of fatty acid translocase (FAT)/CD36, the primary protein carrier-mediated transporter for FFA uptake, as it has been shown that contracting skeletal muscle acutely increased its localization to the plasma membrane,^{92, 93} however, this effect has yet to be confirmed in the heart.

Following uptake, FFAs are esterified to fatty acyl-coenzyme A (CoA) by fatty acyl CoA synthase and either transported into the mitochondria for eventual beta-oxidation or used for the synthesis of triacylglycerides (TAGs). At rest, human isotopic labeling studies measuring $^{14}\text{CO}_2$ production in coronary venous blood indicate that 84% of the FFA taken up by the cell are immediately oxidized, leaving ~26% available for entry into the TAG pool.⁹⁴ This contribution to the TAG pool is not trivial, as intramyocardial TAG turnover occurs in as little as 5 h and contributes 10% to the total energy expenditure of the heart.^{82, 95} Interestingly, myocardial TAG utilization rates were found to be increased considerably under exercise conditions

as measured by a significant release of free glycerol, suggesting increased hydrolysis of intramyocardial stores.⁸⁷ In an *ex vivo* heart preparation, this increase in TAG turnover was a function of increased lactate availability, suggesting that lactate, which is elevated during exercise, stimulates FAO and the turnover of TAG stores. This conclusion was made by the observation that lactate in the perfusion buffer increased intracellular fatty acid content and free glycerol release, while maintaining TAG levels in the myocardium.⁹⁶ With respect to FAO rates, elevated circulating FFA levels, as occurs during exercise, enhances myocardial utilization for beta-oxidation and ATP production as indicated by isotopic labeling studies measuring $^{14}\text{CO}_2$ production.⁹⁴

Lactate: While the heart produces a considerable amount of lactate at rest, ^{14}C -lactate tracer studies calculating arterial-coronary sinus differences indicate the heart consumes an even greater amount of lactate, making the heart a net lactate consumer.^{77, 90, 97, 98} Myocardial lactate uptake, which is positively correlated with arterial lactate concentration, is almost entirely used for oxidation.⁸⁴ Under conditions of elevated arterial concentrations (e.g. exercise), lactate oxidation is significantly enhanced.^{79, 85} This increase in lactate oxidation appears to be correlated with exercise intensity, which determines arterial lactate levels. Under lower exercise intensities, the contribution of lactate oxidation to myocardial oxidative metabolism increased from 13% to 28% and was considerably higher than glucose oxidation (8-14%).⁸⁴ However, during intense exercise lactate levels can increase 10-fold, from 1 mM to 10 mM, and it has been suggested that the

Figure 3: Myocardial metabolism during exercise. Schematic of cardiac metabolism in exercise. Acute exercise results in changes in the bioavailability and utilization of both external (arterial concentrations) and internal (intracellular storage depots) energy substrates for ATP production. Arrows are indicative of changes in both bioavailability and utilization by the myocardium during exercise. For diagrammatic simplicity, the mitochondrion is shown as a single membrane, with CPT1/2 shown as an aggregate of the carnitinylation and reCoAcylation steps required for entry of fats into the mitochondrion for beta oxidation. Similarly, as several GLUT and MCT isoforms are present in the heart, they are diagrammatically represented as a family of transporters. Abbreviations: FFA, free-fatty acids; FAT/CD36, fatty acid translocase; GLUTs, glucose transporters; MCTs, monocarboxylate transporters; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F-1,6-P₂, fructose-1,6-bisphosphate; F-2,6-P₂, fructose-2,6-bisphosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; PFK1, phosphofructokinase 1; PFK2, phosphofructokinase-bisphosphatase 2; LDH, lactate dehydrogenase; PDC, pyruvate dehydrogenase complex; TAG, triacylglyceride; CPT1,2, carnitine palmitoyl transferase 1 and 2.

FIGURE 3

contributions of lactate to total oxidative metabolism under these conditions may in fact be much higher, accounting for 60-90% of substrate utilization.^{85, 99-101}

The effects of exercise training on myocardial lactate metabolism are not well known and is complicated by the fact that a primary effect of training is the reduction of arterial lactate levels for a given sub-maximal intensity.¹⁰² Because lactate utilization is a consequence of its availability, this would suggest that lactate oxidation and its contribution to myocardial oxidative metabolism may be diminished in the trained heart. However, it is likely that training results in metabolic adaptations similar to those seen for fatty acid metabolism. For example, recent studies have suggested that training increases LDH activity,¹⁰³ decreases the K_m for conversion of lactate to pyruvate,¹⁰⁴ and increases the monocarboxylic transporter (MCT1) responsible for lactate uptake,¹⁰⁵ all of which would contribute to enhanced lactate oxidation at a given concentration. Often rarely considered as a myocardial substrate, the importance of lactate to energetics and performance should not be understated. In a recent study by Levy et. al., systemic lactate deprivation resulted in cardiac energy deprivation and dysfunction,¹⁰⁶ highlighting the importance of lactate as an energy source.

Glucose: Under resting conditions, glucose is the most abundant substrate available to the myocardium with a circulating concentration of 4–5.5 mM in healthy individuals. While availability is not an issue, the heart preferentially utilizes FFA for oxidative metabolism with contributions of glucose equal to that of lactate (~17%).¹⁰⁷ This is somewhat surprising considering that glucose is a much more

efficient carbon source than FFAs, indicated by the phosphate to oxygen ratio (P/O) for complete oxidation of substrates,¹⁰⁸ which would make glucose a more efficient source of energy during increased workloads. As with FFAs and lactate, glucose uptake by the myocardium is also dependent on arterial concentrations; however, arterial glucose concentrations are tightly controlled and do not generally fluctuate. During highly intense (e.g. weightlifting) and prolonged endurance exercise, arterial glucose concentrations have been shown to diminish,⁸⁹ while others report that high intensity aerobic exercise resulted in an increase in blood glucose.⁸⁶ Interestingly, this increase in arterial glucose was not associated with an increase in glucose uptake indicated by positron emission tomography utilizing 2-[¹⁸F]fluoro-2-deoxy-D-glucose; however, during lower intensities glucose uptake was increased with no change in arterial levels.⁸⁶ These results of glucose uptake under lower intensities were in agreement with a previous report by Gertz et al.,⁸⁴ however, others have reported diminished glucose uptake during exercise.^{87, 109}

Glucose uptake is facilitated by protein carrier-mediated transport through a family of glucose transporters (GLUT), with the insulin-dependent GLUT4 as the most abundant in cardiac tissue.¹¹⁰ Exercise increases the translocation of GLUT4 to the cellular membrane in part by catecholamine activated β -adrenergic signaling^{111, 112} and through the exercise-induced activation of AMPK.¹¹³ Interestingly, this translocation appears to be insulin-independent as circulating insulin levels are diminished during exercise.¹¹⁴ Activation of AMPK also increases phosphorylation of phosphofructokinase 2 (PFK2) at the S483 residue, which increases the levels of fructose-2,6-bisphosphate (F-2,6-P₂), a potent activator of

PFK1 activity in the heart. If this occurs in the exercising heart, glycolytic rates would likely be elevated. Interestingly, increased utilization of glucose during exercise does not increase lactate release from the myocardium but rather promotes enhanced glucose oxidation,⁸⁴ in part, by increasing muscle pyruvate kinase activity.¹¹⁵ This would suggest that a primary effect of exercise is to increase the coupling of glycolysis to glucose oxidation. Improved coupling of glycolysis and glucose oxidation, especially if glucose uptake is diminished during exercise, would be advantageous as it would increase the proportion of glucose that contributes to energy production.

Another source of glucose for the myocardium is the intracellular storage of glycogen. Although the glycogen pool is only 20% of that of skeletal muscle, myocardial glycogen turnover can be high.¹¹⁶ Interestingly, a small amount of glucose that enters the glycogen pool is immediately cycled back out for eventual conversion to pyruvate;¹¹⁷ the significance of this cycling, however, and its relevance in exercise, is not well understood. The effects of glycogen content are more well defined under increased workloads. Acute increases in work stimulated by epinephrine increase glycogen breakdown via glycogen phosphorylase,^{83, 117, 118} which is preferentially used for oxidation rather than lactate production as shown by isotopically (¹³C) saturating the glycogen pool.^{118, 119} Conversely, glycogen synthesis by glycogen synthase, increases during fasting¹²⁰⁻¹²² and when competing substrates (FFA and lactate) predominate in their oxidation.^{123, 124} Because catecholamines, FFA, and lactate are all elevated during exercise, the effects of exercise on glycogen content are likely subject to duration, intensity, and

the time at which such measurements are made. For instance, it is likely that at the onset of exercise, prior to an increase in substrate availability, glycogen is broken down to meet the abrupt increase in ATP demand. Once arterial FFA and lactate levels rise over time, glycogen breakdown is inhibited and synthesis occurs. Because of these competing effectors of glycogen metabolism, a well-controlled study is required to fully understand the effects of exercise intensity and duration on glycogen metabolism.

Ketones and Amino Acids: Ketone bodies, acetoacetate and β -hydroxybutyrate, can contribute to oxidative metabolism through their conversion to acetyl-CoA, and similar to FFA and carbohydrates, uptake and oxidation is positively correlated to their arterial concentration.¹²⁵ However, ketone bodies themselves are insufficient to meet the energetic need of the heart, both *in vivo*¹²⁶ and *ex vivo*.¹²⁷ While ketone bodies may play a significant role in energy provisions in the pathologic heart,¹²⁸ their contributions to the exercising and physiologically hypertrophied heart are likely minimal.

Amino acids play an important role in contributing several carbon-based intermediates to the TCA cycle. For instance, alanine can undergo transamination to form pyruvate for oxidation, glutamine can enter at the point of α -ketoglutarate, and aspartate can be converted to oxaloacetate, all providing TCA intermediates. Additionally, the malate-aspartate shuttle is important in transferring the reducing equivalent NADH generated from cytosolic glycolysis and lactate to pyruvate conversion, into the mitochondria for the ETC. This reaction in turn regenerates

cofactor levels of NAD^+ , required for glycolysis and LDH activity. In the working heart, only leucine, a branched chain amino acid, has been shown to contribute to oxidative substrate utilization, albeit to a minimal (0.4%) degree.¹²⁹ Similar to ketone bodies, amino acid metabolism in the context of exercise is not well understood.

While understanding how exercise affects the metabolism of individual substrates provides invaluable information, integration of the metabolism of all substrates becomes much more complex, but provides a more complete picture. A more complete and thorough interrogation of myocardial metabolism can be obtained by measuring the utilization of all substrates and the regulation each imposes on the other. For example, in a study by Schonekess, hearts perfused at a constant workload had significantly diminished contributions of glycolysis and glucose oxidation to ATP production when FFA levels were increased to a physiologic range associated with exercise; interestingly, this observation was also made when lactate in the perfusion buffer was increased to physiologic exercising levels.⁷⁹ In a similar *ex vivo* study, workload was increased by epinephrine in the presence of exercising levels of lactate and FFAs, which blunted the increase in glucose utilization.⁷⁸ Similar results from *in vivo* studies further support that although an increase in cardiac workload is typically associated with an increase in glucose uptake and oxidation in the myocardium,^{78, 83, 84, 86} when concentrations of lactate and FFAs were increased, glucose uptake and oxidation were diminished.^{78, 86, 87, 109}

The diminishment in glucose utilization most likely occurs through allosteric inhibition of key glucose-metabolizing enzymes. For instance, acetyl-CoA derived from the oxidation of FFA in the mitochondria inhibits PDH activity, reducing the oxidation of glucose-derived pyruvate. Furthermore, the subsequent increase in citrate is inhibitory to the rate-limiting and committed step of glycolysis, phosphofructokinase 1, and to a lesser extent glucose uptake. The magnitude of inhibition increases along the glycolytic pathway with PDH inhibition being the most severe and glucose uptake the least.^{130, 131} Lactate oxidation also acts to inhibit glycolytic flux by reducing the bioavailability of NAD^+ , a cofactor for glyceraldehyde-3-phosphate dehydrogenase, and decreasing H^+ extrusion which is inhibitory to PFK1. Collectively these results suggest that during exercise, when arterial lactate and FFAs increase, glucose utilization is likely diminished.

Metabolism as a Contributing Factor to Growth

In tissues such as skeletal muscle, adaptive changes in gene expression are thought to be initiated by the repetitive bouts of metabolic stress triggered by strenuous physical activity.⁸ As acute and chronic exercise alter cardiac metabolism, it could be hypothesized that these changes could contribute to the adaptive response; however, understanding the mechanisms and pathways involved is difficult due to the complex and integrative nature of metabolism. Of particular interest is the metabolism of glucose via glycolysis, which is highly regulated during exercise. A central pathway of intermediary metabolism, the fate of glucose is not only limited to pyruvate or lactate for energy production, but it also

contributes to several ancillary pathways of glucose metabolism, such as the pentose phosphate pathway (PPP) and the glycerolipid synthesis pathway (GLP). These pathways provide the first rationale for how changes in glucose utilization could contribute to the cardiac adaptation to exercise.

The simplest explanation as to why changes in metabolism would precede and contribute to cardiac growth is by providing a material cause necessary for that growth. This material cause comes in the form of the DNA, amino acids, proteins, and phospholipids that are required for cellular growth. For example, in the oxidative phase of the PPP, the metabolism of glucose-6-phosphate (G6P) reduces NADP^+ to NADPH which is required for glutathione recycling and anabolic reactions. End products of the non-oxidative phase, however, are critical in the generation of ribose 5-phosphate, the precursor for purine and pyrimidine nucleotide biosynthesis. These nucleotides are the organic structure serving as the basis for the synthesis of DNA, RNA, and nucleic acids, without which growth cannot occur. In the heart, the PPP is activated during pathological hypertrophy¹³²⁻¹³⁴ and heart failure,¹³⁵ and modulating pathway activity regulates the severity of cardiac pathology.¹³⁶⁻¹⁴¹ Similarly, growth of a cell cannot occur without expansion of the cellular membrane which requires increased bioavailability of phospholipids derived from the GLP. Although less is known about the GLP, increased pathway activity may in fact contribute to pathologic cardiac hypertrophy.¹⁴² In the exercising heart, very little is known with regards to pathway flux through the PPP and GLP. However, during exercise when FFA and lactate oxidation increase,

allosteric inhibition at the point of PFK1 may in fact coordinate flux through these pathways, as has been shown in several non-cardiac cell types.¹⁴³⁻¹⁴⁶

Another biosynthetic pathway branching off glycolysis is the hexosamine biosynthetic pathway (HBP), which could also be influenced by changes in PFK and glycolytic activity. Requiring a sugar donor, the synthesis of O-linked β -*N*-acetylglucosamine (O-GlcNAc), a monosaccharide donor for nuclear and cytosolic proteins, can alter protein function, gene transcription, and modulates cell survival under conditions of stress.¹⁴⁷⁻¹⁵⁰ While several molecular signaling pathways are well understood in the exercised heart, post-translational O-GlcNAcylation is a relatively new area of investigation. In rats, an acute bout of swim training resulted in a 30% increase in skeletal muscle UDP-hexosamine concentrations that persisted for 16 h following exercise;¹⁵¹ however, in mouse hearts following 15 mins of treadmill exercise, O-GlcNAc levels were diminished. Interestingly, in these same mice, de-O-GlcNAcylation of O-GlcNAc transferase (OGT), the enzyme responsible for catalyzing the addition of the GlcNAc moiety to proteins, resulted in dissociation from the repressor element 1 silencing transcription factor complex and initiation of physiological growth signaling.¹⁵² Following training, the physiological hypertrophied heart is associated with decreased O-GlcNAc levels and a reduced expression of several regulatory enzymes in the HBP.^{153, 154} The results from these studies suggest that exercise-induced changes in metabolism and coordinate changes in HBP flux could contribute to the physiologic growth program.

Another potential role for metabolism in regulating the cardiac adaptive response to exercise is through direct intracellular signaling of metabolites themselves. For instance, an increase in G6P levels in *ex vivo* hearts subjected to increased workload, which is likely to occur *in vivo* during exercise when glucose utilization rates are diminished, leads to the activation of mTOR.¹⁵⁵ Another example of a metabolite acting in a direct signaling manner is 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), an intermediate of the PPP. An analog of adenosine monophosphate (AMP), AICAR stimulates AMPK, increasing its activation state.¹⁵⁶ As mTOR and AMPK are implicated to play a role in the exercise-induced adaptation, metabolites as direct signalers may play a critical role in the activation of the physiologic growth program.

Based on the literature discussed within this section, there appears to be ample evidence to suggest that metabolism may play a contributing role to the adaptive response to exercise, preceding and triggering physiologic growth of the heart. This raises several questions: How does exercise acutely and chronically affect myocardial metabolism?; Do changes in metabolism contribute to the exercise-induced adaptation of the heart?; and if so, how does metabolism activate the programs of exercise-induced physiologic growth? These questions and fundamental concepts led to the development of the following studies, which sought to identify the role of metabolism in exercise-induced cardiac remodeling.

CHAPTER II

FVB/NJ MICE ARE A USEFUL MODEL FOR EXAMINING CARDIAC ADAPTATIONS TO TREADMILL EXERCISE

Introduction

Regular exercise improves cardiovascular health,⁵⁻⁷ augments musculoskeletal function,⁸ and increases both healthspan⁸⁻¹⁰ and lifespan.^{5, 11-13} Nevertheless, the molecular mechanisms by which exercise promotes health are poorly understood.¹⁵⁷ Exercise studies commonly use murine models, which are valuable for identifying critical gene programs that contribute to exercise adaptation, primarily, because they offer the benefit of relatively rapid and controlled genetic modification [e.g.,^{25, 40, 158, 159}]. Nevertheless, sources of variability in such studies could confound our understanding of how exercise mitigates disease or increases overall health. Minimization of confounding factors is an important consideration for designing exercise studies as well as for interpreting the results obtained.

Although mouse models cannot perfectly recapitulate the complex physiological changes occurring in humans with physical activity, they can phenocopy particular aspects of physiological adaptation. For this purpose, three

models of mouse exercise are used frequently: treadmill training, forced swimming, and voluntary wheel running. While each of these models have advantages and limitations,^{158, 160} treadmill training provides the investigative advantage of controlling the amount of work performed in each training session, which is critical for understanding dependency of (patho)physiological adaptations on exercise workload and becomes increasingly important in studies of mice having different masses (e.g., obesity studies). Unlike the treadmill modality, work cannot be calculated easily in swimming or voluntary wheel exercise. For these reasons, treadmill training is a reliable, well-controlled, and often superior model of exercise for research studies. Nevertheless, several factors contribute to treadmill exercise (non)compliance and to exercise-induced adaptations. These include, but are not limited to, mouse strain, exercise environment, acclimatization, motivation, and assessments of exhaustion.^{52, 161}

Of these, mouse strain is of principal importance. Inbred strains of mice and rats have pronounced differences in their ability to exercise, or their choice to do so.¹⁶²⁻¹⁶⁶ A preponderance of genetic mouse models are on the C57BL/6J or the FVB/NJ background^{167, 168}; however, these strains show strikingly different preferences and capacities for exercise. For instance, C57BL/6J mice appear to be poor treadmill runners, yet display superior capacity on voluntary exercise wheels, while the opposite is true for FVB/NJ mice.^{163, 166} The reason(s) for these differences in training modality preference remain unclear. One reason could relate to the fact that laboratory mice are primarily nocturnal animals, demonstrating the highest activity and food consumption during the night cycle¹⁶⁹⁻¹⁷¹; however, to our

knowledge, the impact of time of exercise (day or night) on treadmill exercise capacity and compliance has not been investigated. The goals of this study were: (1) to test for differences in treadmill exercise capacity and compliance between C57BL/6J and FVB/NJ mice; (2) to delineate whether diurnal or nocturnal training influences exercise compliance and adaptive responses to training; and (3) to assess systemic and cardiac-specific exercise adaptations in mice compliant with treadmill exercise protocols. Our study demonstrates that FVB/NJ mice are a superior strain for treadmill exercise and that the timing of treadmill exercise, at least in this strain, does not influence compliance or exercise-induced adaptation.

Experimental Procedures

Animals. All procedures were approved by the University of Louisville Institutional Animal Care and Use Committee. C57BL/6J and FVB/NJ mice were ordered from Jackson Laboratory (Bar Harbor, ME) at 12 weeks of age and allowed to acclimate at the University of Louisville animal facility for three weeks. At 15 weeks of age, male mice were assigned randomly by strain to sedentary (SED) or exercise (EXE-Day or EXE-Night) groups. Food and water were provided *ad libitum*, and the mice were maintained on a 12:12-h light-dark schedule. Because the majority of published studies regarding exercise in mice have utilized male mice, we chose this gender for our study, which enables comparison with the literature. At the conclusion of the study and 24 h after the last exercise session, the mice were fully anaesthetized with sodium pentobarbital (40 mg/kg, i.p.), followed by euthanasia

via excision of the heart. These procedures are consistent with the AVMA *Guidelines on Euthanasia*.

Metabolic phenotyping. To assess for differences in basal metabolism and diurnal/nocturnal behavior, metabolic cage analyses were performed in naïve 15-week-old C57BL/6J and FVB/NJ mice, essentially as described.^{172, 173} Body weight was recorded prior to the initial and final exercise capacity tests to assess for changes in total body mass. Oxygen consumption rates, carbon dioxide production rates, respiratory exchange ratios, food consumption, water consumption and activity (sum of ambulatory and fine movements) were measured using a physiological/metabolic cage system (TSE PhenoMaster System, Bad Homberg, Germany) as previously described.^{172, 173}

Exercise capacity testing. We performed exercise familiarization and capacity testing in a manner similar to that outlined previously,¹⁶⁶ with minor modifications (**Fig. 4A,B**). Mice were familiarized to the motorized rodent treadmill (Columbus Instruments, Columbus OH) on the Wednesday and Thursday before the first week of training. Familiarization consisted of an initial 10 min period where the treadmill speed and incline were set to zero with shock grid settings of 25 V, 0.34 mA, and 2 Hz. The treadmill speed was then increased steadily to 10 m/min (Wed) and 12 m/min (Thurs) for an additional 10 min.

On the Friday immediately following familiarization to the treadmill, we subjected mice to an exercise capacity test (**Fig. 4B**). For this, the mice were

acclimated to the treadmill for 10 min, with the speed and incline set initially to zero. The treadmill speed was then increased to 8.5 m/min with an angle of inclination set to 0° for 9 min. Next, the treadmill speed and incline were increased to 10 m/min and 5°, respectively, for 3 min. The speed was then increased by 2.5 m/min every 3 min to a maximum speed of 40 m/min, while inclination increased by 5° every 9 min until a maximum incline of 15° was achieved.

We developed strict *a priori* criteria for exercise-induced exhaustion. These criteria were: 1) 10 consecutive seconds on the electric grid; 2) spending more than 50% of time on the grid; and/or 3) lack of motivation to manual prodding. Each mouse was removed immediately from their respective lane once one or more of these criteria was reached. Following the protocol, the mice were housed separately for 30 min to avoid noticeable aggressive behavior following exercise.

Following 4 weeks of training, we repeated this testing protocol to assess changes in exercise capacity. Exercise capacity was measured using the parameters of distance run (meters achieved prior to exhaustion) and work accomplished [calculated as the product of body weight (kg) and vertical distance (m); vertical distance = (distance run)(sin θ), where θ = the angle of inclination of the treadmill from 0°-15°] as outlined previously.¹⁶⁶

Exercise training. Mice assigned to exercise training groups were subjected to a 4-week protocol of forced treadmill running. The training protocol commenced the Monday after the initial exercise capacity testing with mice exercising 5 d/wk (Mon-Fri) at 70 or 75% of the maximal speed achieved during the initial exercise capacity

Figure 4: Exercise testing and training design. Schematic of the familiarization, testing, and treadmill training design: **(A)** General treadmill training design including: familiarization, pre- and post-exercise capacity testing, and exercise training regimen; **(B)** Exercise capacity testing protocol; and **(C)** Treadmill training regimen including: warm-up and exercise training intensity and durations, and post-training housing.

FIGURE 4

A

General Study Design:

B

Exercise Capacity Test:

	0° incline		5° incline		10° incline				15° incline					
Speed (m/min)	8.5	10	12.5	15	17.5	20	22.5	25	27.5	30	32.5	35	37.5	40
	9 min		3 min											

C

Exercise Training Protocol:

	10° incline		
Speed (m/min)	12	70 or 75% of maximal speed attained	Separate Housing
	10 min	Week 1, 40 min; Week 2, 50 min; Weeks 3 & 4, 60 min	30 min

test and an inclination appropriate to the speed (**Fig. 4C**). Prior to each training bout, we provided mice with a “warm-up” period of 10 min at 0 m/min and 10 min at 12 m/min to promote exercise protocol compliance and to minimize risk of injury. For strain comparison and night versus day training groups, training intensity was set at 20.8 m/min, 10° incline for FVB/NJ mice and 16.4 m/min, 5° incline for C57BL/6J mice, which corresponded to 70% of the maximal speed and the appropriate incline at the calculated speed for each strain during the initial exercise capacity test. In subsequent studies of FVB/NJ mice, mice were exercised only during the day, and in these groups, we implemented a more intensive training protocol to further examine systemic, cardiometabolic and skeletal muscle adaptations to treadmill running. For this, training intensity was set at 75% of the maximal initial exercise capacity, which corresponded to 22.3 m/min at a 10° incline. In all groups, we progressively increased the workload of the mice, such that they trained for 40 min during week 1, 50 min during week 2, and 60 min during weeks 3 and 4. We chose this progressive intensity protocol to prevent training plateau and to stimulate systemic as well as cardiovascular and skeletal muscle adaptations.^{166, 174-176}

Assessment of protocol compliance. To prevent injury and record protocol compliance, we monitored the mice carefully during each exercise session. Upon meeting pre-established indicators of exhaustion, mice were removed from the treadmill, and the time run was recorded (See **Table 1**). We used the percentage of total sessions and total minutes completed throughout the 4-week training

program as a measure of compliance. Before and after the initial exercise capacity test, we measured blood lactate levels, which provided a biochemical indicator of exercise-induced exhaustion at or near maximal oxygen consumption (VO_2max).¹⁷⁷⁻¹⁸⁰ We recorded lactate concentration in 0.7 μl of blood from a small tail clip (Lactate Plus meter; Nova Biomedical) prior to the protocol and upon meeting the exhaustion criteria defined above. High lactate levels increase confidence in a successful exercise capacity test by ensuring that failure to continue is due to exhaustion at or near VO_2max and not a failure to comply with the protocol.¹⁸¹⁻¹⁸³

Histology. Following euthanasia, tissue was excised and rapidly fixed for immunohistochemical analysis or immediately snap frozen in liquid nitrogen and stored at -80°C . Tissue was fixed in 10% formalin, paraffin embedded, and sectioned at 4 μm . Heart cross-sections were stained with 4'6-diamidino-2-phenylindole (DAPI; Invitrogen) and wheat germ agglutinin (WGA; ThermoFisher) for quantification of cardiomyocyte cross-sectional area. Quantitative measurements were determined using Nikon Elements software.

Relative mitochondrial DNA measurements. Mitochondrial abundance in heart tissue was estimated by measuring mitochondrial DNA (mtDNA) abundance relative to nuclear DNA (nDNA), similar to our previous studies.^{172, 184} Briefly, total DNA was isolated using a QIAamp DNA Mini Kit (Qiagen). A 25-mg aliquot of the tissue was homogenized, followed by overnight digestion in proteinase K at 55°C .

Following isolation, relative amounts of mtDNA and nDNA were compared using quantitative real-time PCR, using 2 ng of the isolated DNA. Primers for cytochrome *b* (mtDNA) and β -actin (nDNA) were used. The sequences are: cytochrome *b*, 5'-TTGGGTTGTTTGATCCTGTTTCG-3' and 5'-CTTCGCTTTCCACTTCATCTTACC-3'; and β -actin, 5'-CAGGATGCCTCTCTTGCTCT-3' and 5'-CGTCTTCCCCTCCATCGT-3'.

Statistical analysis: Unpaired or paired Student's *t* test was used for direct comparisons; multiple groups were compared by one-way and two-way ANOVA followed by Bonferroni or Sidak Multiple Comparison test, as appropriate. For ratio-based statistical comparisons, the data were log-transformed, and unpaired Student's *t* test was applied for assessing statistical significance between groups; we used a one-sample *t* test for intragroup differences from a ratio of 1 (used to determine significant chronobiological differences in metabolic cage endpoints, i.e., food and water intake, physical movement, VO_2 , VCO_2 , RER). A *p* value of ≤ 0.05 was considered statistically significant.

Results

FVB/NJ mice are not nocturnal. Laboratory mice, in general, are a nocturnal species,¹⁶⁹⁻¹⁷¹ and they typically choose to participate in voluntary exercise at night.¹⁸⁵ Thus, the time at which treadmill protocols are executed could be critical for ensuring exercise regimen compliance and adaptation. Therefore, we first examined the circadian characteristics of the mouse strains by measuring their

food and water intake, locomotion, VO_2 , VCO_2 and RER by placing untrained (naïve) mice in metabolic chambers. To assess differences between day and night behavior, a ratio (night values:day values) was calculated for each parameter. Although overall food consumption was not different between the two strains, the C57BL/6J mice consumed, on average, 3-fold more food at night. In contrast, the FVB/NJ mice showed no difference in food consumption in the night compared with the day (**Fig. 5A**). Cumulative water intake was not different between the strains; however, C57BL/6J mice showed significantly higher water intake at night (**Fig. 5B**). Similarly, C57BL/6J mice were more active at night compared with FVB/NJ mice (**Fig. 5C**). In general, FVB/NJ mice had fewer total beam breaks per hour (C57BL/6J = 2583 ± 711 ; FVB/NJ = 1736 ± 582 ; $p < 0.01$), indicating that cage behavioral activity is lower in this strain.

Metabolic analysis yielded analogous results. In C57BL/6J mice, oxygen consumption (VO_2 ; **Fig. 5D**), carbon dioxide production (VCO_2 ; **Fig. 5E**), and respiratory exchange ratios (RER; **Fig. 5F**) were higher at night compared with the day. Conversely, the FVB/NJ mice did not show metabolic differences in the day versus the night. Thus, the behavioral and metabolic qualities of C57BL/6J mice are supportive of their known nocturnal nature and are in stark contrast to FVB/NJ mice which demonstrate a relative lack of features characteristic of the nocturnal phenotype. Additionally, FVB/NJ mice had higher rates of oxygen consumption (C57BL/6J = $3,713 \pm 327$ ml/h/kg; FVB/NJ = 4058 ± 346 ml/h/kg; $p < 0.05$) which contributed to lower respiratory exchange ratios (C57 BL/6J = 0.93 ± 0.02 ; FVB/NJ = 0.86 ± 0.02 ; $p < 0.0001$).

Figure 5: Chronobiological characteristics of C57BL/6J and FVB/NJ mice.

Ratios of the average night and day values from metabolic cage analysis: **(A)** Food intake, **(B)** Water intake, **(C)** Total activity, **(D)** VO_2 , **(E)** VCO_2 , and **(F)** Respiratory exchange ratio (RER). n = 10 per group. (*) significance between C57BL/6 and FVB/NJ, (#) significance from a ratio of 1; *,#p < 0.05, **,##p < 0.01, ***,###p < 0.001.

FIGURE 5

Effect of strain and time of treadmill running on initial exercise capacity. To determine the effects of mouse genetic background on initial exercise capacity, we subjected 15-week-old C57BL/6J and FVB/NJ mice to exercise capacity testing. The mice were exercised either during the day (i.e., between 9 am–12 pm) under normal laboratory lighting or at night (i.e., between 7:00 pm–10:00 pm) under dark room conditions (safelight red lamp). We chose the latter time based on metabolic cage activity data, which showed increased voluntary locomotor activity starting at 6 pm. Compared with C57BL/6J mice, the FVB/NJ strain ran ~1.5-fold farther (**Fig. 6A**) and demonstrated ~2-fold greater initial capacity for treadmill work (**Fig. 6B**), regardless of when the mice were tested. Despite an apparent trend toward lower initial exercise capacity at night, we found no statistically significant differences between C57BL/6J mice exercised during the day or the night (**Fig. 6B&C**); however, FVB/NJ mice showed significantly lower levels of work performed at night (**Fig. 6B**).

In preliminary assessments, we observed that C57BL/6J mice received more shocks than FVB/NJ mice during treadmill exercise. Therefore, to confirm that reliable exercise capacity values were obtained and to rule out non-compliance to the testing protocol, we recorded blood lactate levels prior to and immediately following the initial exercise capacity test. Resting blood lactate values were similar in C57BL/6J and FVB/NJ mice (**Fig. 6C**). Although it should be noted that the night C57BL/6J group had a slightly higher resting lactate level, in general, blood lactate increased by 2–4-fold in both strains upon meeting criteria for exhaustion (**Fig. 6C**); both resting and maximal lactate abundances are within the

Figure 6: FVB/NJ mice display a higher initial exercise capacity than C57BL/6J mice. Measurements of exercise capacity and fatigue in 15-week old C57BL/6J and FVB/NJ mice tested during the day or at night: **(A)** Comparison of distance achieved; **(B)** comparison of work accomplished; **(C)** Blood lactate levels in C57BL/6J and FVB/NJ mice basally and following fatigue from exercise testing. n = 10 mice per group, *p < 0.05, ****p < 0.0001.

FIGURE 6

ranges previously published for resting mice and mice exercising at or near their VO₂max, respectively.¹⁷⁸⁻¹⁸⁰ Collectively, these results suggest that FVB/NJ mice demonstrate a superior ability to perform treadmill work compared with C57BL/6J mice, and they have higher initial exercise capacities when tested during the day.

Effect of strain and time of training on exercise compliance and adaptation. To determine if mouse strain or the time of training (i.e., day vs. night) influences exercise protocol compliance and adaptive responses to exercise, C57BL/6J and FVB/NJ mice were subjected to a 4-week training program, with one group from each strain training at night, and one group training during the day. Each day, we recorded compliance to the training program for individual mice (**Table 1**). The C57BL/6J mice completed only 50–60% of their training sessions (**Fig. 4A&C**), resulting in less time exercising in general (**Fig. 7D**). Interestingly, compliance in C57BL/6J mice diminished progressively with duration of the training protocol (**Fig. 4A, Table 1**). While FVB/NJ mice were generally compliant when exercised during the day or the night, 100% of the day FVB/NJ group complied with the protocol, whereas FVB/NJ mice exercising at night appeared to show modestly compromised compliance (**Fig. 7B&D**); however, this did not achieve statistical significance.

Pre-training and post-training exercise capacity tests showed that, while both FVB/NJ exercise groups significantly increased exercise capacity after four weeks of training, the C57BL/6J mice showed either no improvement or a

Figure 7: Compliance of FVB/NJ and C57BL/6J to the treadmill training protocol. Compliance to a four-week treadmill training protocol in mice: **(A,B)** Compliance curves indicating the % of mice that completed each training session; **(C)** Compliance measured as the % of total sessions; or **(D)** total minutes completed by mice throughout the exercise training program. n = 10 per group. * $p < 0.05$, *** $p < 0.001$.

FIGURE 7

Table 1. Exercise compliance chart. Daily records of minutes completed each day during the 4 wk training program.

TABLE 1

Strain		Animal ID#	Group	Mon	Tues	Wed	Thurs	Fri	Minutes Completed this week	Mon	Tues	Wed	Thurs	Fri	Minutes Completed this week	Mon	Tues	Wed	Thurs	Fri	Minutes Completed this week	Mon	Tues	Wed	Thurs	Fri	Minutes Completed this week	Mon	Tues	Wed	Thurs	Fri	Minutes Completed this week					
S57B163	S57B163	S2001	Day	✓	✓	✓	✓	✓	200	✓	✓	✓	✓	✓	212	✓	✓	✓	✓	✓	120	✓	✓	✓	✓	✓	145	✓	✓	✓	✓	✓	145					
			Day	✓	✓	✓	✓	✓	200	✓	✓	✓	✓	✓	✓	200	✓	✓	✓	✓	✓	120	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	213					
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			Day	✓	✓	✓	✓	✓	200	✓	✓	✓	✓	✓	✓	200	✓	✓	✓	✓	✓	120	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	216				
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		Day	✓	✓	✓	✓	✓	200	✓	✓	✓	✓	✓	✓																								

TABLE 1

			Totals					Totals	
Strain	Animal ID#	Group	Total Possible Run Time	Total Time Ran	% Time Completed	Total Sessions Completed	Total Sessions Possible	% of Sessions Completed	
C57Bl/6J	B2001	Day	1110	729	66%	11	21	52%	
	B2002	Day	1110	1083	98%	20	21	95%	
	B2003	Day	1110	982	88%	17	21	81%	
	B2004	Day	1110	688	62%	11	21	52%	
	B2005	Day	1110	652	59%	10	21	48%	
	B2006	Day	1110	506	46%	6	21	29%	
	B2007	Day	1110	554	50%	9	21	43%	
	B2008	Day	1110	853	77%	13	21	62%	
	B2009	Day	1110	663	60%	10	21	48%	
	B2010	Day	1110	943	85%	17	21	81%	
	B2011	Night	1110	862	78%	15	21	71%	
	B2012	Night	1110	1076	97%	20	21	95%	
	B2013	Night	1110	513	46%	7	21	33%	
	B2014	Night	1110	938	85%	16	21	76%	
	B2015	Night	1110	833	75%	13	21	62%	
	B2016	Night	1110	483	44%	5	21	24%	
	B2017	Night	1110	367	33%	5	21	24%	
	B2018	Night	1110	367	33%	5	21	24%	
	B2019	Night	1110	453	41%	6	21	29%	
	B2020	Night	1110	583	53%	11	21	52%	
Strain	Animal ID#	Group	1110	1110	100%	21	21	100%	
FVB/NJ	B2031	Day	1110	1110	100%	21	21	100%	
FVB/NJ	B2032	Day	1110	1110	100%	21	21	100%	
FVB/NJ	B2033	Day	1110	1110	100%	21	21	100%	
FVB/NJ	B2034	Day	1110	1110	100%	21	21	100%	
FVB/NJ	B2035	Day	1110	1110	100%	21	21	100%	
FVB/NJ	B2036	Day	1110	1110	100%	21	21	100%	
FVB/NJ	B2037	Day	1110	1110	100%	21	21	100%	
FVB/NJ	B2038	Day	1110	1110	100%	21	21	100%	
FVB/NJ	B2039	Day	1110	1110	100%	21	21	100%	
FVB/NJ	B2040	Day	1110	1110	100%	21	21	100%	
FVB/NJ	B2041	Night	1110	1027	93%	18	21	86%	
FVB/NJ	B2042	Night	1110	1075	97%	18	21	86%	
FVB/NJ	B2043	Night	1110	1011	91%	20	21	95%	
FVB/NJ	B2044	Night	1110	1031	93%	18	21	86%	
FVB/NJ	B2045	Night	1110	1075	97%	19	21	90%	
FVB/NJ	B2046	Night	1110	1110	100%	21	21	100%	
FVB/NJ	B2047	Night	1110	1059	95%	20	21	95%	
FVB/NJ	B2048	Night	1110	1110	100%	21	21	100%	
FVB/NJ	B2049	Night	1110	814	73%	14	21	67%	
FVB/NJ	B2050	Night	1110	1061	96%	20	21	95%	

decrease in the distance run or the work accomplished following training (**Fig. 8A&B**). This lack of response to exercise in the C57BL/6J mice is likely due to poor compliance to the protocol.

To determine if compliance of C57BL/6J mice to the protocol correlated with improvements in exercise capacity, we plotted the percent improvement in distance and work against the percent time completed during the exercise regimen. As shown in **Fig. 8C&D**, there is a significant, albeit weak, correlation between the percent time completed and exercise capacity in this strain. Collectively, these results show that, unlike FVB/NJ mice, C57BL/6J mice are not compliant with this extended treadmill exercise protocol, and that the time at which FVB/NJ mice exercise does not markedly influence their exercise capacity.

Physiological cardiac growth is a common endpoint used to verify cardiometabolic adaptation to exercise.²⁷ Exercise training increased heart weight to tibia length (HW/TL) in both FVB/NJ exercise groups, whereas the C57BL/6J mice showed no changes in heart size (**Table 2**). We did not find a significant correlation between exercise time completed and HW/TL in the C57BL/6J strain (data not shown). These results indicate that exercise causes cardiac growth in FVB/NJ mice and that C57BL/6J mice, likely due to lack of compliance to this treadmill protocol, fail to demonstrate physiological cardiac growth.

FVB/NJ mice display robust adaptations following exercise training. It is known that, compared with the C57BL/6J strain, FVB/NJ mice can achieve higher critical

Figure 8: Treadmill training-induced improvements in exercise capacity in C57BL/6J and FVB/NJ mice. Changes in exercise capacity in the mouse strains: (A) Distance run and (B) work accomplished during initial and final exercise testing following four weeks of treadmill training. n = 10 per group. *p < 0.05, ***p < 0.001. Panels C and D show correlations of compliance and (C) % distance and (D) % work improved (in the C57BL/6J strain). n = 20 mice (includes the C57BL/6J day and the C57BL/6J night groups)

FIGURE 8

Table 2: Measurements of exercise-induced cardiac growth. Mice were trained for 4 weeks at 70% of their initial exercise capacity. Values are means $\pm$ SD; n=10/group. HW/BW, heart weight-to-body weight ratio; HW/TL, heart weight-to-tibia length ratio. Statistical comparisons are between SED and EXE-Day and SED and EXE-Night. n = 10 per group. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

TABLE 2

	C57BL/6J	FVB/NJ
Body Mass, g		
SED	28.2±2.3	30.5±2.6
EXE-Day	27.3±1.4	29.9±1.7
EXE-Night	26.4±1.8	28.5±1.0
Heart Mass, mg		
SED	123.7±15.0	115.4±6.1
EXE-Day	118.7±10.1	135.8±5.5 ^{***}
EXE-Night	118.0±13.0	130.5±9.1 [*]
HW/BW, mg/g		
SED	4.4±0.4	3.8±0.3
EXE-Day	4.3±0.2	4.6±0.2 ^{****}
EXE-Night	4.5±0.4	4.6±0.2 ^{****}
HW/TL, mg/mm		
SED	6.9±0.9	6.3±0.3
EXE-Day	6.6±0.5	7.6±0.3 ^{***}
EXE-Night	6.6±0.7	7.3±0.4 [*]

running speeds on the treadmill,¹⁸¹ which indicates that their training regimen could be intensified further to evoke more robust adaptations to exercise. To test this, we trained an independent group of FVB/NJ mice at a slightly higher intensity (75% of their initial maximum exercise capacity) for 4 weeks. Compared with pre-training exercise capacity values, this more intensive protocol yielded a ~1.7-fold improvement in distance run and a ~2.4-fold increase in work in the mice (**Fig. 9A&B**). As expected, cardiac size was significantly higher in exercised mice (**Fig. 9C&D**). In exercise-adapted hearts, myocyte cross sectional area was found to be 22% higher than that found in hearts of sedentary mice, and the myocyte area distribution curves were shifted to the right (**Fig. 9E-G**), indicating that the exercise-induced cardiac growth in this strain is due primarily to an increase in cardiomyocyte size. Furthermore, mitochondrial biogenesis, estimated using the ratio of relative mtDNA to nDNA content, was 53% higher in the exercise-adapted mice (**Fig. 9H**). These findings indicate that treadmill training in FVB/NJ mice promotes robust increases in systemic exercise capacity and elicits physiological cardiac growth. Collectively, these results indicate that treadmill exercise in FVB/NJ mice elicits robust systemic and cardiac muscle adaptations.

Discussion

The goals of this study were to examine treadmill exercise capacity, compliance, and adaptation in two commonly used strains of laboratory mice. We found that C57BL/6J mice have significantly lower treadmill exercise capacity compared with FVB/NJ mice. C57BL/6J mice, in our hands, became progressively non-compliant

Figure 9: FVB/NJ mice display robust cardiometabolic adaptations to treadmill exercise training. Adaptations to a 4-week treadmill training program in FVB/NJ mice, where the training regimen was set based on 75% of their initial exercise capacity: **(A)** Distance run and **(B)** work accomplished during exercise testing; **(C)** Representative images of hearts from Sed and Exe mice; **(D)** HW/TL; **(E)** Representative cross section; **(F,G)** quantification of cross sectional area in heart sections stained with WGA and DAPI; and **(H)** Mitochondrial biogenesis, as indicated by relative mitochondrial (mt) DNA content. n = 6-10 mice per group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

FIGURE 9

with the exercise protocol, which is a likely reason underlying their lack of adaptation to exercise. Conversely, FVB/NJ mice demonstrated near perfect compliance and showed robust increases in exercise capacity as well as cardiac adaptations. Because laboratory mice are typically nocturnal, we also compared night and day behavior in the strains as well as exercise compliance and capacity. Our metabolic cage data suggest that, contrary to the C57BL/6J strain, FVB/NJ mice have no proclivity for activity and feeding at night, and that their metabolic phenotype lacks circadian variation. Moreover, despite the nocturnal nature of C57BL/6J mice, exercising them at night did not improve compliance or exercise capacity. Collectively, these results indicate that FVB/NJ mice are a superior model for examining systemic and tissue-specific adaptation to treadmill exercise and that diurnal training appears to promote higher exercise compliance and capacity.

The increase in exercise capacity and cardiac adaptations in FVB/NJ mice are likely a consequence of their remarkable compliance as well as their superior ability to perform treadmill work. Indeed, other studies show that the FVB/NJ strain has a higher critical running speed¹⁸¹ and is able to run farther than C57BL/6J mice.^{163, 166} To compare exercise capacity and adaptations between the strains in a controlled manner, we used equivalent relative intensities of training in the strains, which equated to 70% of the speed achieved during initial exercise capacity tests. These experiments showed that FVB/NJ mice could improve their exercise capacity and that exercise in this strain causes physiologic cardiac growth. Training FVB/NJ mice at a higher workload (initiated at 75% of their initial maximum speed) appeared to evoke superior improvements in distance run and

work and provoked physiologic cardiac growth. These results indicate that the FVB strain is useful for examining intensity-dependent adaptations to exercise.

The usefulness of the treadmill model lies in its ability to control the level of exercise-associated work and to relate biochemical and physiological adaptations to workload. This is, of course, dependent on compliance to the treadmill protocol. We show that C57BL/6J mice completed the protocol during the first week of training, but that they become increasingly unwilling to run thereafter. It is unlikely that this decrease in compliance is due to overtraining because it occurred upon only the second week of training (and after a two-day rest period), and the mice did not resume their ability to exercise, even after long periods of refusing to comply with the protocol. Furthermore, the mice did not lose weight during the protocol, which can be an indicator of overtraining.¹⁸⁶ Although it remains unclear why the C57BL/6J mice choose to run less after the first week, it could be that their tendency to receive more shocks is a negative stimulus for running, or that they associate the shocks with a positive outcome, i.e., removal from the treadmill. Interestingly, C57BL/6J mice are prone to footshock-induced analgesia,^{187, 188} and they show progressively decreased shock avoidance behavior compared with other strains.¹⁸⁹ It is also conceivable that this strain is more susceptible to negative stress in the brain caused by forced running,¹⁹⁰ as opposed to voluntary running. Thus, it is possible that this strain acclimates to the repeated treadmill shocks that they receive during training.

Escape behavior is another potential contributing factor to the lack of compliance of C57BL/6J mice. A study by Mori and Makino showed that C57BL/6J

mice escape and avoid shock by moving to an adjacent compartment in an L-type movement pattern rather than an R-type (rearing and jumping forward) pattern.¹⁹¹ This is of particular importance because the R-type pattern must be invoked to escape shock in our treadmill apparatus. Regardless, it would appear that the lack of both physiologic cardiac growth and improvements in exercise capacity in C57BL/6J mice is a result of non-compliance with the treadmill protocol.

We also show that FVB/NJ mice lack biological rhythmicity compared with C57BL/6J mice and that the time of day does not appear to affect compliance to the treadmill protocol. Lack of circadian behavior in FVB/NJ mice is a likely consequence of expression of the *retinal degenerative (rd)* mutation, which renders them blind to visual images and appears to underlie aberrant circadian wheel running behavior and spatial awareness.¹⁹² Conversely, C57BL/6J mice appear to see well: they demonstrate entrainment to a 12:12 h light:dark cycle, and they re-entrain to phase advances.¹⁹² Of note, while FVB/NJ mice are blind, C57BL/6J mice become deaf, progressively losing their hearing during the first year of life.¹⁹³⁻¹⁹⁵ Nevertheless, despite the impaired senses of these strains, the lack of circadian variation in the FVB/NJ strain, and the presence of a nocturnal phenotype in C57BL/6J mice, the timing at which the mice were tested and trained did not appear to have a strong impact. However, we do show that, for FVB/NJ mice, running during the day is associated with a higher initial exercise capacity. Because calculations for the 4-week training regimen are based on this initial capacity, it is possible that testing initial exercise capacities of mice during the day

could result in a higher training workload, which could equate to increased physiological adaptations.

Although it is unclear whether the mutations that underlie loss of sight or hearing in FVB/NJ or C57BL/6J mice contribute to their compliance to exercise protocols, it is clear that C57BL/6J and FVB/NJ mice have different exercise capabilities and preferences. Consistent with previous studies,^{163, 166} we found that C57BL/6J mice have lower treadmill exercise capacity, which did not improve even when they were tested at night. Nevertheless, several studies show that C57BL/6J mice are quite adept at voluntary wheel exercise [e.g.,^{163, 166, 196-201}], even load bearing wheels which simulate resistance training²⁰²; and, consistent with their nocturnal behavior, wheel activity in C57BL/6J mice peaks during the night.¹⁹² This suggests that the C57BL/6J strain is more amenable to the wheel running modality than treadmill exercise. While several investigators have exercised C57BL/6J mice using a treadmill protocol successfully [reviewed in¹⁶¹], only a few studies document robust increases in exercise capacity or physiological adaptations to extended treadmill training in this strain.²⁰³⁻²⁰⁵ Most studies show adaptations that appear relatively minimal compared with other strains [e.g.,^{166, 206, 207}] or demonstrate that additional exercise mimetics (e.g., AMPK or PPAR δ agonists) may be required to promote robust physiological adaptations to treadmill exercise in C57BL/6J mice.²⁰⁸

Several limitations of our study deserve mention. We did not measure VO_2 _{max} or identify anaerobic thresholds, which can delineate metabolic crossover points²⁰⁹; however, we used stringent criteria for determining fatigue (i.e.,

behavioral criteria decided upon *a priori*) as well as blood lactate measurements to ensure that mice meeting these criteria were fatigued and not simply incompliant with the exercise capacity protocol. Although we show that FVB/NJ mice are proficient treadmill runners, their lack of circadian behavior suggests that they may not be suitable for investigating chronobiological changes associated with exercise (e.g., exercise-induced changes in *clock* genes). In addition, we did not test whether lower speeds in C57BL/6J mice or different motivating stimuli would increase compliance to the protocol. Some studies in rats,²¹⁰ as well as C57BL/6J mice,²⁰³ use chocolate as a reward-based incentive to comply with the protocol. We did not test the use of reward in our study, nor did we examine whether alternative aversive stimuli (e.g., air puffs instead of shocks) would improve exercise compliance in the C57 strain. Thus, we do not rule out the possibility that C57BL/6J mice could be coaxed to run with an intensity and compliance similar to that of FVB/NJ mice; however, it is clear to us that improved treadmill compliance in C57BL/6J mice would appear to require a different protocol than that used here, or a reward that encourages continual compliance.

In summary, our findings indicate that FVB/NJ mice are a useful strain for testing treadmill exercise-mediated adaptations. This strain complies well with forced treadmill training and shows a robust capacity for cardiac exercise adaptation. Unlike C57BL/6J mice, FVB/NJ mice do not have a strong nocturnal nature, and they appear to show higher initial exercise capacities and comply better when trained during daytime hours. We also found that, in our hands, C57BL/6J mice show poor compliance to the treadmill exercise regimen,

regardless of when they exercise. These findings demonstrate that FVB/NJ mice are a suitable and robust model for understanding the mechanisms underlying cardiac adaptations to exercise.ⁱ

ⁱ This chapter previously appeared as an article in the journal *Frontiers in Physiology*. The original citation is as follows: Gibb AA, et al. FVB/NJ mice are a useful model for examining cardiometabolic adaptations to treadmill exercise. *Front Physiol*, 2016.

CHAPTER III

EXERCISE-INDUCED CHANGES IN GLUCOSE METABOLISM PROMOTE PHYSIOLOGIC CARDIAC GROWTH

Introduction

Exercise promotes cardiovascular wellness,²¹¹ augments musculoskeletal function,⁸ and lengthens lifespan.²¹² These salutary effects of exercise are dependent on the ability of the body to adapt to recurrent bouts of physical activity. Cumulative adaptation of the circulatory, pulmonary, and musculoskeletal systems augment exercise capacity,²¹³ which is the strongest indicator of cardiovascular health and a robust predictor of mortality.²¹⁴ Cardiovascular adaptations to exercise are particularly critical to enable adequate oxygen and nutrient delivery to peripheral tissues, and they help preserve circulatory system health with age.^{215,}
²¹⁶ Physiological cardiac growth due to exercise is a key component of these cardiovascular adaptations and contributes not only to exercise capacity, but protects the heart from injury as well.²¹⁷ Nevertheless, the mechanisms by which regular exercise triggers and sustains cardiac adaptation remain unclear. Understanding how exercise promotes healthy cardiac remodeling could provide the knowledge required to delay the progression of heart disease or reverse tissue pathology.

Previous studies have identified signaling pathways and gene programs essential for cardiac adaptation.^{27, 218} For example, C/EBP β and CITED4 are known to be critical for regulating the transcriptional programs that instigate cardiac growth^{45, 46, 219}; however, the mechanisms by which such transcriptional programs are activated with exercise are unclear. In tissues such as skeletal muscle, adaptive changes in gene expression are thought to be initiated by the repetitive bouts of metabolic stress triggered by strenuous physical activity.⁸ While it follows that the heart should conform to this paradigm, this has not been tested and it remains unclear which metabolic pathways and flux control steps may be important to provoke tissue adaptation to exercise.

In this study, we examined exercise-induced changes in glucose metabolism and their relationship to cardiac remodeling. We provide evidence that exercise regulates the activity of myocardial phosphofructokinase 1 (PFK1) and that the resulting changes in metabolism are sufficient to activate a transcriptional program influencing exercise-induced cardiac growth. Nonetheless, our data also indicate that chronically low or high relative levels of glycolysis, such as occurs in type II diabetes or heart failure, are damaging to mitochondria. These findings suggest that changes in myocardial glucose metabolism, brought about by regular exercise, regulate transcriptional programming and physiologic remodeling of the heart and that maintenance of metabolic flexibility is required to preserve mitochondrial health.

Experimental Procedures

Experimental Animals. All procedures were approved by the University of Louisville Institutional Animal Care and Use Committee. The mice used in this study include transgenic mice expressing a kinase-deficient (kd) form of PFK2 under the control of the α -MHC promoter (i.e., Glyco^{Lo} mice), transgenic mice expressing a phosphatase-deficient form of PFK2 under the control of the α -MHC promoter (i.e., Glyco^{Hi} mice), and wild-type (WT) littermates.^{220, 221} Male mice on the FVB/NJ background were used for all studies, except for F-2,6-P₂ measurements in isolated cardiomyocytes, where both male and female mice were used. All studies began at 15 weeks of age; food and water were provided *ad libitum*; and the mice were maintained on a 12:12-h light-dark schedule, as described previously.⁶³ At the conclusion of animal experiments, and following a 6 h fast, mice were anaesthetized with sodium pentobarbital (150 mg/kg, i.p.), followed by euthanasia via excision of the heart.

Exercise Capacity Testing. Mice were familiarized to the motorized rodent treadmill (Columbus Instruments, Columbus OH) on the Wednesday and Thursday before the first week of training. Familiarization consisted of an initial 10 min period where the treadmill speed and incline were set to zero with shock grid settings of 25 V, 0.34 mA, and 2 Hz. The treadmill speed was then increased steadily to 10 m/min (Wed) and 12 m/min (Thurs) for an additional 10 min.

On the Friday immediately following treadmill familiarization, we subjected mice to an exercise capacity test. For this, the mice were acclimated to the

treadmill for 10 min, with the speed and incline set initially to zero. The treadmill speed was then increased to 8.5 m/min with an angle of inclination set to 0° for 9 min. Next, the treadmill speed and incline were increased to 10 m/min and 5°, respectively, for 3 min. The speed was then increased by 2.5 m/min every 3 min to a maximum speed of 40 m/min, while inclination increased by 5° every 9 min until a maximum incline of 15° was achieved.

A priori criteria for exercise-induced exhaustion were: 10 consecutive seconds on the electric grid; spending more than 50% of time on the grid; and/or lack of motivation to manual prodding. Each mouse was removed immediately from their respective lane once one or more of these criteria was reached. Blood lactate levels provided a biochemical indicator of exercise-induced exhaustion at or near maximal oxygen consumption (VO_2max).¹⁷⁷⁻¹⁸⁰ We recorded lactate concentration in 0.7 μl of blood from a small tail clip (Lactate Plus meter; Nova Biomedical) prior to the protocol and upon meeting the exhaustion criteria defined above. High lactate levels increase confidence in a successful exercise capacity test by ensuring that failure to continue is due to exhaustion at or near VO_2max and not a failure to comply with the protocol.¹⁸¹⁻¹⁸³

Following 4 weeks of training, we repeated this testing protocol to assess improvements in exercise capacity. Exercise capacity was measured using the parameters of distance run (meters achieved prior to exhaustion) and work accomplished [calculated as the product of body weight (kg) and vertical distance (m); vertical distance = (distance run)($\sin\theta$), where θ = the angle of inclination of the treadmill from 0°-15°] as outlined previously.^{63, 166}

Exercise Training. Mice assigned to exercise training groups were subjected to a 4-week protocol of forced treadmill running. The training protocol commenced the Monday after the initial exercise capacity testing with mice exercising 5 d/wk (Mon–Fri) at 75% of their maximal speed achieved during the initial exercise capacity test and an inclination appropriate to the speed. Prior to each training bout, we provided mice with a “warm-up” period of 10 min at 0 m/min and 10 min at 12 m/min to promote exercise protocol compliance and to minimize risk of injury. In all groups, we progressively increased the workload of the mice, such that they trained for 40 min during week 1, 50 min during week 2, and 60 min during weeks 3 and 4. We chose this progressive intensity protocol to prevent training plateau and to stimulate systemic and cardiac adaptations.⁶³

To test the effects of chronic exercise in the adapted and recovery state, mice were euthanized 24 h or 1 h after the last training session of the 4 wk training regimen, respectively. To interrogate the effects of an acute, single-bout of exercise on cardiac metabolism, mice were subjected to familiarization as previously outlined and euthanized immediately following the completion of a 1 d, single exercise training session; 40 min at 75% intensity. Mice were excluded from the study if they were non-compliant with the exercise protocol or if they were found to be injured or in poor health, e.g., due to fighting, etc.

Echocardiographic Assessment. Transthoracic echocardiography of the left ventricle was performed as previously described.^{222, 223} Measurements were made

on the Vevo 770 echocardiography instrument. Body temperature was maintained (36.5–37.5°C) using a rectal thermometer interfaced with a servo-controlled heat lamp. Mice were anesthetized with 2% isoflurane, maintained under anesthesia with 1.5% isoflurane, and examined. Using short-axis B-modes and endocardial values, stroke volume (SV) was calculated as: diastolic volume – systolic volume; ejection fraction (EF%) as $SV/\text{diastolic volume} \times 100\%$; cardiac output as $SV \times \text{heart rate (HR)}$. Left ventricular diameters during diastole (LVIDd), left ventricular diameters during systole (LVIDs), and heart rate were determined from long-axis M-modes. Relative wall thickness was calculated as $(\text{diastolic posterior wall thickness} + \text{diastolic anterior wall thickness})/\text{LVIDd}$. To prevent bias, the individuals performing echocardiography and image tracing were unaware of the group assignments.

Radiometric measurement of myocardial glucose utilization. Hearts were excised and perfused in non-recirculating Langendorff mode with Krebs-Henseleit (KH) buffer (37°C; 5% CO₂/95% O₂; containing in mmol/L: 118.5 NaCl, 25 NaHCO₃, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 2.5 CaCl₂ and 5 mM glucose; pH 7.4). Glycolytic flux was assessed by measuring the amount of ³H₂O released through the metabolism of exogenous [5-³H]glucose (Perkin Elmer), as previously described.^{220, 221} Briefly, following a 20 min period of perfusion (equilibration), KH buffer containing 25 µCi/ml of [5-³H]glucose was infused into the cannula via a syringe infusion pump (Harvard Apparatus) at a proportion 1:100 to that of the flow rate (final concentration = 0.25 µCi/ml). The perfusate was collected in two separate 5-min

intervals. From the collected perfusate, 400 μ l of perfusate was added to 25 μ l of 0.6 M HCl in a microcentrifuge tube. The microcentrifuge tube was then placed in a scintillation vial containing 2 ml of H₂O to allow for evaporation diffusion of [³H]₂O in the microcentrifuge tubes into the water contained in the scintillation vial. To account for incomplete equilibration of [³H]₂O, in parallel vials, known amounts (μ Ci) of [5-³H]glucose and [³H]₂O (Moravek Biochemicals) were placed in microcentrifuge tubes, and placed into separate scintillation vials containing 0.5 ml dH₂O. After 72 h incubation at 37°C, 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). Glucose utilization was then calculated using the formula reported by Ashcroft,²²⁴ with considerations for: the specific activity of [5-³H]glucose; incomplete equilibration and background; the dilution of [5-³H]- to unlabeled-glucose; and scintillation counter efficiency. Glucose utilization was normalized to total heart weight and represented as a fold change to the control group. To prevent bias, the individual assisting in hanging the hearts for perfusion was unaware of the group assignments.

Measurement of blood glucose and lactate. Blood lactate concentration was measured in 0.7 μ l of blood from a small tail clip using a Lactate Plus meter (Nova Biomedical). Blood glucose concentration was measured from the same tail clip using the Accu-Check Aviva blood glucose monitoring meter (Roche).

Assessment of plasma free fatty acid.: Circulating plasma free fatty acids (FFA) were assessed via a colorimetric enzymatic assay, per manufacturers guidelines (Sigma-Aldrich). Briefly, plasma was incubated with acyl-CoA synthetase for 30 min at 37°C, in a 96-well microplate. A master reaction mix was added to each well and samples were again incubated for 30 min at 37°C. Absorbance was then measured at 570 nm wavelength and the concentration of FFA in each sample was calculated based on a standard curve.

Measurement of plasma IGF-1. Plasma IGF-1 was quantified using a mouse/rat IGF-1 quantikine ELISA kit (R&D Systems, Minneapolis, MN). Briefly, plasma was diluted 500-fold, added to microplate, and incubated for 2 h at room temperature with constant shaking. The microplate was then washed 5× followed by addition of secondary antibody and incubation at room temperature for 2 h with constant shaking. The microplate was then washed five times. For colorimetric determination, substrate solution was added to each well for 30 min, followed by addition of stop solution and determination of color intensity by measuring at 450 nm with wavelength correction at 540 nm. The concentration of IGF-1 was calculated using a standard curve.

Mitochondrial function. Heart mitochondria were isolated and subjected to respiratory function assays using the Seahorse XF24, similar to that described previously.²²⁵ Briefly, whole hearts were washed 5× with cold buffer A (220 mM mannitol, 70 mM sucrose, 5 mM MOPS, 1 mM EDTA; pH 7.2 with KOH) followed

by homogenization using a glass-col homogenizer in 2 ml of buffer A containing 0.2% fatty acid-free BSA. Homogenate was then subjected to centrifugation at 800g for 10 min followed by supernatant collection and centrifugation at 10,000g for 10 min. The pellet containing mitochondria was then resuspended in 1 ml fresh buffer A (without BSA) and centrifuged at 10,000g, with this step repeated once. The washed mitochondrial pellet was then resuspended in 150 µl respiration buffer (120 mM KCl, 25 mM Sucrose, 10 mM HEPES, 1 mM MgCl₂, 5 mM KH₂PO₄; pH 7.2 with KOH) and kept on ice.

To determine mitochondrial function, samples were diluted to a concentration of 5 µg (protein) in 50 µl respiration buffer per well, and centrifuged onto XF24 microplates at 500g for 3 min at 4°C. State 3 respiration in response to substrates were measured after injection of pyruvate + malate (5.0 mM + 2.5 mM, final concentrations), succinate + rotenone (10 mM + 1 µM, final concentrations), or palmitoylcarnitine + malate (50 µM + 0.5 mM, final concentrations). The oxygen consumption rates recorded after injection of oligomycin (1 µg/ml), an inhibitor of ATP synthase, served as a measure of State 4 respiration. Respiratory control ratios, state 3/state 4, was calculated as a measure of the coupling of oxygen consumption to ATP production.

Measurement of F-2,6-P₂ metabolite concentration. Metabolite levels of F-2,6-P₂ were measured in isolated cardiac myocytes as outlined previously.^{220, 221}

Glycogen Assay. To assess myocardial glycogen content, 15 mg of heart tissue powder was added to 100 μ l of ice-cold dH₂O, homogenized using a glass col homogenizer, boiled for 10 min, and centrifuged at 16,000g for 10 min. Glycogen was then hydrolyzed in a 384-well plate for 1 h at 37°C followed by addition of reaction mix containing the OxiRed probe for 1 h at 37°C. Absorbance was then measured at 590 nm. The amount of glycogen was determined against a standard curve and normalized to tissue wet weight.

Histology. Following euthanasia, the heart tissue was excised, flushed with 1 M KCl solution, and fixed in 10% formalin, embedded in paraffin, and sectioned at 4 μ m. Heart cross-sections were stained with 4'6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA) and wheat germ agglutinin (WGA; ThermoFisher, Waltham, MA) for quantification of cardiomyocyte cross-sectional area. Additionally, sections were stained with isolectin B4 (Vector Laboratories, Burlingame, CA) to assess capillary density and sirius red for fibrosis. Quantitative measurements were determined using Nikon Elements software, with no less than 100 myocytes with centrally located nuclei assessed for area measurements. To prevent bias, the individual analyzing all histology was unaware of the group assignments. A subset of slides from each group was used based on power calculations to detect a 20% difference in cross-sectional area and capillary:myocyte ratio at $\alpha < 0.05$.

Protein and mRNA Expression Analysis. For measuring protein abundance, tissues and cells were homogenized in buffer containing 50 mM HEPES (pH 7.0), 2 mM EDTA, 1% NP-40, 10% glycerol, 1 mM MgCl₂, 1 mM CaCl₂, and 150 mM NaCl; fresh phosphatase and protease inhibitors were added immediately prior to tissue homogenization. The homogenates were sonicated on ice and centrifuged at 13,000g for 20 min. Proteins in the supernatant were separated by SDS-PAGE, electroblotted to PVDF membranes, and probed according to the manufacturers' protocols. A horseradish peroxidase-linked secondary antibody (Cell Signaling Technology, Danvers, MA) was used to detect immunoreactive proteins. Images were recorded using a Fujifilm LAS-300 imager, and relative protein abundance was measured via densitometric analysis using TotalLab software.

For transcriptional expression analysis via RT-PCR, RNA was extracted from tissues and cells using the RNeasy Plus Universal Kit (Qiagen, Germantown, MD), followed by cDNA synthesis. Real-time PCR amplification was performed with SYBR Green quantitative PCR (qPCR) Master Mix (SA Biosciences, Germantown, MD) using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster, CA). Relative expression was determined by the $2^{-\Delta\Delta Ct}$ method. A list of the antibodies and primers used can be found below.

Antibodies used in these studies.

Antibodies	Source	Identifier
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	ThermoFisher	Cat#D1306
WGA (Wheat germ agglutinin, Alexa Fluor 647 conjugate)	ThermoFisher	Cat#W32466
Fluorescein labeled griffonia simplicifolia lectin I (GSL I) isolectin B4	Vector Laboratories	Cat#FL-1201

Sirius Red	Sigma-Aldrich	Cat#365548
Rabbit monoclonal anti-p-Akt (S473)	Cell Signaling Technology	Cat#4060
Rabbit polyclonal anti-Akt	Cell Signaling Technology	Cat#9272
Rabbit monoclonal anti-p-PFK2 (S483;PFKFB2)	Cell Signaling Technology	Cat#13064
Rabbit polyclonal anti-PFK2 (PFKFB2; heart isoform)	Bethyl Labs	Cat#A304-286A-M
Rabbit monoclonal anti-HK1	Cell Signaling Technology	Cat#2024
Rabbit monoclonal anti-HK2	Cell Signaling Technology	Cat#2867
Rabbit polyclonal anti-PFK1	Santa Cruz Biotechnology	Cat#sc-67028
Rabbit polyclonal anti-PFK2 (PFKFB1; liver isoform)	Novus Biologicals	Cat#NB100-92391
Rabbit polyclonal anti-PFK2 (PFKFB3; ubiquitous isoform)	Proteintech	Cat#13763-1-AP
Rabbit monoclonal anti-PKM1/2	Cell Signaling Technology	Cat#3106
Rabbit polyclonal anti-GFAT1	Santa Cruz Biotechnology	Cat#sc-134894
Rabbit polyclonal anti-GS	Cell Signaling Technology	Cat#3893
Mouse monoclonal anti-LDHB	Abcam	Cat#ab85319

PCR primers used in these studies.

Sequence-Based Reagents		
Gene of Interest	Forward	Reverse
Cited4	CATGGACACCGAGCTCATC	CTGACCCCAGGTCTGAGAAG
Cebpb	GGGGTTGTTGATGTTTTGGT	TCGAAACGGAAAAGGTTCTCA
Nfatc2	CACAGATACGGTGACCCCT	GTGGGATCGGGTTCTTCTTC
HPRT	AGGACCTCTCGAAGTGTTGG	AGGGCATATCCAACAACAAAC

Analysis of cardiac ultrastructure. Transmission electron microscopy analysis, left ventricular tissue was dissected into 3 mm³ pieces and fixed in 2.5% glutaraldehyde overnight. The samples were then post-fixed in 2% aqueous osmium tetroxide, dehydrated in ascending concentrations of ethanol and embedded in Araldite 502 (Electron Microscopy Sciences, Hatfield PA). One-micron thick sections were stained with toluidine blue for screening under a light

microscope. Selected blocks in which the muscle had a longitudinal orientation were cut into 800 Å thick sections, mounted on 200-mesh copper grids, doubly stained with uranyl acetate and lead citrate, and examined in a Phillips CM-10 electron microscope with a LaB 6 cathode (Phillips Electronic Instruments Co., New York, NY) operating at 80 kV.

Metabolomic Analysis. Hearts were freeze-clamped *in situ* using liquid nitrogen-cooled Wollenberger Tongs, which helps to capture the metabolome as it is in the beating mouse heart *in vivo*. Samples were prepared by Metabolon using their automated MicroLab STAR® system (Hamilton Company, Reno, NV). First, tissue homogenates were made in water at a ratio of 5 uL per mg of tissue. For quality control, several recovery standards were added prior to the first step in the extraction process. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were then precipitated with methanol (final concentration 80% v/v) under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. For quality assurance and control, a pooled matrix sample was generated by taking a small volume of each experimental sample to serve as a technical replicate throughout the data set. Extracted water samples served as process blanks. A cocktail of standards known not to interfere with the measurement of endogenous compounds was spiked into every analyzed sample, allowing instrument performance monitoring and aiding chromatographic alignment.

The extract was divided into fractions for analysis by reverse phase (RP)/UPLC-MS/MS with positive ion mode electrospray ionization (ESI), by RP/UPLC-MS/MS with negative ion mode ESI, and by HILIC/UPLC-MS/MS with negative ion mode ESI. Samples were placed briefly on a TurboVap[®] (Zymark) to remove the organic solvent. All methods utilized a Waters ACQUITY UPLC and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was reconstituted in solvents compatible with each MS/MS method. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 μ m) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). For more hydrophobic compounds, the extract was gradient eluted from the aforementioned C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA. Aliquots analyzed using basic negative ion optimized conditions were gradient eluted from a separate column using methanol and water, containing 6.5 mM ammonium bicarbonate (pH 8). The last aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 μ m) using a gradient consisting of water and acetonitrile with 10 mM ammonium formate (pH 10.8). The

MS analysis alternated between MS and data-dependent MSⁿ scans using dynamic exclusion. The scan range covered 70–1000 *m/z*.

Raw data were extracted, peak-identified and processed using Metabolon's proprietary hardware and software. Compounds were identified by comparison to library entries of purified, authenticated standards or recurrent unknown entities, with known retention times/indices (RI), mass to charge ratios (*m/z*), and chromatographic signatures (including MS/MS spectral data). Biochemical identifications were based on three criteria: retention index within a narrow RI window of the proposed identification, accurate mass match to the library ± 10 ppm, and the MS/MS forward and reverse scores between experimental data and authentic standards. Proprietary visualization and interpretation software (Metabolon, Inc., Durham, NC) was used to confirm the consistency of peak identification among the various samples. Library matches for each compound were checked for each sample and corrected, if necessary. Area under the curve was used for peak quantification.

Original scale data (raw area counts) were analyzed using Metaboanalyst 3.0 software (<http://www.metaboanalyst.ca/>).²²⁶ Metabolites with greater than 50% of the values missing were omitted from the analysis, and missing values were imputed by introducing values with half the minimum positive value in the original data. An interquartile range filter was used to identify and remove variables unlikely to be of use when modeling the data. The data were log-transformed and auto-scaled (mean-centered and divided by the standard deviation of each variable). Univariate (e.g., volcano plots) and multivariate (e.g., PCA) analyses

were then performed. For multiple comparison testing, q values were calculated as in R using a method embedded within the Metaboanalyst software, controlling for the false discovery rate.²²⁷ Radar charts were constructed in R using average Z-scores of significantly different metabolites from each biochemical pathway.

Transcriptomic analyses. Hearts of male WT, Glyco^{Lo} and Glyco^{Hi} mice (15–16 weeks of age) were subjected to transcriptomic analysis using the GeneChip[®] Mouse Genome 430 2.0 Array (Affymetrix, Inc.). Array data were analyzed using the affy²²⁸ and limma²²⁹ packages of R for Robust Multi-array Average (RMA) for normalization and for moderate t-statistics to derive p-values, respectively. The p-values were adjusted using the Benjamini and Hochberg method for false discovery rate control.²²⁷ For the genes with multiple probe set IDs, the probe set with the highest standard deviation among all samples was kept for further analysis. Gene Ontology analysis was performed using DAVID (<https://david.ncifcrf.gov/home.jsp>).²³⁰

Data and Software Availability. Cytoscape software was used to visualize changes in metabolic pathways and networks and was facilitated using the MetaboLync Cytoscape Plugin (Metabolon, Inc). Metabolomic data were analyzed using Metaboanalyst 3.0 software. Gene array data has been deposited in the Gene Expression Omnibus databank (GSE100176) and will be made available to readers upon publication.

Statistical analysis. Statistical parameters including the value of n (number of mice), the definition of center, dispersion and precision measures (mean $\pm$ SEM or SD), and statistical significance is reported in the Figures and Figure Legends. A *p* value of ≤ 0.05 was considered statistically significant. In the Figures, asterisks denote statistical significance as calculated by unpaired or paired Student's *t* test (for direct comparisons); Levene's test was used to assess the equality of group variances between groups, and if found to be significantly different, a Welch-modified *t*-test was used to assess significance. Multiple comparisons were assessed by one-way or two-way ANOVA followed by Bonferroni or Sidak Multiple Comparison tests (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$), as appropriate. Statistical analysis was performed using GraphPad Prism 7, Metaboanalyst, and/or the R program.

Results

Exercise promotes physiologic cardiac growth. Wild-type mice subjected to 4 weeks of treadmill training showed a 48% increase in distance run and a 77% increase in work accomplished (**Fig. 10A, B**); successful exercise testing was validated by elevated blood lactate levels, indicating that the mice ran to exhaustion (**Fig. 10C**). In comparison with untrained mice, trained mice had 15% higher levels of circulating plasma IGF-1 (**Fig. 10D**), further demonstrating systemic adaptation to the training regimen. Exercise-adapted mice displayed physiologic cardiac growth characterized by a 10% increase in heart weight to tibia

Figure 10: Exercise training improves exercise capacity. Systemic adaptation to a 4-wk treadmill training protocol: Pre- and post-training **(A)** distance and **(B)** work; **(C)** Validation of a successful high-intensity exercise capacity test as indicated by levels of blood lactate measured at rest and immediately after the criteria for exercise-induced fatigue were met; and **(D)** Plasma levels of insulin-like growth factor-1 (IGF-1). Sed, sedentary; Exe, exercise. Data represented as mean $\pm$ SEM; *** $p < 0.001$, **** $p < 0.0001$; $n = 6$ per group.

FIGURE 10

Figure 11: Exercise training promotes physiologic cardiac growth. Effect of treadmill training on the exercise-induced cardiac growth program: **(A)** Gravimetric measurements of cardiac size (HW/TL, heart weight/tibia length); **(B)** Representative myocardial sections stained with wheat germ agglutinin (red) and isolectin B4 (green). Nuclei are stained with DAPI (blue); **(C)** Cardiomyocyte cross-sectional area; **(D)** Capillary-to-myocyte ratio; **(E)** Phosphorylation of AKT (Ser473) 24 h following the last training session; **(F)** Relative Mrna expression of *Cebpb*, *Cited4*, and *Nfatc2*. Data are represented as mean $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n = 5–6 per group.

FIGURE 11

Table 3: Echocardiographic data from sedentary and exercised WT mice.

After acclimatization and an initial exercise capacity test, male FVB/NJ mice (15 weeks of age) were subjected to exercise training 5 d a week for 4 wk. One day following the final exercise capacity test, the mice were anesthetized and cardiac structure and function were assessed by echocardiography. Abbreviations: Sed, sedentary; Exe, exercised; HR, heart rate; EDV, end-diastolic volume; ESV, end-systolic volume; SV, stroke volume; EF, ejection fraction; CO, cardiac output; LVIDd, left ventricular internal diameter in diastole; LVIDs, left ventricular internal diameter in systole; LVPWd, left ventricular posterior wall in diastole; LVPWs, left ventricular posterior wall in systole; LVAWd, left ventricular anterior wall in diastole; LVAWs, left ventricular anterior wall in systole; RWT, relative wall thickness.

* $p < 0.05$, ** $p < 0.01$ vs. Sed.

TABLE 3

	WT-Sed			WT-Exe		
	Mean		SD	Mean		SD
HR (bpm)	482	±	32	492	±	25
<u>Endocardial Values</u>						
EDV (μl)	38	±	3	45	±	6**
ESV (μl)	11	±	3	13	±	2
SV (μl)	27	±	3	32	±	5**
EF (%)	71	±	7	72	±	4
CO (ml/min)	13	±	2	16	±	2**
<u>Chamber Diameter</u>						
LVIDd (mm)	3.7	±	0.2	4.0	±	0.2**
LVIDs (mm)	2.2	±	0.2	2.5	±	0.2**
<u>Wall Thickness</u>						
LVPWd (mm)	0.8	±	0.1	0.9	±	0.1*
LVPWs (mm)	1.2	±	0.1	1.3	±	0.1*
LVAWd (mm)	0.8	±	0.1	0.8	±	0.1
LVAWs (mm)	1.1	±	0.2	1.1	±	0.2
RWT	0.46	±	0.07	0.42	±	0.07
n	12			10		

length (HW/TL; **Fig. 11A**), a 24% increase in cardiomyocyte cross-sectional area, and a 35% increase in capillary-to-myocyte ratio (**Fig. 11B–D**). Furthermore, exercise increased chamber diameter and wall thicknesses, improved indices of cardiac function (EDV, SV, and CO; **Table 3**), and activated the pro-hypertrophic signaling intermediate protein kinase B (AKT)^{35, 39-42} (**Fig. 11E**). Exercise training downregulated *Cebpb* and upregulated *Cited4*, an observation which is consistent with previous findings showing necessary involvement of these transcriptional mediators in regulating physiologic growth.^{45, 46, 217-219} Moreover, the expression of *Nfatc2*, the deletion of which ameliorates pathological hypertrophy and heart failure but does not prevent physiological hypertrophy,²³¹ was markedly lower in hearts from exercise-adapted mice (**Fig. 11F**). Collectively, these results indicate that exercise training elicits robust cardiac adaptation in mice.

Metabolomic changes in the exercise-adapted heart at steady state. To examine global metabolic changes in exercise-adapted hearts, we first compared the relative abundance of 424 metabolites in hearts isolated from sedentary and exercise-adapted mice. To capture the metabolic state of the adapted heart as accurately as possible, the hearts were freeze-clamped *in situ* 24 h after the final exercise bout (**Fig. 12A**). Principal component analysis (PCA) showed little separation between the groups (**Fig. 12B**), and only 20 metabolites (out of 424 measured) were found to be significantly different (**Fig. 12C**). Of these, only two metabolites had a *q* value below 0.05 (**Table 4**). These findings demonstrate that

Figure 12: Metabolomic changes in the exercise-adapted mouse heart. (A)

Mice were either exercised for 4-wk (EXE) or remained sedentary (SED) under similar handling conditions. Following a 6 h fast and 24 h after the final exercise session, hearts were freeze-clamped *in situ*. The relative abundance of metabolites in the hearts was examined by LC/MS/MS; **(B)** 2D PCA analysis; **(C)** Volcano plot showing metabolites that changed ≥ 1.25 -fold ($p \leq 0.05$) in Exe hearts compared with Sed hearts. A list of metabolites found to be significantly different in exercise-adapted hearts can be found in Table S2. N = 9 Sed and 11 Exe mouse hearts per group.

FIGURE 12

Table 4: Significantly different metabolites in hearts from exercise-adapted mice. Hearts from WT, exercise-adapted (EXE) mice [(and corresponding WT sedentary controls (SED)] were freeze-clamped *in situ*, 24 h after the final exercise bout. Metabolites extracted from the hearts were subjected to LC/MS analysis. Raw area counts from each identified metabolite were log-transformed, autoscaled, and then subjected to t-test analysis. Missing values were imputed using half the minimum value of observed raw area count data, and metabolites with >50% values missing were omitted from the analysis. Asterisks (*) indicate compounds that were not officially confirmed based on a standard, but whose identity matches the expected exact mass using the UHPLC/MS/MS² accurate mass platform. Shown are those metabolites with a *p* value threshold of 0.05 and a fold change threshold of 1.25. n = 9 SED mice and n = 10 EXE mice.

TABLE 4

Metabolite	Identifier (KEGG or HMDB)	Relative Fold Change (EXE/SED)	p value	q value	Super Pathway	Metabolic Subpathway(s)
hexanoylglycine	HMDB00701	1.88	0.005	0.241	Lipid	Fatty Acid Metabolism (Acyl Glycine)
p-cresol-glucuronide*	HMDB11686	1.77	0.001	0.161	Amino Acid	Phe and Tyr Metabolism
3-hydroxybutyrylcarnitine	HMDB13127	1.47	0.023	0.380	Lipid	Fatty Acid Metabolism (Acyl Carnitine)
3-hydroxylaurate	HMDB00387	1.44	0.008	0.256	Lipid	Fatty Acid, Monohydroxy
2-hydroxyadipate	HMDB00321	1.44	0.013	0.300	Lipid	Fatty Acid, Dicarboxylate
3-hydroxymyristate	-	1.43	0.026	0.389	Lipid	Fatty Acid, Monohydroxy
prolylglycine	HMDB11178	1.42	0.010	0.278	Peptide	Dipeptide
3-hydroxydecanoate	HMDB02203	1.41	0.011	0.282	Lipid	Fatty Acid, Monohydroxy
cytidine-5'-diphosphoethanolamine	HMDB01564	1.38	0.011	0.282	Lipid	Phospholipid Metabolism
oleoylcholine	-	1.37	0.003	0.241	Lipid	Phospholipid Metabolism
galactitol (dulcitol)	HMDB00107	1.32	0.002	0.161	Carbohydrate	Fructose, Mannose and Galactose Metabolism
cytidine 5'-diphosphocholine	HMDB01413	1.32	0.025	0.389	Lipid	Phospholipid Metabolism
N-monomethylarginine	HMDB29416	1.31	0.021	0.363	Amino Acid	Urea cycle; Arg and Pro Metabolism
3-hydroxyoctanoate	HMDB01954	1.27	0.038	0.434	Lipid	Fatty Acid, Monohydroxy
lysine	HMDB00182	1.26	<0.001	0.003	Amino Acid	Lys Metabolism
fumarate	HMDB00134	1.25	0.032	0.422	Energy	TCA Cycle
N-delta-acetylornithine	-	0.78	0.021	0.363	Amino Acid	Urea cycle; Arg and Pro Metabolism
pipecolate	HMDB00070	0.71	0.019	0.363	Amino Acid	Lys Metabolism
4-ethylphenyl sulfate	C13637	0.53	0.033	0.422	Xenobiotics	Benzoate Metabolism
4-vinylphenol sulfate	HMDB04072	0.13	<0.001	0.003	Xenobiotic	Benzoate metabolism

the steady-state abundance of most metabolites in the exercise-adapted heart is unchanged compared with that of sedentary mice.

Exercise induces periodicity in glucose metabolism. Because the steady-state abundance of biochemical pathway intermediates may not be reflective of pathway flux,^{222, 232} and because several studies show that exercise changes cardiac metabolism (e.g.,^{40, 66}), we next measured the metabolism of [5-³H]glucose in hearts from sedentary and exercise-adapted mice. Radiometric assays of perfused hearts showed a nearly 2-fold increase in glucose utilization in exercise-adapted hearts compared with hearts from sedentary mice (**Fig. 13A**). Glycolytic enzymes were found in similar abundances in exercise-adapted hearts relative to hearts from sedentary mice (**Fig. 14**). These data indicate that changes in enzyme abundance are unlikely to contribute to the increase in glycolysis that occurs in the exercise-adapted heart.

Because glycolysis is influenced by rate-limiting steps, such as occurs at the phosphofructokinase reaction, we next examined factors that regulate PFK1—the rate-limiting and committed step of glycolysis.^{233, 234} The PFK1 reaction exerts more control on glycolysis than any other step²³⁵ and is dependent on several factors including: the levels of fructose-2,6-bisphosphate (F-2,6-P₂), substrate availability, energy charge, the levels of citrate and ATP, and pH. Because PFK2 regulates the levels of F-2,6-P₂, a potent activator of PFK1, we examined the phosphorylation status of the myocardial isoform of PFK2, PFKFB2. Phosphorylation of PFKFB2 at S483 can increase F-2,6-P₂ levels and is known to

be under the influence of insulin-like growth factor-1/AKT signaling.^{35, 236-238} Notably, both cardiac AKT phosphorylation and plasma insulin-like growth factor-1 were elevated in exercise-adapted mice (see **Fig. 11E** and **Fig. 10D**). In agreement with radiometric findings, S483 phosphorylation of PFKFB2 (p-PFK2) was 50% higher in exercise-adapted hearts compared with hearts of sedentary mice (**Fig. 13B, upper panel and 13C, left panel**). Taken together, the radiometric and immunologic data provide convergent evidence that glycolysis is increased in the exercise-adapted heart and indicate that this increase in glycolytic activity may be attributable at least in part to PFK activation.

Because exercise is a dynamic challenge that leads to rapid changes in metabolism, we next examined glycolytic changes that occur early after exercise, i.e., in the trained state during recovery. Because *ex vivo* measurements of flux in the recovery period following an exercise session are unlikely to provide meaningful data (e.g., due to lack of a metabolic steady-state), we measured levels of p-PFK2 as a possible indicator of phosphofructokinase activity and glucose utilization through glycolysis. Compared with corresponding sedentary controls, hearts excised from exercise-adapted mice 1 h after the last exercise session showed significantly diminished PFK2 phosphorylation (**Fig. 13B, lower panel and 13C, right panel**).

To determine whether these changes in metabolism precede cardiac adaptation, we eliminated the variable of extended training and examined PFK2 activation immediately after a single bout of exercise. For this, a separate group of

Figure 13: Exercise dynamically regulates PFK2 and glycolysis in the heart.

Measurements of glucose metabolism in the heart: **(A)** Measurement of relative myocardial glucose utilization in isolated perfused hearts from mice that remained sedentary (Sed) or that were exercised for 4 weeks (Exe). Measurements were performed on hearts excised 24 h after the last exercise session; **(B)** Representative immunoblots from a separate cohort of mice of S483 phosphorylation of the cardiac isoform of PFK2 (PFKFB2) in the sedentary and the exercise-adapted state: upper panel, levels of PFK2 phosphorylation 24 h after the last bout of exercise; lower panel, PFK2 phosphorylation occurring 1 h after the last exercise bout. **(C)** Quantification of PFK2 phosphorylation from blots represented in panel B; **(D)** Representative immunoblots of S483 phosphorylation of PFK2 in hearts of mice exercised for 1 d; these hearts were excised immediately after exercise, which would appear to be indicative of the state of PFK2 occurring during or immediately after exercise; **(E)** Schematic representation of glycolysis showing relationship of PFK activity with glycogen storage; **(F)** Myocardial glycogen content in hearts of Sed mice and in hearts excised immediately after 1 bout of exercise; Circulating levels of **(G)** blood glucose, **(H)** blood lactate, and **(I)** plasma free fatty acids (FFA) in mice subjected to a single bout of exercise; **(J)** Schematic illustrating changes in glucose utilization occurring with acute and chronic exercise, i.e., exercise-induced metabolic periodicity. Data are represented as mean $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$; $n = 5-8$ per group.

FIGURE 13

Figure 14: Exercise training does not alter the abundance of key glucose-metabolizing enzymes. Western blot analysis of enzymes in glycolysis and glucose metabolism: **(A)** Representative western blots of hexokinase 2 (HK2), phosphofructokinase (PFK1), phosphofructo-kinase/fructose-bisphosphatase 2 (PFKFB2; PFK2), phosphofructo-kinase/fructose-bisphosphatase 3 (PFKFB3; PFK2), pyruvate kinase (PKM1/2), pyruvate dehydrogenase kinase 1 (PDHK1), glutamine:fructose-6-phosphate aminotransferase 1 (GFAT1), glycogen synthase (GS), and lactate dehydrogenase b (LDHB); **(B)** Quantification of western blots from A. Data are represented as mean $\pm$ SEM; n = 6 per group; in all cases, $p > 0.05$ after Bonferroni correction.

FIGURE 14

mice was subjected to a single bout of exercise for 40 min. Immediately following this exercise bout, mice were euthanized and the hearts were excised for biochemical analysis. We found that p-PFK2 levels in these hearts were remarkably lower than those in sedentary controls (**Fig. 13D**), suggesting that a bout of exercise might acutely decrease the rate of glycolysis in the heart. Because measurement of glycolytic rate *ex vivo* would likely obfuscate the metabolic changes that occur during or shortly after a bout of exercise (due to loss of hemodynamic, metabolic, and paracrine [e.g. catecholamine] influence), we chose to measure glycogen content as an additional surrogate of PFK activity and glycolysis. Thus, we hypothesized that, during exercise, PFK-mediated decreases in glycolytic rate should promote glycogen accumulation (**Fig. 13E**), which is consistent with the notion that decreases in PFK activity promote glycogenesis.²³⁹ Indeed, glycogen content was elevated >5-fold in hearts collected immediately after a single bout of exercise (**Fig. 13F**). Notably, this elevation in cardiac glycogen is not related to the short (6 h) fasting period occurring in sedentary and exercised mice because independent experiments showed that fasting mice for this duration does not affect myocardial glycogen content (unfasted, 0.17 ± 0.03 $\mu\text{g}/\text{mg}$ tissue; 6 h fasted, 0.18 ± 0.05 $\mu\text{g}/\text{mg}$ tissue; $n=7-8$ per group).

Because substrate availability and delivery are key determinants of myocardial substrate preference and utilization,^{75, 78, 79, 100} we measured the circulating levels of glucose, lactate, and free fatty acids in mice immediately following a bout of exercise. Compared with pre-exercise levels, mice subjected to 40 min of treadmill exercise showed a 50% decrease in blood glucose and a 2.5-

fold increase in blood lactate (**Fig. 13G,H**). Moreover, plasma free fatty acids were significantly elevated (**Fig. 13I**), which along with the changes in blood glucose and lactate, would stimulate lactate and fat oxidation and diminish overall glucose utilization. These data are concordant with our biochemical measurements and provide convergent evidence suggesting that, during and immediately after exercise, the rate of myocardial glycolysis is diminished; however, upon adaptation and full recovery, the steady-state rates of glycolysis appear to be increased in the heart (**Fig. 13J**).

Phosphofructokinase is a strong regulator of the glucose-fatty acid cycle in the heart. We next examined whether exercise-induced periodicity in glucose metabolism might be essential for structural and functional cardiac adaptation. This hypothesis is consistent with the notion that repetitive bouts of exercise are a trigger for the transcriptional adaptation in skeletal muscle.⁸ As tools to examine the relationship between changes in myocardial glucose metabolism and cardiac remodeling, we used genetically modified mice expressing kinase-deficient (Glyco^{Lo}) or phosphatase-deficient (Glyco^{Hi}) point-mutant isoforms of PFK2 under the control of the α -MHC promoter, which constitutively decrease or increase PFK1 activity and myocardial glycolytic rate.^{220, 221} Compared with cardiomyocytes isolated from WT mice, the abundance of F-2,6-P₂ was >2-fold lower in Glyco^{Lo} cardiomyocytes and >5-fold higher in Glyco^{Hi} cardiomyocytes (**Fig. 15A**). The rate of glycolysis in perfused hearts was in accordance with transgene-induced alterations in F-2-6-P₂ (**Fig. 15B**). Consistent with the notion that PFK activity

regulates glycogen storage,²³⁹ Glyco^{Lo} hearts showed 1.6-fold higher myocardial glycogen content, and Glyco^{Hi} hearts demonstrated 4-fold lower levels of glycogen (**Fig. 15C**).

To determine how glycolysis affects myocardial metabolism, we measured the steady-state abundance of myocardial metabolites in both Glyco^{Lo} and Glyco^{Hi} hearts (**Fig. 15D**). PCA showed that the relative abundances of metabolites could discriminate between the genotypes (**Fig. 15E**). Volcano plot analysis demonstrated that, out of the 424 metabolites measured, 108 metabolites in Glyco^{Lo} hearts (**Fig. 16A, Table 5**) and 109 metabolites in Glyco^{Hi} hearts (**Fig. 16B, Table 6**) were significantly different from WT hearts. Visualization of changes in the metabolic network demonstrated the greatest impact on the lipid superfamily, with acylcarnitines (subfamily 1) showing robust, diametrically opposite changes in the Glyco^{Lo} and Glyco^{Hi} hearts (**Fig. 15F, G**). Z-score analyses illustrate remarkable increases in medium and long chain acylcarnitines in Glyco^{Hi} hearts; the corresponding species were lower in abundance in Glyco^{Lo} hearts (**Fig. 17A and Tables 5, 6**). In addition, long chain fatty acids (saturated and unsaturated) were lower in Glyco^{Lo} mice, while many of the same species were elevated in Glyco^{Hi} mice (**Fig. 15F, G, Fig. 17A, and Tables 5, 6**). Interestingly, Glyco^{Lo} hearts demonstrated elevated levels of aconitate, homocitrate, and α -ketoglutarate (**Fig. 15F, G and Table 5**), suggesting that high rates of lipid oxidation in these hearts might flood the Krebs cycle. Glycolytic intermediates were lower in Glyco^{Hi} mice, while most glycolytic intermediates were unchanged in abundance in Glyco^{Lo}

Figure 15: Cardiac F-2,6-P₂ regulates myocardial glucose and lipid metabolism. Metabolic phenotyping of Glyco^{Lo} and Glyco^{Hi} mice: **(A)** Levels of F-2,6-P₂ in cardiomyocytes isolated from WT, Glyco^{Lo}, and Glyco^{Hi} mice. N = 3–6 per group; **(B)** Relative myocardial glucose utilization in isolated perfused hearts. n = 5–7 per group; **(C)** Myocardial glycogen content. N = 7–9 per group; **(D)** Schematic of 106hosphor106ics analysis of WT, Glyco^{Lo}, and Glyco^{Hi} hearts; **(E)** 2D PCA analysis; **(F)** Metabolic network visualization of metabolites in Glyco^{Lo} and Glyco^{Hi} hearts, compared with corresponding WT hearts. Circle size is indicative of the magnitude of change (blue and red circles indicate significantly decreased or increased metabolites abundance compared with WT controls, respectively). Numbers represent metabolites in the following subpathways: 1: Acylcarnitines; 2: Monacylglycerols; 3: Monohydroxy fatty acids; 4: Phospholipid metabolism; 5: Lysolipids; 6: Polyunsaturated fatty acids; 7: Sphingolipids; 8: Dicarboxylic fatty acids; 9: Long chain fatty acids; 10: Glycolysis; 11: Fructose, Mannose, Galactose metabolism; 12: Pentose metabolism; 13: TCA cycle; 14: Purine metabolism, (hypo)xanthine/inosine; 15: Purine metabolism, adenine; 16: Gly, Ser, Thr metabolism; 17: BCAA metabolism; 18: Met, Cys, SAM, Tau metabolism; **(G)** Overview of changes in major metabolic subpathways in Glyco^{Lo} and Glyco^{Hi} hearts (F). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. the indicated group.

FIGURE 15

Figure 16: Metabolomic changes in the Glyco^{Lo} and Glyco^{Hi} mouse hearts.

Following a 6 h fast, hearts were freeze-clamped *in situ*. The relative abundance of metabolites in the hearts was measured by LC/MS/MS. Volcano plot showing metabolites that changed ≥ 1.25 -fold ($p \leq 0.05$) in **(A)** Glyco^{Lo} and **(B)** Glyco^{Hi} hearts compared with WT hearts; A list of metabolites found to be significantly different in these hearts can be found in Tables S3 and S4. N = 9 WT, 10 Glyco^{Lo}, 10 Glyco^{Hi} mouse hearts per group.

FIGURE 16

Table 5: Significantly different metabolites in hearts from WT and Glyco^{Lo} mice. Metabolites extracted from the hearts of WT and cardiac-specific kd-PFK2 transgenic mice (Glyco^{Lo} mice) were subjected to LC/MS analysis. Raw area counts from each identified metabolite were log-transformed, autoscaled, and then subjected to statistical analyses (t-test). Missing values were imputed using half the minimum value of observed raw area count data, and metabolites with >50% values missing were omitted from the analysis. Asterisks (*) indicate compounds that were not officially confirmed based on a standard, but whose identity matches the expected exact mass using the UHPLC/MS/MS² accurate mass platform. Shown are those metabolites with a *p* value threshold of 0.05 and a fold change threshold of 1.25. *n* = 9 WT mice and *n* = 10 Glyco^{Lo} mice. #S-methylmethionine was not detected in any of the WT samples, and campesterol was detected only in one WT sample; thus, the relative fold change values are based upon imputed values (i.e., half the minimum value reported for the particular metabolite).

TABLE 5

Metabolite	Identifier (KEGG or HMDB)	Relative Fold Change (Glyco ¹⁰ /WT)	p value	q value	Super Pathway	Metabolic Subpathway(s)
S-methylmethionine	C05319	12.28	<0.0001	<0.0001	Amino acid	Met, Cys, SAM, Tau metabolism
maltotriose	HMDB01262	4.29	0.0054	0.0304	Carbohydrate	Glycogen metabolism
ribonate	HMDB00867	3.49	<0.0001	0.0004	Carbohydrate	Pentose metabolism
N-acetylaspartate (NAA)	HMDB00812	2.26	<0.0001	<0.0001	Amino acid	Ala, Asp metabolism
aconitate [cis or trans]	HMDB00072	2.20	0.0018	0.0137	Energy	TCA cycle
p-cresol-glucuronide*	HMDB11686	1.90	0.0031	0.0197	Amino acid	Phe, Tyr metabolism
p-cresol sulfate	HMDB11635	1.89	0.0015	0.0120	Amino acid	Phe, Tyr metabolism
1-linoleoyl-2-arachidonoyl-GPE (18:2/20:4)*	-	1.81	0.0391	0.1349	Lipid	Phospholipid
orotidine	HMDB00788	1.74	<0.0001	<0.0001	Nucleotide	Pyrimidine metabolism, orotate containing
methyl glucopyranoside (alpha + beta)	-	1.61	0.0002	0.0032	Xenobiotic	Food component
1-stearoyl-2-oleoyl-GPC (18:0/18:1)	-	1.61	<0.0001	<0.0001	Lipid	Phospholipid
hexanoylglycine	HMDB00701	1.60	0.0299	0.1094	Lipid	Fatty acid metabolism, acylglycine
glutamate	HMDB00148	1.53	<0.0001	<0.0001	Amino acid	Glutamate metabolism
stachydrine	HMDB04827	1.52	0.0152	0.0632	Xenobiotic	Food component
maltose	HMDB00163	1.52	0.0133	0.0577	Carbohydrate	Glycogen metabolism
kynurenine	HMDB00684	1.52	0.0262	0.1028	Amino acid	Tryptophan metabolism
1-(1-enyl-palmitoyl)-2-oleoyl-GPC (p-16:0/18:1)*	-	1.49	<0.0001	0.0004	Lipid	Plasmalogen
deoxycarnitine	HMDB01161	1.45	0.0009	0.0088	Lipid	Carnitine metabolism
2-methylcitrate/homocitrate	-	1.42	0.0068	0.0367	Energy	TCA cycle
mannitol/sorbitol	HMDB00247	1.40	0.0007	0.0080	Carbohydrate	Fructose, mannose, galactose metabolism
gulonic acid*	-	1.37	0.0107	0.0508	Cofactor/Vitamin	Ascorbate/Aldehyde metabolism
1-stearoyl-2-arachidonoyl-GPE (18:0/20:4)	-	1.36	0.0017	0.0133	Lipid	Phospholipid
alpha-ketoglutarate	HMDB00208	1.36	0.0017	0.0133	Energy	TCA cycle
1-palmitoyl-2-arachidonoyl-GPE (16:0/20:4)*	HMDB05323	1.34	0.0007	0.0080	Lipid	Phospholipid
N-acetylmethionine	HMDB11745	1.33	0.0035	0.0214	Amino acid	Met, Cys, SAM, Tau metabolism
methylsuccinate	HMDB01844	1.32	0.0098	0.0481	Amino acid	BCAA metabolism
1,2-dioleoyl-GPC (18:1/18:1)*	-	1.32	0.0019	0.0137	Lipid	Phospholipid
1-palmitoyl-2-palmitoleoyl-GPC (16:0/16:1)*	-	1.29	0.0009	0.0088	Lipid	Phospholipid
glycerophosphoglycerol	C03274	1.28	0.0223	0.0885	Lipid	Glycerolipid
nicotinamide ribonucleotide (NMN)	HMDB00229	1.28	<0.0001	0.0015	Cofactor/Vitamin	Nicotinate/Nicotinamide metabolism
1,2-dilinoleoyl-GPE (18:2/18:2)*	-	1.28	0.0062	0.0339	Lipid	Phospholipid
methionine sulfone	-	1.27	0.0269	0.1038	Amino acid	Met, Cys, SAM, Tau metabolism
1-stearoyl-2-oleoyl-GPE (18:0/18:1)	-	1.27	0.0002	0.0032	Lipid	Phospholipid
thiamin monophosphate	HMDB02666	1.26	0.0012	0.0106	Cofactor/Vitamin	Thiamine metabolism
4-hydroxy-2-nonenal	HMDB04362	1.25	0.0131	0.0577	Lipid	Fatty acid, oxidized
beta-hydroxyisovalerate	HMDB00754	0.80	0.0139	0.0594	Amino acid	BCAA metabolism
palmitate (16:0)	HMDB00220	0.78	0.0019	0.0137	Lipid	Long chain fatty acid

TABLE 5

Metabolite	Identifier (KEGG or HMDB)	Relative Fold Change (Glyco ⁰ /WT)	p value	q value	Super Pathway	Metabolic Subpathway(s)
N6-carboxyethyllysine	-	0.78	0.0104	0.0506	Amino acid	Lysine metabolism
5-dodecenoate (12:1n7)	HMDB00529	0.77	0.0113	0.0528	Lipid	Medium chain fatty acid
12-HEPE	HMDB10202	0.76	0.0036	0.0216	Lipid	Eicosanoid
azelate (nonanedioate)	HMDB00784	0.76	0.0132	0.0577	Lipid	Fatty acid, dicarboxylate
1-linoleoyl-GPC (18:2)	HMDB10386	0.76	0.0028	0.0187	Lipid	Lysolipid
2-methylmalonyl carnitine	HMDB15133	0.75	<0.0001	0.0002	Lipid	Fatty acid synthesis
oleate/vaccenate (18:1)	-	0.75	0.0132	0.0577	Lipid	Long chain fatty acid
octadecanedioate	HMDB00782	0.74	0.0052	0.0299	Lipid	Fatty acid, dicarboxylate
3-hydroxyoctanoate	HMDB01954	0.74	0.0320	0.1151	Lipid	Fatty acid, monohydroxy
nonadecanoate (19:0)	HMDB00772	0.74	0.0019	0.0137	Lipid	Long chain fatty acid
argininosuccinate	HMDB00052	0.74	0.0474	0.1525	Amino acid	Urea cycle; Arg/Pro metabolism
1-palmitoyl-GPC (16:0)	HMDB10382	0.73	<0.0001	<0.0001	Lipid	Lysolipid
homocysteine	HMDB00670	0.73	0.0166	0.0678	Amino acid	Urea cycle; Arg/Pro metabolism
1-methylimidazoleacetate	HMDB002820	0.72	0.0446	0.1466	Amino acid	Histidine metabolism
succinylcarnitine	-	0.71	<0.0001	<0.0001	Energy	TCA cycle
1-oleoyl-GPG (18:1)*	-	0.70	0.0048	0.0285	Lipid	Lysolipid
1-palmitoyl-2-linoleoyl-glycerol (16:0/18:2)*	-	0.69	0.0424	0.1408	Lipid	Diacylglycerol
arachidonate (20:4n6)	HMDB01043	0.68	0.0050	0.0296	Lipid	Long chain fatty acid
1-palmitoyl-GPE (16:0)	HMDB11503	0.68	0.0407	0.1373	Lipid	Lysolipid
2-hydroxyadipate	HMDB00321	0.67	0.0083	0.0419	Lipid	Fatty acid, dicarboxylate
glycerol	HMDB00131	0.67	0.0008	0.0087	Lipid	Glycerolipid metabolism
1-arachidonoyl-GPI (20:4)*	HMDB61690	0.67	0.0079	0.0407	Lipid	Lysolipid
1-linoleoyl-GPE (18:2)*	HMDB11507	0.67	0.0449	0.1466	Lipid	Lysolipid
15-HETE	HMDB02110	0.65	0.0147	0.0617	Lipid	Eicosanoid
10-nonadecenoate (19:1n9)	HMDB13622	0.64	0.0008	0.0086	Lipid	Long chain fatty acid
linolenate [alpha or gamma; (18:3n3 or 6)]	HMDB03073	0.64	0.0078	0.0407	Lipid	Polyunsaturated fatty acid
1-oleoyl-3-linoleoyl-glycerol (18:1/18:2)	-	0.63	0.0344	0.1217	Lipid	Diacylglycerol
13-HODE + 9-HODE	-	0.63	0.0009	0.0088	Lipid	Fatty acid, monohydroxy
arachidate (20:0)	HMDB02212	0.63	<0.0001	0.0002	Lipid	Long chain fatty acid
1-stearoyl-GPE (18:0)	HMDB11130	0.63	0.0398	0.1364	Lipid	Lysolipid
linoleate (18:2n6)	HMDB00673	0.63	0.0004	0.0053	Lipid	Polyunsaturated fatty acid
stearidonate (18:4n3)	HMDB06547	0.63	0.0286	0.1075	Lipid	Polyunsaturated fatty acid
trans-4-hydroxyproline	HMDB00725	0.63	0.0004	0.0051	Amino acid	Urea cycle; Arg/Pro metabolism
isovaleryl carnitine	HMDB00688	0.62	0.0478	0.1526	Amino acid	BCAA metabolism
2-hydroxyglutarate	HMDB00606	0.62	0.0054	0.0304	Lipid	Fatty acid, dicarboxylate

TABLE 5

Metabolite	Identifier (KEGG or HMDB)	Relative Fold Change (Glyco ¹⁰ /WT)	p value	q value	Super Pathway	Metabolic Subpathway(s)
N6-carboxyethyllysine	-	0.78	0.0104	0.0506	Amino acid	Lysine metabolism
5-dodecenoate (12:1n7)	HMDB00529	0.77	0.0113	0.0528	Lipid	Medium chain fatty acid
12-HEPE	HMDB10202	0.76	0.0036	0.0216	Lipid	Eicosanoid
azelate (nonanedioate)	HMDB00784	0.76	0.0132	0.0577	Lipid	Fatty acid, dicarboxylate
1-linoleoyl-GPC (18:2)	HMDB10386	0.76	0.0028	0.0187	Lipid	Lysolipid
2-methylmalonyl carnitine oleate/vaccenate (18:1)	HMDB13133	0.75	<0.0001	0.0002	Lipid	Fatty acid synthesis
octadecanedioate	-	0.75	0.0132	0.0577	Lipid	Long chain fatty acid
3-hydroxyoctanoate	HMDB00782	0.74	0.0052	0.0299	Lipid	Fatty acid, dicarboxylate
nonadecanoate (19:0)	HMDB01954	0.74	0.0320	0.1151	Lipid	Fatty acid, monohydroxy
argininosuccinate	HMDB00772	0.74	0.0019	0.0137	Lipid	Long chain fatty acid
1-palmitoyl-GPC (16:0)	HMDB00052	0.74	0.0474	0.1525	Amino acid	Urea cycle, Arg/Pro metabolism
homocysteine	HMDB10382	0.73	<0.0001	<0.0001	Lipid	Lysolipid
1-methylimidazoleacetate	HMDB00670	0.73	0.0166	0.0678	Amino acid	Urea cycle, Arg/Pro metabolism
succinylcarnitine	HMDB02820	0.72	0.0446	0.1466	Amino acid	Histidine metabolism
1-oleoyl-GPG (18:1)*	-	0.71	<0.0001	<0.0001	Energy	TCA cycle
1-palmitoyl-2-linoleoyl-glycerol (16:0/18:2)*	-	0.70	0.0048	0.0285	Lipid	Lysolipid
arachidonate (20:4n6)	HMDB01043	0.69	0.0424	0.1408	Lipid	Diacylglycerol
1-palmitoyl-GPE (16:0)	HMDB11503	0.68	0.0050	0.0296	Lipid	Long chain fatty acid
2-hydroxyadipate	HMDB00321	0.68	0.0407	0.1373	Lipid	Lysolipid
glycerol	HMDB00131	0.67	0.0083	0.0419	Lipid	Fatty acid, dicarboxylate
1-arachidonoyl-GPI (20:4)*	HMDB01690	0.67	0.0008	0.0087	Lipid	Glycerolipid metabolism
1-linoleoyl-GPE (18:2)*	HMDB11507	0.67	0.0079	0.0407	Lipid	Lysolipid
15-HETE	HMDB02110	0.65	0.0449	0.1466	Lipid	Lysolipid
10-nonadecenoate (19:1n9)	HMDB13622	0.64	0.0147	0.0617	Lipid	Eicosanoid
linolenate [alpha or gamma: (18:3n3 or 6)]	HMDB03073	0.64	0.0008	0.0086	Lipid	Long chain fatty acid
1-oleoyl-3-linoleoyl-glycerol (18:1/18:2)	-	0.63	0.0078	0.0407	Lipid	Polyunsaturated fatty acid
13-HODE + 9-HODE	-	0.63	0.0344	0.1217	Lipid	Diacylglycerol
arachidate (20:0)	HMDB02212	0.63	0.0009	0.0088	Lipid	Fatty acid, monohydroxy
1-stearoyl-GPE (18:0)	HMDB11130	0.63	<0.0001	0.0002	Lipid	Long chain fatty acid
linoleate (18:2n6)	HMDB00673	0.63	0.0398	0.1364	Lipid	Lysolipid
stearidonate (18:4n3)	HMDB06547	0.63	0.0004	0.0053	Lipid	Polyunsaturated fatty acid
trans-4-hydroxyproline	HMDB00725	0.63	0.0286	0.1075	Lipid	Polyunsaturated fatty acid
isovaleryl carnitine	HMDB00688	0.62	0.0004	0.0051	Amino acid	Urea cycle, Arg/Pro metabolism
2-hydroxyglutarate	HMDB00606	0.62	0.0478	0.1526	Amino acid	BCAA metabolism
		0.62	0.0054	0.0304	Lipid	Fatty acid, dicarboxylate

Table 6: Significantly different metabolites in hearts from WT and Glyco^{Hi} mice. Metabolites extracted from the hearts of WT and cardiac-specific pd-PFK2 transgenic mice (Glyco^{Hi} mice) were subjected to LC/MS analysis. Raw area counts from each identified metabolite were log-transformed, autoscaled, and then subjected to t-test analysis. Missing values were imputed using half the minimum value of observed raw area count data, and metabolites with >50% values missing were omitted from the analysis. The asterisk (*) indicates compounds that were not officially confirmed based on a standard, but whose identity matches the expected exact mass using the UHPLC/MS/MS² accurate mass platform. Shown are those metabolites with a *p* value threshold of 0.05 and a fold change threshold of 1.25. *n* = 9 WT mice and *n* = 10 Glyco^{Hi} mice.

TABLE 6

Metabolite	Identifier (KEGG or HMDB)	Relative Fold Change (Glyco ^W /WT)	p value	q value	Super Pathway	Metabolic Subpathway(s)
cis-4-decenoyl carnitine	-	4.63	0.0060	0.0330	Lipid	Acylcarnitine
linoleoylcarnitine*	HMDB06469	3.38	0.0007	0.0070	Lipid	Acylcarnitine
palmitoleoylcarnitine*	-	3.34	0.0009	0.0080	Lipid	Acylcarnitine
myristoleoylcarnitine*	-	3.27	0.0004	0.0048	Lipid	Acylcarnitine
butyrylcarnitine	HMDB02013	3.26	0.0033	0.0215	Lipid/Amino acid	Acylcarnitine/BCAA metabolism
decanoylcarnitine	HMDB00651	3.07	0.0024	0.0177	Lipid	Acylcarnitine
myristoylcarnitine	HMDB05066	2.63	0.0026	0.0180	Lipid	Acylcarnitine
isovalerylcarnitine	HMDB00688	2.62	0.0018	0.0141	Amino acid	BCAA metabolism
laurylcarnitine	HMDB02250	2.56	0.0016	0.0127	Lipid	Acylcarnitine
1,2-dilinoleoyl-GPE (18:2/18:2)*	-	2.53	<0.0001	<0.0001	Lipid	Phospholipid
palmitoylcarnitine	HMDB00222	2.52	0.0038	0.0226	Lipid	Acylcarnitine
octanoylcarnitine	HMDB00791	2.51	0.0008	0.0075	Lipid	Acylcarnitine
cystathionine	HMDB00099	2.46	<0.0001	<0.0001	Amino Acid	Met, Cys, SAM, Tau metabolism
1-stearoyl-2-linoleoyl-GPE (18:0/18:2)*	-	2.14	<0.0001	<0.0001	Lipid	Phospholipid
1-myristoylglycerol (14:0)	HMDB11561	2.13	0.0247	0.1000	Lipid	Monooacylglycerol
hexanoylcarnitine	HMDB00705	2.08	0.0003	0.0039	Lipid	Acylcarnitine
1-palmitoleoyl-2-linoleoyl-GPC (16:1/18:2)*	-	2.08	<0.0001	<0.0001	Lipid	Phospholipid
oleoylcarnitine	HMDB05065	1.98	0.0099	0.0500	Lipid	Acylcarnitine
stearoylcarnitine	HMDB00848	1.97	0.0030	0.0200	Lipid	Acylcarnitine
1-oleoyl-2-linoleoyl-GPC (18:1/18:2)*	-	1.96	<0.0001	<0.0001	Lipid	Phospholipid
saccharopine	HMDB00279	1.89	0.0037	0.0226	Amino acid	Lysine metabolism
1,2-dilinoleoyl-GPC (18:2/18:2)	-	1.86	<0.0001	<0.0001	Lipid	Phospholipid
isobutyrylcarnitine	HMDB00736	1.85	0.0233	0.0962	Amino acid	BCAA metabolism
2-myristoylglycerol (14:0)	-	1.81	0.0496	0.1649	Lipid	Monooacylglycerol
2-methylbutyrylcarnitine (C5)	HMDB00378	1.77	0.0070	0.0298	Amino acid	BCAA metabolism
1-stearoyl-2-oleoyl-GPC (18:0/18:1)	-	1.76	<0.0001	<0.0001	Lipid	Phospholipid
1-palmitoyl-2-linoleoyl-GPG (16:0/18:2)	-	1.76	<0.0001	0.0008	Lipid	Phospholipid
adenosine 5'-monophosphate (AMP)	HMDB00045	1.76	0.0393	0.1330	Nucleotide	Purine, Adenine containing
glycerophosphoglycerol	C03274	1.74	0.0002	0.0027	Lipid	Glycerolipid
1-oleoyl-2-linoleoyl-GPE (16:0/20:4)*	HMDB05349	1.74	<0.0001	<0.0001	Lipid	Phospholipid
1-palmitoyl-2-arachidonoyl-GPE (16:0/20:4)*	HMDB05323	1.73	<0.0001	<0.0001	Lipid	Phospholipid
beta-hydroxyisovalerylcarnitine	-	1.72	0.0005	0.0051	Amino acid	BCAA metabolism
1-palmitoyl-2-palmitoleoyl-GPC (16:0/16:1)*	-	1.72	<0.0001	<0.0001	Lipid	Phospholipid
1-palmitoyl-2-linoleoyl-GPE (16:0/18:2)	HMDB05322	1.71	<0.0001	0.0003	Lipid	Phospholipid
1-stearoyl-2-arachidonoyl-GPC (18:0/20:4)	-	1.70	<0.0001	<0.0001	Lipid	Phospholipid
1-arachidonoyl-GPE (20:4n6)*	HMDB11517	1.68	0.0007	0.0071	Lipid	Lysolipid

TABLE 6

Metabolite	Identifier (KEGG or HMDB)	Relative Fold Change (Glyco ^W /WT)	p value	q value	Super Pathway	Metabolic Subpathway(s)
1-stearoyl-2-linoleoyl-GPC (18:0/18:2)*	-	1.67	<0.0001	0.0001	Lipid	Phospholipid
1-stearoyl-2-arachidonoyl-GPE (18:0/20:4)	-	1.66	<0.0001	<0.0001	Lipid	Phospholipid
1-linoleoyl-2-arachidonoyl-GPC (18:2/20:4n6)*	-	1.64	0.0002	0.0024	Lipid	Phospholipid
erucate (22:1n9)	HMDB020668	1.57	0.0140	0.0661	Lipid	Long chain fatty acid
1-(1-enyl-palmitoyl)-2-arachidonoyl-GPC (P-16:0/20:4)*	-	1.50	0.0004	0.0048	Lipid	Plasmalogen
N6-succinyladenosine	HMDB00912	1.49	0.0488	0.1637	Nucleotide	Purine, Adenine containing
pyridoxal	HMDB01545	1.46	0.0037	0.0226	Cofactor/Vitamin	Vitamin B6 metabolism
benzoate	HMDB01870	1.45	0.0365	0.1330	Xenobiotic	Benzoate metabolism
gamma-aminobutyrate (GABA)	HMDB00112	1.45	0.0067	0.0362	Amino acid	Glutamate metabolism
1-(1-enyl-stearoyl)-2-arachidonoyl-GPE (P-18:0/20:4)*	HMDB05779	1.44	0.0020	0.0146	Lipid	Plasmalogen
oxalate (ethanedioate)	HMDB02329	1.44	0.0012	0.0082	Cofactor/Vitamin	Ascorbate/Aldehyde metabolism
stachydrine	HMDB04827	1.44	0.0120	0.0877	Xenobiotic	Food component
1-palmitoyl-2-arachidonoyl-GPC (16:0/20:4)	-	1.44	<0.0001	0.0011	Lipid	Phospholipid
1-palmitoyl-2-linoleoyl-GPC (16:0/18:2)	-	1.42	<0.0001	<0.0001	Lipid	Phospholipid
glycine	HMDB00123	1.41	0.0013	0.0120	Amino acid	Gly, Ser, Thr metabolism
2-oleoylglycerol (18:1)	-	1.41	0.0232	0.0646	Lipid	Monacylglycerol
1,2-di-oleoyl-GPC (18:1/18:1)*	-	1.38	0.0007	0.0071	Lipid	Phospholipid
ornithine	HMDB03374	1.38	0.0260	0.1038	Amino acid	Urea cycle; Arg, Pro metabolism
1-palmitoyl-2-oleoyl-GPC (16:0/18:1)	-	1.37	<0.0001	<0.0001	Lipid	Phospholipid
succinylcarnitine	-	1.37	<0.0001	<0.0001	Energy	TCA cycle
pinelate (heptanedioate)	HMDB00857	1.36	0.0005	0.0053	Lipid	Fatty acid, dicarboxylate
homostachydrine*	HMDB33433	1.36	0.0416	0.1442	Xenobiotic	Food component
Nglycylneuraminate	HMDB00833	1.35	0.0014	0.0120	Xenobiotic	Food component
cytidine-5'-diphosphoethanolamine	HMDB01564	1.35	0.0052	0.0298	Lipid	Phospholipid metabolism
1-(1-enyl-palmitoyl)-2-linoleoyl-GPC (P-16:0/18:2)*	-	1.33	0.0001	0.0022	Lipid	Plasmalogen
betaine	HMDB00043	1.32	0.0016	0.0127	Amino acid	Gly, Ser, Thr metabolism
2-methylmalonyl carnitine	HMDB13133	1.31	0.0001	0.0023	Lipid	Fatty acid synthesis
4-hydroxy-2-nonenal	HMDB04362	1.31	0.0042	0.0226	Lipid	Fatty acid, oxidized
N6-carboxyethyllysine	-	1.30	0.0038	0.0226	Amino acid	Lysine metabolism
1-(1-enyl-palmitoyl)-2-arachidonoyl-GPE (P-16:0/20:4)*	-	1.29	0.0032	0.0207	Lipid	Plasmalogen
1-stearoyl-2-arachidonoyl-GPI (18:0/20:4)	-	1.29	0.0002	0.0024	Lipid	Phospholipid
sedoheptulose	HMDB03219	1.28	0.0104	0.0519	Carbohydrate	Pentose metabolism
phosphoethanolamine	HMDB00224	1.28	0.0029	0.0198	Lipid	Phospholipid metabolism
asparagine	HMDB00168	1.27	0.0019	0.0141	Amino acid	Ala/Asp metabolism
myo-inositol	HMDB00211	0.79	0.0135	0.0650	Lipid	Inositol metabolism
4-cholesten-3-one	HMDB00921	0.79	0.0022	0.0164	Lipid	Sterol

TABLE 6

Metabolite	Identifier (KEGG or HMDB)	Relative Fold Change (Glyco ^W /WT)	p value	q value	Super Pathway	Metabolic Subpathway(s)
N1-methyladenosine	HMDB03331	0.78	0.0049	0.0287	Nucleotide	Purine, Adenine containing
1-palmitoyl-GPC (16:0)	HMDB10382	0.77	<0.0001	0.0002	Lipid	Lysolipid
maleamate	C01596	0.77	0.0193	0.0873	Cofactor/Vitamin	Nicotinate/Nicotinamide metabolism
2-piperidinone	-	0.76	0.0127	0.0621	Xenobiotic	Food component
mannose	HMDB00169	0.76	0.0025	0.0180	Carbohydrate	Fructose, mannose, galactose metabolism
N-delta-acetylornithine	-	0.76	0.0094	0.0485	Amino acid	Urea cycle; Arg, Pro metabolism
1-(1-enyl-palmitoyl)-GPC (P-16:0)*	-	0.74	0.0013	0.0114	Lipid	lysoplasmalogen
stearoyl sphingomyelin (d18:1/18:0)	HMDB01348	0.74	0.0001	0.0023	Lipid	Sphingolipid
sphingomyelin (d18:1/20:0, d16:1/22:0)*	-	0.74	0.0004	0.0045	Lipid	Sphingolipid
citruiline	HMDB00904	0.73	0.0077	0.0375	Amino acid	Urea cycle; Arg, Pro metabolism
1-stearoyl-GPG (18:0)	-	0.72	0.0303	0.1185	Lipid	lysolipid
hippurate	HMDB00714	0.71	0.0439	0.1484	Xenobiotic	Benzoate metabolism
thiamin (Vitamin B1)	HMDB00235	0.69	<0.0001	0.0007	Cofactor/Vitamin	Thiamine metabolism
fructose	HMDB00660	0.68	0.0035	0.0223	Carbohydrate	Fructose, mannose, galactose metabolism
arabitol/xylitol	-	0.68	0.0204	0.0887	Carbohydrate	Pentose metabolism
dihydroxyacetone phosphate (DHAP)	HMDB01473	0.66	<0.0001	<0.0001	Carbohydrate	Glycolysis
lactate	HMDB00190	0.63	0.0015	0.0120	Carbohydrate	Glycolysis
homocitrulline	HMDB00679	0.62	0.0165	0.0765	Amino acid	Urea cycle; Arg, Pro metabolism
mannitol/sorbitol	HMDB00247	0.59	<0.0001	0.0005	Carbohydrate	Fructose, mannose, galactose metabolism
3-phosphoglycerate	HMDB00807	0.58	0.0200	0.0880	Carbohydrate	Glycolysis
1-palmitoyl-GPG (16:0)*	-	0.58	0.0014	0.0120	Lipid	Lysolipid
1-oleoyl-GPI (18:1)	-	0.48	0.0003	0.0035	Lipid	Lysolipid
carnosine	HMDB00033	0.46	0.0002	0.0024	Peptide	Dipeptide derivative
1-palmitoyl-GPI (16:0)*	HMDB61695	0.46	<0.0001	<0.0001	Lipid	Lysolipid
anserine	HMDB00194	0.45	0.0004	0.0048	Peptide	Dipeptide derivative
phosphoenolpyruvate (PEP)	HMDB00263	0.36	0.0016	0.0127	Carbohydrate	Glycolysis
maltotetraose	HMDB01296	0.27	0.0336	0.1256	Carbohydrate	Glycogen metabolism

Fig 17: Modulation of cardiac glycolytic rate regulates lipid, carbohydrate, and amino acid metabolism. Spider charts showing average z-scores of (A) lipid (B) carbohydrate and (C) amino acid and peptide metabolites that were significantly different in Glyco^{Lo} or Glyco^{Hi} hearts compared with WT controls. N = 9 WT, 10 Glyco^{Lo}, 10 Glyco^{Hi} mouse hearts per group.

FIGURE 17

FIGURE 17

B

C

— Glyco^{Hi}
— Glyco^{Lo}

mice (**Fig. 15F, G and Fig. 17B**). Changes in the steady-state abundances of glycolytic and lipid metabolites are most consistent with increased propensity for glucose oxidation in Glyco^{Hi} hearts and increased lipid oxidation in Glyco^{Lo} hearts. These changes would be sufficient to explain the accumulation of acylcarnitines and diminished levels of glycolytic intermediates occurring in Glyco^{Hi} hearts, and the lower levels of acylcarnitines in Glyco^{Lo} hearts. Polarization of myocardial metabolism also resulted in modest changes in amino acid metabolites (see **Fig. 17C and Tables 5, 6**). Collectively, these results show the high degree to which phosphofructokinase regulates the glucose-fatty acid cycle in the heart.

Constitutively low glycolytic activity promotes structural and transcriptional changes consistent with the exercise-adapted heart. To examine whether PFK activity regulates cardiac growth, we assessed the structure and function of Glyco^{Lo} and Glyco^{Hi} hearts. Both the Glyco^{Lo} and Glyco^{Hi} hearts showed a mild increase in cardiac size, which was associated with increased cardiomyocyte cross-sectional area (**Fig. 18A-C, E, F**); however, significantly increased capillary-to-myocyte ratio was observed only in Glyco^{Lo} hearts (**Fig. 18A, D**). Picrosirius red staining showed no noteworthy myocardial fibrosis in either strain (**Fig. 18A**). Interestingly, Glyco^{Lo} hearts showed elevated EDV and slightly increased wall thicknesses compared with WT mice (**Table 7**), which is similar to that found in WT, exercise-adapted mice (see **Table 3**). In contrast, Glyco^{Hi} hearts had

Figure 18: Constitutive changes in glycolysis promote cardiac growth and hypertrophy. Structural and molecular indices of cardiac growth/hypertrophy: **(A)** Representative myocardial sections stained for: left panels, wheat germ agglutinin (red), isolectin B4 (green) and DAPI (blue); and right panels, picrosirius red; **(B,E)** Gravimetric measurement of cardiac size (HW/TL, heart weight/tibia length); **(C,F)** Cardiomyocyte cross sectional area; **(D,G)** Capillary-to-myocyte ratio; **(H)** Basal phosphorylation of AKT (Ser473); **(I,J)** Relative Mrna expression of genes associated with the physiological growth program. Data are represented as mean $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; n = 6–12 per group.

FIGURE 18

Table 7: Echocardiographic parameters from Glyco^{Lo} and Glyco^{Hi} mice compared with WT controls. Cardiac structure and function were evaluated in 20-week-old, male, cardiac-specific, kd-PFK2 mice (Glyco^{Lo} mice), pd-PFK2 mice (Glyco^{Hi} mice) and WT littermates by echocardiography. Abbreviations: HR, heart rate; EDV, end-diastolic volume; ESV, end-systolic volume; SV, stroke volume; EF, ejection fraction; CO, cardiac output; LVIDd, left ventricular internal diameter in diastole; LVIDs, left ventricular internal diameter in systole; LVPWd, left ventricular posterior wall in diastole; LVPWs, left ventricular posterior wall in systole; LVAWd, left ventricular anterior wall in diastole; LVAWs, left ventricular anterior wall in systole; RWT, relative wall thickness. *p<0.05, **p<0.01 vs. corresponding WT controls.

TABLE 7

	WT			Glyco ^{Lo}			WT			Glyco ^{Hi}		
	Mean		SD	Mean		SD	Mean		SD	Mean		SD
HR (bpm)	486	±	22	486	±	26	492	±	52	494	±	38
<u>Endocardial Values</u>												
EDV (μl)	44	±	4	49	±	5*	50	±	4	54	±	7
ESV (μl)	12	±	3	15	±	4	15	±	2	20	±	5**
SV (μl)	32	±	3	34	±	3	35	±	4	34	±	4
EF (%)	72	±	4	70	±	6	70	±	4	63	±	5**
CO (ml/min)	15	±	2	16	±	2	17	±	2	17	±	2
<u>Chamber Diameter</u>												
LVIDd (mm)	3.9	±	0.2	4.0	±	0.3	4.1	±	0.2	4.4	±	0.3*
LVIDs (mm)	2.3	±	0.3	2.3	±	0.3	2.5	±	0.3	2.9	±	0.3**
<u>Wall Thickness</u>												
LVPWd (mm)	0.8	±	0.1	0.9	±	0.1*	1.0	±	0.2	1.0	±	0.1
LVPWs (mm)	1.3	±	0.2	1.4	±	0.2	1.5	±	0.2	1.4	±	0.1
LVAWd (mm)	0.9	±	0.1	0.9	±	0.1	1.1	±	0.2	1.2	±	0.2
LVAWs (mm)	1.3	±	0.2	1.3	±	0.2	1.6	±	0.3	1.6	±	0.2
RWT	0.43	±	0.07	0.47	±	0.10*	0.51	±	0.10	0.45	±	0.07
(n=)	10			10			11			11		

elevated ESV, lower EF%, and larger chamber diameters, suggesting a more dilated cardiac phenotype (Table S5).

To determine whether the signaling mediators and transcriptional programs underlying exercise-induced cardiac growth^{43, 45, 46, 218} are affected by constitutive changes in glucose utilization, we next examined the phosphorylation of AKT and the relative expression of *Cebpb* and *Cited4*. While no differences in basal p-AKT (S473) were observed in either strain (**Fig. 18H**), *Cebpb* expression was lower and *Cited4* expression was higher in Glyco^{Lo} hearts compared with WT counterparts (**Fig. 18I**). Expression of these genes was unchanged in Glyco^{Hi} hearts (**Fig. 18J**). Although *Nfatc2* showed a trend toward lower expression in Glyco^{Lo} hearts, this did not reach statistical significance. These data suggest that constitutively low or high rates of glycolysis cause mild cardiac hypertrophy and that a low rate of glycolysis, in the absence of AKT activation, is sufficient to promote a structural, functional, and transcriptional phenotype resembling the exercise-adapted heart.

Metabolic inflexibility causes mitochondrial structural and functional abnormalities.

Because constitutive polarization of metabolism toward either glucose or fat oxidation could have deleterious effects on mitochondrial structure and function, we examined mitochondrial ultrastructure by transmission electron microscopy and measured respiration in mitochondria isolated from the hearts of both Glyco^{Lo} and Glyco^{Hi} mice. As shown in **Fig. 19A**, qualitative analysis of electron micrographs showed disorganization of mitochondrial cristae and diminished cristae density compared with WT littermates. Consistent with this observation,

mitochondria from both Glyco^{Lo} and Glyco^{Hi} hearts showed lower respiration in the presence of pyruvate+malate or succinate (**Fig. 19B,C**). In addition, respiratory control ratios (RCR) were found to be lower in cardiac mitochondria from both transgenic strains, suggesting diminished coupling of mitochondrial electron transport to ATP synthesis (**Fig. 19D**). Thus, while constitutively low glycolysis imparts some structural, functional, and transcriptional features characteristic of the exercise-adapted heart, the mitochondrial compartment is damaged by metabolic inflexibility.

Constitutively low glycolysis is sufficient for maximal cardiac growth. With the observation that Glyco^{Lo} hearts have a cardiac phenotype partially resembling the exercise-adapted heart, we next asked whether these mice are capable of performing more work or adapting to a greater degree than their WT counterparts. To address this question, we subjected both WT and Glyco^{Lo} mice to exercise testing prior to and following 4 weeks of treadmill training. Although Glyco^{Lo} mice were able to adapt systemically to the training regimen, they were not able to run farther or perform more work than WT mice (**Fig. 20A,B**). Unlike WT mice, which showed a ~15% increase in cardiac size, Glyco^{Lo} mice showed no further increase in cardiac growth following training (**Fig. 20C**). Similarly, myocardial expression of *Cebpb* and *Cited4* were not found to be different in exercised Glyco^{Lo} mice compared with sedentary Glyco^{Lo} mice (**Fig. 20D**), suggesting that their baseline cardiac phenotype is sufficient to meet the increased cardiovascular demands brought about by exercise. These results suggest that constitutively low glycolytic

Figure 19: Metabolic inflexibility disrupts cristae structure and causes mitochondrial dysfunction. (A) Electron micrographs of WT, Glyco^{Lo}, and Glyco^{Hi} hearts. Arrows indicate areas of decreased cristae organization and density compared to WT controls; (B) State 3 and (C) State 4 respiration in isolated cardiac mitochondria measured using complex I (Pyruvate+Malate)- and complex II (Succinate)-specific substrates; (D) Respiratory control ratios. Data are represented as mean $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$; n = 3 per group.

FIGURE 19

Figure 20: Low myocardial glycolytic rates are sufficient for maximal cardiac growth. Effects of exercise training on exercise capacity, cardiac growth, and mitochondrial function in WT and Glyco^{Lo} mice: Pre- and post-training (**A**) distance and (**B**) work; (**C**) Percent cardiac growth in exercise-adapted (Exe) mice compared to sedentary (Sed) controls. N = 10–11 per group; (**D**) Relative Mrna expression of genes associated with the physiological growth program. N = 5 per group; (**E**) State 3 respiration in isolated cardiac mitochondria using complex I (Pyruvate+Malate)-, complex II (Succinate)-, and fatty acid oxidation (Palmitoylcarnitine+malate)-specific substrates. N = 3 per group. Data are represented as mean $\pm$ SEM; *p<0.05, ****p<0.0001.

FIGURE 20

rate is sufficient to maximally activate physiologic cardiac growth.

Because exercise could have beneficial effects on mitochondrial function,³⁹ we next posited that it might restore mitochondrial function in Glyco^{Lo} hearts. In WT mice, exercise did not affect state 3 respiration in the presence of any substrate tested, and, in Glyco^{Lo} mice, exercise did not reverse mitochondrial dysfunction caused by their metabolic phenotype (**Fig. 20E**).

Glycolysis regulates gene expression in the heart. To gain further insight into the extent to which glycolysis might regulate myocardial gene expression, we assessed relative transcript abundance in Glyco^{Lo} and Glyco^{Hi} hearts. Results of Genechip[®] arrays showed that the expression of 2001 genes were significantly different in Glyco^{Lo} and Glyco^{Hi} hearts compared with hearts of corresponding WT mice (Up: Glyco^{Lo}, 7; Glyco^{Hi}, 309; Down: Glyco^{Lo}, 22; Glyco^{Hi}, 295 genes); 14 differentially expressed genes overlapped in Glyco^{Lo} and Glyco^{Hi} hearts). As shown in **Fig. 21A**, the top 100 genes include genes critical for: intermediary metabolism, neurotransmission and ion channel activity/transport, DNA damage/repair, cell proliferation/differentiation, cell signaling, gene transcription, inflammation and stress responses, detoxification and xenobiotic metabolism, and cytoskeletal/contractile function. We were particularly interested in genes that are differentially regulated by glycolysis, i.e., affected in diametric opposition with respect to low or high glycolytic activity (**Fig. 21B**). In addition to *Cited4*, we found that glycolysis regulates the expression of genes involved in one-carbon metabolism and downstream methylation reactions (*Pah*, *Phgdh*, *Mtr*), lipid

Figure 21: Myocardial glycolysis coordinately regulates gene transcription in the heart. Results of transcriptomic analyses: (A) Robust Multi-array Average (RMA) analysis of top 100 genes that were found to be the most different based on the standard deviation across the samples; (B) Genes found to be coordinately regulated by glycolysis: Bars indicate genes with fold-changes at least 1.25-fold lower or higher in PFK2 mutant mice compared with WT controls. All genes shown were significantly different between Glyco^{Lo} and Glyco^{Hi} mice at $p < 0.05$, after adjustment for the false discovery rate. Significant differences from the WT controls (dotted line) using the same statistical analysis is shown by the asterisks (*FDR-adjusted p -value < 0.05); (C) Gene ontology analysis of processes significantly regulated by low rates of glycolysis; and (D) Gene ontology analysis of processes significantly regulated by high rates of glycolysis. The 15 most significant processes are shown. $n = 3$ per group.

A

WT Glyco^{Lo} Glyco^{Hi}

B

WT

Relative gene expression (fold change)

C

D

metabolism (*Insg1*, *Angptl3*), gluconeogenic activity (*Fbp2*), cell division (*Cenpf*), osmoregulation (*Cenpf*, *Aqp4*), transcription (*Tox3*), G-protein signaling (*Ramp1*), calcium-related processes (*Rcan1*, *Cacna1g*). Gene Ontology analysis showed that low rates of glycolysis had a strong influence on genes involved in oxidation-reduction processes, lipid metabolism, and responses to cAMP (**Fig. 21C**). High glycolytic rates also influenced genes involved in metabolism, responses to cAMP, responses to hormonal stimuli, and transcription. Additionally, genes involved in angiogenesis as well as differentiation showed highly significant changes (**Fig. 21D**). Collectively, the findings of this study indicate that the activity of phosphofructokinase has pervasive effects on the metabolic, transcriptional, structural, and functional characteristics of the adult heart.

Discussion

In this study, we provide evidence suggesting that exercise dynamically regulates myocardial glycolytic activity at the level of phosphofructokinase. To assess the importance of exercise-induced periodicity in glucose metabolism, we used transgenic mice expressing mutant transgenes that constitutively increase or decrease phosphofructokinase activity in the heart. Expression of a dominant-negative form of PFK2 (Glyco^{Lo}) in the heart decreased glycolytic rate and was sufficient to modulate the *Cebpb/Cited4* transcriptional program that regulates physiologic cardiac growth.^{45, 46, 219} Mice in which myocardial glycolytic rate is constitutively high also showed slight hypertrophy, but these hearts did not engage the physiologic growth gene program. The corollary of these findings is that

exercise-induced changes in phosphofructokinase activity are necessary to activate transcriptional programs dictating cardiac growth and hypertrophy. This is supported by our transcriptomics data from both Glyco^{Lo} mice and Glyco^{Hi} mice, which revealed coordinated regulation of not only the *Cited4* gene, but genes important for epigenetic regulation, lipid metabolism, gluconeogenic activity, contractility, and angiogenesis as well. Nevertheless, constitutive changes in myocardial metabolism incited damage to the mitochondrial compartment. Collectively, these results suggest that episodic decreases in glycolytic activity caused by exercise are required for physiologic cardiac remodeling and that metabolic flexibility is important to maintain mitochondrial health in the heart.

Our findings in the heart are reminiscent of exercise-induced changes in skeletal muscle, where transient and repetitive changes in gene expression, likely initiated by acute disruption of metabolic equanimity during exercise, stimulate adaptation.⁸ Although testing the role of metabolic periodicity in the heart is difficult, the post-translational regulation of PFK2 that we found to occur with exercise provided a unique opportunity to test not only how glucose utilization affects the cardiac phenotype, but how restricting fuel flexibility regulates cardiac structure, function, and gene expression as well. The dominant-negative approach to overcome the activity of endogenous myocardial PFK2, i.e., expression of the kinase-deficient form of PFK2, was sufficient to trigger a physiologic cardiac growth phenotype: Glyco^{Lo} hearts showed lower *Cebpb* and higher *Cited4* expression, which mirrored the transcriptional changes found in the exercise-adapted heart. This transcriptional signature was complemented by a cardiac growth phenotype

replete with enlarged myocytes and increased capillary density as well as cardiac chamber volumes, wall thicknesses, and functional values that were nearly identical to that found in the exercise-adapted heart. Glyco^{Lo} hearts appear to have a maximal functional and structural physiologic growth phenotype because they could not further activate the physiologic growth gene program or undergo additional cardiac growth in response to the exercise regimen. Importantly, the phenotype of Glyco^{Lo} hearts developed in the absence of AKT activation, which has been suggested to underlie exercise-induced downregulation of *Cebpb* and upregulation of *Cited4* and the general cardiac growth phenotype.^{27, 218} Thus, these data help form a model in which exercise-induced modulation of glucose metabolism is a proximal regulator of physiologic cardiac growth.

Several factors appear to contribute to acute, exercise-induced decreases in glycolysis and phosphofructokinase activity. The lower circulating blood glucose levels and increased levels of blood lactate and free fatty acids that we found to occur immediately after a single bout of exercise suggest diminished glycolytic activity occurring in the heart during and in the early recovery period after exercise. Indeed, lactate and fatty acids are preferred substrates of the heart that suppress glucose utilization both *in vivo*^{75, 77, 84} and *ex vivo*.^{78, 79} Moreover, previous studies in humans and animal models suggest that changes in these substrates during exercise contribute to diminished exercise oxygen extraction ratios for glucose and lower glucose uptake and utilization.^{87, 109} Not only do increases in circulating lactate and fatty acids with exercise appear to favor lower rates of glucose utilization, they divert glucose from glycolysis to glycogen synthesis, which is

thought to be caused in part by diminished phosphofructokinase activity.^{86, 130, 240}

Consistent with this idea, our results show diminished phosphorylation of S483 of PFK2 and increased levels of glycogen, which support the hypothesis that PFK1 activity and glycolysis are diminished with prolonged exercise or during the early recovery period. In the context of glucose utilization, changes in PFK1 are particularly important because this node of metabolism represents the major control point of glycolysis, exerting 65% control over flux through the pathway.²³⁵

Although constitutively low glycolytic rates appeared sufficient to drive physiologic growth, metabolic inflexibility in both the Glyco^{Hi} and Glyco^{Lo} hearts appeared sufficient to cause some pathology as well. While Glyco^{Hi} hearts were enlarged, they did not demonstrate significantly increased capillary-to-myocyte ratios; and, functionally, they presented with a mildly diminished ejection fraction, significantly higher end systolic volume, and larger chamber diameters. These findings are in agreement with previous studies²²¹ and suggest that chronically high rates of glycolysis promote a more pathological form of hypertrophy. Furthermore, that mitochondria isolated from both Glyco^{Hi} and Glyco^{Lo} hearts showed mitochondrial dysfunction suggests that deleterious changes in mitochondria occurring in the heart in the context of type II diabetes or heart failure—conditions associated with excessive myocardial reliance on fatty acids or glucose, respectively^{88, 241}—could be in part caused by severe metabolic inflexibility. Although genetic downregulation of *Cebpb* and upregulation of *Cited4* protect against pathologic insults,^{45, 46} mice with constitutively low glycolysis subjected to pressure overload display exacerbated pathological remodeling,²⁴²

which we suggest may be due to poor mitochondrial health caused by metabolic inflexibility.

Although at steady state the exercise-adapted heart showed higher glycolytic activity, we found relatively few changes in metabolite abundance. We speculate that higher myocardial metabolic flux in the absence of changes in the steady state abundance of metabolites may be an indication of a physiological form of metabolic remodeling that occurs in the exercise-adapted heart. However, it should be noted that our results contrast somewhat with a previous finding that showed modestly lower abundances of acylcarnitines and organic acids in hearts of female mice adapted to two months of voluntary wheel running.²⁴³ The discrepancy between these two studies could be due to sex differences, strain differences, differences in the training modality and/or intensity. Additionally, these differences could be due to different methods by which hearts are handled prior to freezing for metabolomics analysis, which is an important consideration given the high-energy utilization of mouse heart.

Although our data support the idea that PFK-mediated modulation of metabolism influences transcriptional programs relating to cardiac growth and remodeling, it is possible that fat utilization or lactate oxidation, which are regulated in concert with glucose metabolism, are also important triggers of cardiac adaptation. Future studies to determine how exercise acutely changes the cardiac metabolome could help answer whether changes in metabolites in each pathway during or shortly after exercise promote biofeedback that coordinates transcriptional programming. Moreover, identifying how exercise intensity and

duration regulate cardiac metabolism and gene transcription would be important for optimizing cardiac adaptations and for further understanding links between metabolism and cardiac growth. Particularly intriguing is the question of how such changes in metabolism may regulate epigenetic modifications. In our study, we found that glycolysis regulated *Phgdh*, *Mthfd2*, and *Mtr*, which code for proteins critical for serine biosynthesis, one-carbon metabolism, and methylation reactions. This could be significant because *Cited4* is epigenetically regulated: hypermethylation of *Cited4*-associated CpG islands causes lower expression of *Cited4* in oligodendroglial tumors.²⁴⁴ While our data do not address this mechanism, they suggest that low glycolytic rates could prevent such methylation events, thereby permitting *Cited4* transcription, especially when appropriate stimuli are present.

In summary, our results indicate that low rates of glycolysis during or shortly after exercise regulate gene programs important for cardiac growth and that invariantly high rates of glycolysis promote a more pathological form of hypertrophy. While metabolic inflexibility is damaging to mitochondria, changes in glucose utilization due to regular exercise appear to be important to maintain mitochondrial health and to derive maximal cardiac benefits from physical activity. In addition, by integrating metabolomic and transcriptomic data, these studies provide new insights into how PFK-mediated changes in metabolism regulate gene expression in the heart. Additional work is needed to further disclose how exercise-mediated changes in metabolism regulate gene transcription and tissue

remodeling and how these changes could be targeted to maximize the beneficial cardiovascular effects of exercise.ⁱⁱ

ⁱⁱ This chapter previously appeared as an article in the journal *Circulation*. The original citation is as follows: Gibb AA, et al. Exercise-induced changes in glucose metabolism promote physiologic cardiac growth. *Circulation*, 2017.

CHAPTER IV

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

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 glycerol(phospho)lipids and serine are also dependent on the production of glycolytic intermediates.²⁴⁵ In the heart, the PPP is activated during hypertrophy¹³²⁻¹³⁴ and heart failure,¹³⁵ and modulating the activity of this pathway regulates the severity of cardiac pathology.¹³⁶⁻¹⁴¹ Similarly, O-linked β -N-acetylglucosamine (O-GlcNAc)-modified proteins, which require synthesis of the sugar donor UDP-GlcNAc via the HBP,^{147, 246} are elevated in hypertrophied and failing hearts.^{223, 247-}

²⁴⁹ Although less is known about the glycerolipid pathway (GLP), increased activity of this pathway may underlie pathologic cardiac hypertrophy.¹⁴² In addition, numerous studies have identified aberrant ancillary pathway behavior in the context of diabetes [e.g., as reviewed in ^{250, 251}].

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., ²⁵²⁻²⁵⁸]. 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 types¹⁴³⁻¹⁴⁶; 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*,^{259, 260} and catecholamines²⁶¹ and pressure overload *in vivo*.²⁶²

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-P₂^{184, 220, 221, 263} and is relevant to mechanisms regulating cardiovascular homeostasis. For example, kinases known to be important for cardiac responses to stress^{27, 264} are strong regulators of PFK2 activity.^{236, 265-267} 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.^{149, 268,}

²⁶⁹ 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^{L_o}) or phosphatase deficient- (S32A and H258A; Glyco^{Hi}) form of PFK2.^{220, 221, 270-272} A 1.4 kb BamHI/Nhe1 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 [5-³H]-glucose as previously described.^{224, 273, 274} 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 [5-³H]-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 dH₂O to allow for evaporation diffusion of [³H]₂O in the microcentrifuge tubes into the scintillation vials. To account for incomplete equilibration of [³H]₂O and background, known amounts (µCi) of [5-³H]-glucose and [³H]₂O (Moravek Biochemicals) were placed into microcentrifuge tubes, and these were placed into separate scintillation vials containing 500 µl dH₂O. After incubation at 37°C 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.*,²²⁴ with considerations for the specific activity of [5-³H]-glucose, incomplete equilibration and background, dilution of [5-³H]- 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 [$^{13}\text{C}_6$]-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,^{184, 275-277} 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,^{184, 277} 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.

[¹³C₆]-glucose and [¹³C₃]-lactate measurements in the media. For quantification of [¹³C₆]-glucose and [¹³C₃]-lactate in cell media, 10 µL of media were mixed with 9990 µL of 5 mM NH₄OH in methanol containing 5 µM [D₂]-glucose and 5 µM [D₂]-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 [¹³C]-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 [¹³C]-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 ¹³C₆-glucose and ¹³C₃-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.^{184, 278}

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.²⁷⁹ For this, 75,000 cells were plated into each well. An hour before each experiment, the media was replaced with 675 μ l 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 37°C, CO₂-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 μ M); Port B, carbonyl cyanide-*p*-(trifluoromethoxy)phenylhydrazone (FCCP; 1 μ M); and Port C, antimycin A and rotenone (AA/Rot; 10 μ M / 1 μ M). The OCR and ECAR values were normalized to the total amount of protein in each well.

Statistical Analysis. All values are mean $\pm$ 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^{Lo}) or phosphatase-deficient (Glyco^{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. 22A**), and the bicistronic vector also contained GFP, used to determine relative transduction efficiency. As shown in **Fig. 22B**, 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^{Lo} or the Glyco^{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. 22C**). Although Glyco^{Hi}-expressing cells routinely displayed a higher level of mutant protein expression relative to Glyco^{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^{Hi} and Glyco^{Lo}

Figure 22: 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 (Glyco^{Lo}) or increase (Glyco^{Hi}) glycolytic rate, respectively; **(B)** GFP expression in cardiomyocytes 48 h after transduction (50 MOI) with adenoviruses expressing GFP only or the Glyco^{Lo} or Glyco^{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 22

constructs, respectively (**Fig. 22D**). 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 [$^{13}\text{C}_6$]-glucose and, after 18 h, measured the relative levels of [$^{13}\text{C}_6$]-glucose and [$^{13}\text{C}_3$]-lactate in the media. As shown in **Fig. 22E**, the levels of [$^{13}\text{C}_6$]-glucose consumed by the cell was 1.8-fold higher in Glyco^{Hi}-expressing cells compared with Ad-GFP, with no difference in Glyco^{Lo}-expressing cells. Levels of [$^{13}\text{C}_3$]-lactate extruded into the media was 2.5-fold higher in Glyco^{Hi} cells compared with GFP control cells and >10-fold higher compared with Glyco^{Lo} virus transduced cells (**Fig. 22F**). As shown in **Fig. 22G**, 29% of [$^{13}\text{C}_6$]-glucose was metabolized to [$^{13}\text{C}_3$]-lactate in GFP control cells, whereas in Glyco^{Lo}-expressing cells, 6% of labeled glucose was metabolized to lactate; in Glyco^{Hi}-expressing cells, 44% of labeled glucose was converted to lactate.

Assessment of [$^{13}\text{C}_6$]-glucose uptake, [$^{13}\text{C}_3$]-lactate production, and [5- ^3H]-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 $^3\text{H}_2\text{O}$. 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. 23A,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^{Lo} cells, yet Glyco^{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. 23B**). Although lactate production was markedly suppressed in Glyco^{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^{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. 23B**).

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^{Lo} or Glyco^{Hi} PFK2

Figure 23. Phosphofructokinase-mediated glucose distribution in isolated cardiomyocytes. Schematics illustrating glucose utilization in myocytes transduced with GFP, Glyco^{Lo} or Glyco^{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 ¹³C-glucose tracing and by glucose utilization measured by radiometric assays, respectively.

FIGURE 23

mutants in cardiomyocytes and then assessed carbon incorporation into end products of ancillary biosynthetic pathways using stable isotope-resolved metabolomics.²⁸⁰ Expression of the Glyco^{Hi} mutant modestly diminished incorporation of glucose-derived carbon into ATP. Lower fractional enrichment of ¹³C in Glyco^{Hi}-expressing cells is attributable to decreased carbon allocation into the phosphoribosyl ring of the nucleotide (*m*+5 isotopologue) (**Fig. 24A, 24B**), suggesting diminished flux of ¹³C-labeled glucose-6-phosphate (G6P) through the oxidative PPP (oxPPP). The higher level of the *m*+0 isotopologue of ATP (i.e., the unlabeled ATP pool) in Glyco^{Hi} cells further indicates that the flux of ¹³C₆-G6P through the oxPPP was lower in Glyco^{Hi} cells. Similar changes were observed in other purines such as GTP (**Fig. 24C**). Myocytes expressing the Glyco^{Lo} mutant showed ¹³C fractional enrichment values into ATP that were similar to GFP-transduced control cells (**Fig. 24A, 24B**), suggesting that low PFK activity does not strongly affect purine synthesis in these cells.

For the pyrimidines CTP and UTP, we found a higher *m*+0 isotopologue value in Glyco^{Hi} cells and a lower *m*+0 isotopologue value in Glyco^{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. 24D, 24E**). Interestingly, both the Glyco^{Lo} and the Glyco^{Hi} 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 Glyco^{Lo} and

Figure 24: Phosphofructokinase coordinates pentose phosphate pathway activity. Isolated cardiomyocytes were transduced with GFP, Glyco^{L0}, or Glyco^{Hi} adenoviruses and incubated with ¹³C₆-glucose for 18 h. Stable isotope tracing of nucleotides in NRCMs incubated with media containing U-¹³C-glucose: **(A)** Atom-resolved map illustrating the biological and biochemical history of ¹³C incorporation into the purine AMP (phosphates in ATP omitted to conserve space); Fractional enrichment values of ¹³C in **(B)** ATP and **(C)** GTP after incubation with ¹³C-glucose; **(D)** Atom-resolved map illustrating the biological and biochemical history of ¹³C incorporation into the pyrimidine UMP; Fractional enrichment values of ¹³C in **(E)** UTP and **(F)** CTP after incubation with ¹³C-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. *,\$ p<0.05, **,\$\$ p<0.01, ***,\$\$\$ p<0.001, ****,\$\$\$\$ p<0.0001.

A

Serine/folate metabolism

$^{13}\text{C}_3$ -Serine

$^{13}\text{C}_1$ -N 10 -formyl-THF

SHMT

$^{13}\text{C}_2$ -Glycine

$^{13}\text{C}_3$ -Phosphoserine

$^{13}\text{C}_3$ -Phosphoglycerol

$^{13}\text{C}_6$ -G6P

$^{13}\text{C}_6$ -Glucose

HK

PRPP

$^{13}\text{C}_5$ -ribose

Purine Biosynthesis

PPP

Glycolysis

B

ATP

Fractional Enrichment

Isotopologue ($m+x$)

GFP

Glyco^{Lo}

Glyco^{Hi}

C

GTP

Fractional Enrichment

Isotopologue ($m+x$)

D

Pyrimidine Biosynthesis

Glutamine + CO₂

CPS II

Carbamoyl phosphate

$^{13}\text{C}_3/^{13}\text{C}_2$ -Ump

$^{13}\text{C}_3/^{13}\text{C}_2$ -OAA

AST

CAC

$^{13}\text{C}_3$ -Ac-CoA

PC

PDH

$^{13}\text{C}_3$ -Pyruvate

$^{13}\text{C}_6$ -G6P

HK

$^{13}\text{C}_6$ -Glucose

Glycolysis

PRPP

$^{13}\text{C}_5$ -ribose

PPP

E

CTP

Fractional Enrichment

Isotopologue ($m+x$)

F

UTP

Fractional Enrichment

Isotopologue ($m+x$)

Glyco^{Hi} 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 ¹³C-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 ¹³C-labeled aspartate to the molecule (see Fig. 3D), show that both the Glyco^{Lo} and Glyco^{Hi} cells have less incorporation of ¹³C, 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 ¹³C₅-labeling of the ribose ring and ¹³C₁ labeling of aspartate, occurring as a result of scrambling during the 2nd turn of the Krebs cycle, as suggested previously.²⁸¹ Overall, these data suggest that high PFK activity modestly diminishes oxPPP flux and ¹³C 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. 25A**). To determine how the glycolytic activity affects glucose-derived carbon allocation into the HBP, we measured fractional enrichment of ¹³C from [¹³C₆]-

glucose into UDP-HexNAc in NRCMs transduced with Glyco^{Lo} or Glyco^{Hi} virus. After 18 h, >90% of the UDP-HexNAc pool integrated ¹³C-glucose-derived carbon (**Fig. 25B**), 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^{Hi} cells indicates that high rates of glycolysis diminish glucose carbon incorporation into UDP-HexNAc. The lower ¹³C 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 ¹³C-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 ¹³C, respectively) demonstrate significantly higher fractional enrichment in Glyco^{Hi} cells. Given that both the Glyco^{Lo} and the Glyco^{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 ¹³C-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 ¹³C 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^{Lo} cells was higher than that in control cells suggests that low

Figure 25: High phosphofructokinase activity diminishes glucose carbon incorporation into UDP-HexNAc. Isolated cardiomyocytes were transduced with GFP, Glyco^{Lo}, or Glyco^{Hi} adenoviruses and incubated with ¹³C₆-glucose for 18 h. Stable isotope tracing of UDP-HexNAc in NRCMs incubated with media containing ¹³C-glucose: **(A)** Atom-resolved map illustrating the biological and biochemical history of ¹³C incorporation into UDP-GlcNAc; **(B)** Fractional enrichment values of ¹³C in UDP-HexNAc (which includes both UDP-GlcNAc and UDP-GalNAc) after incubation with ¹³C₆-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. *,\$ p<0.05, **,\$\$ p<0.01, \$\$\$ p<0.001, \$\$\$\$ p<0.0001.

FIGURE 25

A

B

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. 25A, 25B**). 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,²⁸² myocytes have a relatively low capacity for free glycerol uptake; therefore, there is little contribution of glycerol kinase to G3P synthesis.^{283,}²⁸⁴ However, G3P can be generated from DHAP by glycerol-3-phosphate dehydrogenase (**Fig. 26A**). 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 Glyco^{Lo} cells showed diminished incorporation of ^{13}C from $^{13}\text{C}_6$ -glucose into the glycerol ($m+3$) moiety of phosphatidylcholine (PC; **Fig. 26B**), phosphatidylinositol (PI; **Fig. 26C**), phosphatidylethanolamine (PE; **Fig. 26D**), and phosphatidylserine (PS; **Fig. 26E**). Incorporation of $^{13}\text{C}_6$ -glucose-derived carbon into fatty acids in PC, PI, PE, and PS was not different in Glyco^{Lo} 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. 26D**), PI showed diminished labeling characterized by lower incorporation of ^{13}C into fatty acids. Glycerol ^{13}C labeling in PI from Glyco^{Hi} 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. 26F**, 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, Glyco^{Hi} 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 cataplerosis²⁸⁵ is diminished under conditions of high PFK activity. Because cataplerotic reactions

Figure 26: Phosphofructokinase regulates glycerolipid biosynthesis. Stable isotope tracing of phospholipids and triacylglycerols in cardiomyocytes incubated with media containing $^{13}\text{C}_6$ -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. *,\$ $p < 0.05$, **,,\$ $p < 0.01$, \$\$\$ $p < 0.001$, \$\$\$\$ $p < 0.0001$.

FIGURE 26

depend upon mitochondrial activity, we next measured respiration in cells expressing either the control construct (GFP) or the Glyco^{Hi} or Glyco^{Lo} constructs. Glyco^{Hi}-expressing cells showed 50% lower basal oxygen consumption, whereas Glyco^{Lo}-expressing cells showed significantly higher basal OCR values (**Fig. 27A, 27C**). 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.²⁸⁶⁻²⁸⁹ Although addition of oligomycin showed marginal effects on ATP-linked OCR, proton leak was significantly diminished in Glyco^{Hi} cells. Upon addition of a mitochondrial uncoupler, Glyco^{Lo} and Glyco^{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. 27A, 27C**). 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^{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^{Lo} PFK2 mutant (**Fig. 27B**). 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 CO₂ (i.e.,

Figure 27: 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^{Lo}, or Glyco^{Hi} adenoviruses. n=3 independent isolations per group. *p < 0.05, **p < 0.01.

FIGURE 27

$\text{CO}_2 \rightarrow \text{HCO}_3^- + \text{H}^+$)^{290, 291}; thus, the inconsistency of ECAR with absolute measurements of glycolytic rate and $^{13}\text{C}_3$ -lactate in Glyco^{Lo} 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.²⁹² 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,²³⁵ 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 GLUT4.¹¹⁰ 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,²⁹³ 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 ¹³C 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,²⁹⁴ our comparisons of ¹³C 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,¹⁴⁴ fibroblasts,^{295, 296} and developing embryos²⁹⁷ 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.²³⁵ Similar to findings in neurons,²⁹⁸ 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 ¹³C 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 N¹⁰-formyltetrahydrofolate, CO₂ and glycine, while pyrimidine ring carbons come from carbamoyl phosphate and glutamine, as well as aspartate generated by cataplerosis.²⁹⁹ 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 N¹⁰-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^{Hi} 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^{Hi} cells shows that these pathways were diminished under conditions of high PFK activity. From these data, we inferred that the decrease in *de novo* pyrimidine biosynthesis in the Glyco^{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,²⁸⁵ 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^{Hi} cells, paired with the observation that ¹³C-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 ¹³C isotopologue data for UDP-GlcNAc³⁰⁰ 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.^{147, 246} The fact that the unlabeled pool of UDP-HexNAc was higher in Glyco^{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 ¹³C and ¹⁵N) 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 biphosphatase 2,³⁰¹ 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,^{302, 303} 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 ¹³C incorporation into glycerol was not different, but fatty acyl chain labeling was diminished in Glyco^{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.²³⁹ 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.⁸⁴ 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.⁸⁴ 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.²³⁵ 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.^{220, 221} 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-acetyl-hexosamine(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.ⁱⁱⁱ

ⁱⁱⁱ This chapter previously appeared as an article in the Biochemical Journal. The original citation is as follows: Gibb AA, et al. Integration of flux measurements to resolve changes in anabolic and catabolic metabolism in cardiac myocytes. *Biochem J*. 2017.

CHAPTER V

CONCLUDING DISCUSSION

The studies presented here were designed to develop an understanding of the effects of exercise on cardiac metabolism and the potential role of these changes in regulating the adaptive response, i.e., physiologic growth. We reasoned that, because changes in metabolism present not only a material cause but can also have direct and indirect roles in intracellular signaling, exercise-induced changes in cardiac metabolism are essential for adaptation to training. Our general hypothesis is that exercise-induced changes in glycolytic flux are essential for activation of the physiological growth program. To test this hypothesis, we assessed the effects of acute and chronic exercise on myocardial metabolism, in particular glycolysis, and whether these changes contributed to the physiologic growth of the heart.

To establish a model in which to test our hypothesis, we first examined nocturnal/diurnal behavior, treadmill exercise compliance, and systemic as well as cardiac-specific exercise adaptations in two commonly used mouse strains, C57BL/6J, and FVB/NJ mice. As discussed in Chapter II, metabolic cage analysis indicated a strong nocturnal nature of C57BL/6J mice, whereas FVB/NJ mice showed no circadian element to activity, food or water intake, VO_2 , or VCO_2 . Initial

exercise capacity tests revealed that, compared with C57BL/6J mice, FVB/NJ mice are capable of achieving nearly 2-fold higher workloads prior to exhaustion. FVB/NJ mice tested during the day were capable of achieving significantly more work compared with their night-tested counterparts. Following 4 weeks of training, FVB/NJ mice showed significant increases in exercise capacity as well as physiologic cardiac growth characterized by enlarged myocytes and higher mitochondrial DNA content. C57BL/6J mice showed no increases in exercise capacity or cardiac growth regardless of whether they exercised during the day or the night. This lack of adaptation in C57BL/6J mice was attributable, at least in part, to their progressive loss of compliance to the treadmill training protocol. From these results, we conclude that the FVB/NJ strain is a useful and robust mouse model for examining cardiac adaptations to treadmill exercise and that treadmill training during daytime hours does not negatively affect exercise compliance or capacity.

In Chapter III, we examined the effects of exercise on myocardial metabolism and the role these changes may play in contributing to exercise-induced cardiac growth. As discussed, we found that exercise acutely decreased glucose utilization by modulating circulating substrates and reducing phosphofructokinase activity; however, upon exercise adaptation and recovery there was an increase in myocardial phosphofructokinase activity and glycolysis. To assess the relevance of these changes in glycolytic activity, we determined how cardiac-specific expression of mutant forms of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2) affect cardiac structure, function, metabolism, and

gene programs relevant to cardiac remodeling. Cardiac-specific expression of a kinase-deficient PFK2 transgene (Glyco^{Lo} mice) lowered glycolytic rate and regulated the expression of genes known to promote cardiac growth. Hearts of Glyco^{Lo} mice had larger myocytes, enhanced cardiac function, and higher capillary-to-myocyte ratios. Expression of phosphatase-deficient PFK2 in the heart (Glyco^{Hi} mice) increased glucose utilization and promoted a more pathological form of hypertrophy devoid of transcriptional activation of the physiologic cardiac growth program. Modulation of phosphofructokinase activity was sufficient to regulate the glucose-fatty acid cycle in the heart; however, metabolic inflexibility caused by invariantly low or high phosphofructokinase activity caused modest mitochondrial damage. Transcriptomic analyses showed that glycolysis regulates the expression of key genes involved in cardiac metabolism and remodeling. Collectively, these findings suggest that the exercise-induced decreases in glycolytic activity stimulate physiologic cardiac remodeling and that metabolic flexibility is important for maintaining mitochondrial health in the heart.

To gain insight into how these changes in glycolytic activity could contribute to cardiac growth, we assessed the impact of changes in glycolytic activity on cardiomyocyte anabolic metabolism. In Chapter IV, we show that expression of mutant forms of PFK2 in rat neonatal cardiomyocytes coordinately regulate catabolic and anabolic metabolism. An important finding was that >40% of glucose consumed by myocytes 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 ¹³C fractional

enrichment patterns from stable isotope tracing experiments 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}C incorporation under conditions of high PFK activity; the isotopologue ^{13}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.

Collectively, the data obtained from these studies support the notion that acute and chronic exercise is associated with changes in myocardial metabolism and that the periodic decreases in myocardial glucose utilization occurring during exercise are a stimulus for physiologic cardiac growth. Additionally, metabolic flexibility, maintained in the exercised heart, appears to be a primary distinguishing feature of mitochondrial health in the heart. These findings are summarized in **Fig. 28** and are discussed in greater detail below.

Figure 28: Exercise-induced effects on cardiac metabolism and its role in regulating cardiac growth and mitochondrial health. Acute exercise increases the circulating levels of FFA and lactate which act to decrease the utilization of glucose by the myocardium; however, in the exercise-adapted state sustained increases in circulating hormones contribute in part to enhanced glucose utilization. At the cellular level, allosteric regulation of the rate-limiting step of glycolysis, PFK1, is coordinately affected to and influence glucose utilization both during exercise and in the adapted state. These exercise-induced decreases in glycolysis are sufficient to promote physiological growth of the heart. While decreases in glucose utilization are a key trigger for cardiac growth, exercise-induced metabolic flexibility is essential for the preservation of mitochondrial health in the physiological hypertrophied heart.

FIGURE 28

Metabolic Regulation of Cardiac Growth

While exercise promotes cardiovascular wellness and overall health, the mechanisms by which this is accomplished remain unclear. The three salient findings from our studies, related to the heart, are: (1) that periodic decreases in myocardial glycolytic activity are sufficient to promote physiologic cardiac growth³⁰⁴; (2) that maintenance of metabolic flexibility in the heart is essential for mitochondrial health³⁰⁴; and (3) that glycolytic activity in cardiomyocytes coordinates the biosynthesis of nucleotides, nucleotide sugars, and glycerophospholipids.³⁰⁵ Our findings have several implications, germane to both the basic understanding of cardiac remodeling as well as future targets for therapy. The finding that exercise acutely diminishes glucose utilization and that this change in metabolism activates the gene program for physiologic growth provides new knowledge and a metabolic target that could be leveraged for preventing adverse remodeling in response to stress. In particular, controlled pharmacologic modulation of the PFK node of metabolism could be used to activate the cardiac growth gene program as well as to stimulate the heart to use multiple substrates, which appears to be required for optimal mitochondrial function. Also, the finding that PFK activity regulates ancillary biosynthetic pathway activity rouses new concepts about the importance of coordinated anabolic activity in modulating hypertrophic responses to stress. These findings and their potential impact with respect to myocardial health and remodeling are discussed in detail below.

Myocardial glycolysis coordinates cardiomyocyte biosynthetic activity: A principle finding from our studies was that PFK activity in cardiomyocytes coordinated the biosynthesis of materials required for growth (**Fig. 29**). One pathway affected by PFK and glycolytic activity was the PPP, which contributes to growth by: (1) replenishing NADPH levels required for anabolic reactions and the regeneration of GSH; (2) synthesizing nucleotides for DNA repair and replication; and (3) providing metabolites that can act as direct signaling modulators. Previously it has been shown that glycolysis regulates glucose entry into the PPP in several cell types. In particular, developing embryos increase PPP flux by decreasing PFK enzyme abundance, rewiring glucose metabolism for normal development.²⁹⁷ In the context of the adult heart, pathological hypertrophy¹³²⁻¹³⁴ and heart failure¹³⁵ are both associated with an increase in PPP activity. During short-intensive exercise, the activities of the enzymes of the oxidative part of the PPP have been found to be slightly diminished; however, in the immediate recovery period, an increase in enzymatic activity was observed that persisted for up to 3 h.³⁰⁶ While the effectors of increased PPP activity are not well known, a recent study identified that overexpression of PGC-1 α in skeletal muscle increased the enzymatic activities and end-products of the PPP.³⁰⁷ This finding is important as upregulation of PGC-1 α is well-known to be initiated by acute bouts of exercise.

Our results provide additional mechanistic insight as to how PPP activity is increased in the exercising and recovering heart. Our *in vivo* results show that during and in the early recovery phase of exercise, PFK activity is diminished. This likely results in the redistribution of glucose into other ancillary pathways, such as

Figure 29: Metabolic regulation of the biosynthetic pathways involved in the cardiac growth response.

FIGURE 29

the PPP, that are upstream of PFK. In fact, several lines of evidence support this assumption, such as the increase in myocardial glycogen shown to occur at this time. Our *in vivo* model of diminished PFK activity (i.e., Glyco^{Lo}) validated that regulation at this node of metabolism increases PPP activity as Glyco^{Lo} mice displayed increased abundances of several PPP-derived metabolites. Our *in vitro* studies also suggest that increased PPP activity may be a more coordinated and selective process, as low rates of glycolysis increased the biosynthesis of pyrimidines without affecting the purine pool.

Collectively, these results suggest that an increase in PPP activity occurs when PFK activity is diminished. This results in increased nucleotide biosynthesis for DNA replication and amino acid synthesis as well as regeneration of the NADPH pool for anabolic reactions. As growth is an anabolic event, the cell must appropriately coordinate the biosynthesis of these building blocks, priming itself for the recovery phase when growth takes place. Also, the increase in PPP activity may have a direct role in signaling the growth of the heart. Results from our metabolomic screening showed that AICAR, an AMP-analog derived from the PPP, was significantly elevated in Glyco^{Lo} mice compared to WT controls based on a simple unpaired t-test. This increase in AICAR was associated with a significant increase in AMPK activation (data not shown). This result could suggest that changes in PPP activity are not only important for the building blocks for growth, but also for regulating signaling pathways that instigate the growth program.

Our *in vivo* and *in vitro* data also collectively suggest that an increase in PFK activity, such as occurs during the homeostatic phase in the exercise-adapted heart, would be important for the synthesis of glycerol(phospho)lipids. In particular, the synthesis of the glycerol moiety via the GLP is required to provide the backbone for fatty acid esterification and phospholipid synthesis. We found that the synthesis of abundant phospholipids in the cardiomyocyte, such as phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol,³⁰⁸ appear to be regulated strongly by PFK-mediated changes in glycolytic activity.³⁰⁵ Higher levels of these phospholipids would be required for *de novo* expansion of cellular membranes as well as for maintaining larger myocytes, in response to stressors such as exercise.

We also found that changes in glycolytic activity cause significant phospholipid remodeling. This observation is important as it has been shown that changes in phospholipid composition, as occurs when phosphatidylethanolamine synthesis is reduced, results in impaired cardiac function.³⁰⁹ Similarly, chronic heart failure and diabetic cardiomyopathy, conditions in which metabolism becomes inflexible, are associated with disturbances in cardiac sarcolemmal membrane phospholipid homeostasis.³¹⁰ In the exercise-adapted heart, proper phospholipid homeostasis is maintained, with little to no change in phospholipid composition.³⁰⁸ This could be a result of the acute periodic episodes of altered PFK and glycolytic activity instigated by exercise as opposed to the constitutive changes observed in disease.

Metabolic flexibility and mitochondrial health: Another key finding from our studies is that metabolic flexibility appears to be essential for maintaining mitochondrial health. While it is generally accepted that mitochondrial dysfunction develops in pathologic forms of hypertrophy, where metabolism becomes inflexible, the exact mechanisms driving dysfunction remain ill-defined. Several proposed mechanisms that have been suggested to contribute to mitochondrial dysfunction include mitochondrial DNA depletion and swelling,³¹¹ disorganization and reduced density of the cristae structures,³¹² increased oxidative stress,³¹³ and mitochondrial uncoupling.³¹⁴ Our findings *in vivo* indicate that invariant inflexibility of cardiac metabolism is sufficient to promote a mild form of mitochondrial dysfunction, suggesting that the metabolic phenotypes of the heart in pathology (e.g., chronic heart failure and diabetic cardiomyopathy) may in fact initiate the decline in mitochondrial health.

In line with this idea, several of the manifestations of mitochondrial dysfunction were identified in our *in vivo* mouse models. In both Glyco^{Lo} and Glyco^{Hi} hearts, we observed disorganization and a decreased density of mitochondrial cristae. Additionally, results from our metabolomics data suggest that metabolic inflexibility results in chronic oxidative stress, indicated by elevations in the lipid peroxidation product, 4-hydroxy-2-nonenal (4-HNE). Data from our *in vitro* studies may provide insight as to why this oxidative stress occurs, as increased rates of cardiomyocyte glycolysis diminished the biosynthesis of glutathione (data not shown), a key antioxidant. Lastly, it is possible that the mitochondria are more uncoupled which could contribute to the dysfunction

observed. As reviewed by Lopashuk, the oxidation of FFA contributes to mitochondrial uncoupling.⁸² Therefore, in instances of excessive FAO such as occurs in the Glyco^{Lo} heart, chronic uncoupling of the mitochondria could be detrimental. Our data indicate that the mitochondria from Glyco^{Lo} hearts are, in fact, more uncoupled than control hearts.

Mitochondrial dysfunction is also likely a consequence of changes in phospholipid homeostasis. As already discussed, both our *in vivo* and *in vitro* data indicate that changes in PFK and glycolytic activity affect phospholipid metabolism. Therefore, changes in one or several of the phospholipids found in the mitochondrial membrane could affect mitochondrial health. One of the most abundant phospholipids of the inner mitochondrial membrane is cardiolipin, which plays an important role in regulating the enzymatic activity of the electron transport chain.^{315, 316} In heart failure, mitochondrial cardiolipin content is reduced and is correlated with mitochondrial dysfunction.³¹⁷⁻³¹⁹ While we did not measure the cardiolipin abundance or its biosynthesis, identifying whether cardiolipin metabolism is affected by changes in glycolytic activity could provide novel mechanistic insights into mitochondrial damage in disease. Taken together, these results provide evidence that metabolic flexibility is essential for the maintenance of a healthy mitochondrial pool. Furthermore, our results provide a potential therapeutic target, the PFK node of glycolysis, to increase metabolic flexibility and improve mitochondrial health in disease.

Periodic decreases in myocardial glycolysis activate the cardiac growth program:

As discussed, our data collectively indicate that changes in PFK and glycolytic activity coordinate biosynthetic pathways (e.g., PPP, GLP) that are required for cardiomyocyte growth. In addition, our data also suggest that metabolism plays a critical role in the activation of gene sets that promote cardiac growth. Most notably, our findings suggest that decreases in myocardial glycolytic rate, such as occurs during exercise, is sufficient to activate the *Cebpb/Cited4* axis. While it has previously been shown that the activation of AKT was required for this transcriptional regulation,⁴⁵ we found that low rates of glycolysis were sufficient to activate the physiologic gene program, suggesting that changes in metabolism are likely a proximal cause of cardiac growth.

Results from our studies suggest that PFK activity regulates anabolic pathways required for the synthesis of the amino acids alanine, serine, and glycine. The stimulation of the serine biosynthetic pathway (SBP), in particular, would not only increase serine/glycine biosynthesis, but it would control the one-carbon metabolic reactions (i.e., those involving methyl tetrahydrofolate metabolites) that are known to modulate the epigenetic landscape.³²⁰ Our data show not only that the abundance of these metabolites are changed by PFK activity (i.e., increased in Glyco^{Hi}), but that glycolysis regulates the transcription of enzymes involved in one-carbon metabolism (e.g., *Phgdh*). This could be significant because *Cited4* is epigenetically regulated whereby hypermethylation of its CpG island causes lower expression.²⁴⁴ Our results are in line with the general consensus showing that altered metabolism regulates the epigenetic landscape.³²¹⁻³²³ While additional

work establishing the mechanistic link between metabolism and epigenetics is required, our results provide new insights into the regulation of gene expression via metabolism.

Expanding on the observation that metabolites involved in one-carbon metabolism (e.g., serine, methionine, cysteine) are affected by PFK activity, the metabolic flexibility induced by exercise at this same metabolic node could contribute to the regulation of gene transcription. As these metabolites are involved in the methylation events of DNA and RNA, controlled modulation of the SBP pathway, by exercise-induced changes in PFK activity, could readily increase and decrease methylating agents such as 5-methyl-THF, altering gene transcription at the level of epigenetics. Our results support this notion as several genes, including *Cited4*, were coordinately regulated; however, we have yet to address whether changes in the SBP contribute to the physiologic gene program. The idea that PFK activity affects the epigenetic landscape is important, as it provides a potential therapeutic target that can be leveraged to prevent adverse remodeling.

Future Directions

Our results clearly demonstrate a key role for metabolism in regulating the cardiac growth response to stress; however, several questions remain unanswered. For instance, an experiment that would help to further identify how metabolic pathways integrate to promote the physiologic growth program would be to identify which pathways are similarly altered in the Glyco^{Lo} and acutely exercising heart. For example, if one-carbon metabolites involved in methylation

are similarly reduced in their abundance, this would provide further evidence of the metabolic regulation of epigenetic modifications. Similarly, if flux through the HBP, which we show to be affected by PFK and glycolytic activity, is similarly affected in exercising and Glyco^{Lo} hearts, identification of the proteins that are analogously post-translationally modified could provide additional targets to further dissect the mechanisms that promote physiologic growth of the heart. Furthermore, identifying how exercise intensity and duration regulate these metabolic pathways and gene transcription would be important for optimizing cardiac adaptations and for further understanding links between metabolism and cardiac growth.

Understanding how modulation of other nodes of glucose metabolism may also provide insight as to the regulation imposed by metabolism on cardiac growth. For example, cardiac-specific pyruvate dehydrogenase complex deficiency results in a mild form of cardiac hypertrophy in mice,³²⁴ an interesting observation as it could be reasoned that this genetic modification likely results in a phenotype similar to our Glyco^{Lo} mouse. In mice subjected to pressure overload, cardiomyocyte overexpression of hexokinase resulted in the attenuation of pathological hypertrophy.¹³⁹ Lastly, an interesting clinical finding in patients with glycogen storage diseases is the presentation of a mild form of hypertrophic cardiomyopathy.³²⁵ This suggests that myocardial glycogen contributes to the growth of the heart and may be relevant to our findings as we see that exercise and diminished PFK activity increase myocardial glycogen content. Therefore, it would be interesting to determine whether glycogen accumulation during exercise contributes to the cardiac growth response. These studies, in addition to our

results, provide ample evidence of the role of metabolism in regulating the growth responses to stress and indicate additional nodes of glucose metabolism that potentially coordinate the biosynthetic pathways.

The conclusions from our studies also open up the possibility to explore the contributions of metabolism in other forms of cardiac growth and hypertrophy. For instance, little is known about the metabolic changes that occur in pregnancy-induced physiological hypertrophy. In healthy women, pregnancy is associated with a transient increase in cardiac size, similar to that observed in the exercised heart.³²⁶ This growth response has been linked to the activation of AKT and ERK1/2 signaling pathways, both known to influence and control cellular metabolism.³²⁷⁻³²⁹ with this knowledge in hand, it would be interesting to test whether pregnancy-induced cardiac growth is associated with similar changes in PFK activity and transcription regulation of *Cebpb* and *Cited4* as we observed in our exercise model.

The transcriptomics results from our study provided a large data set in which to ask new and exciting questions in the field of cardiac health in disease. For example, the expression of gluconeogenic genes, fructose-1,6-bisphosphatase 2 (*Fbp2*) and phosphoenolpyruvate (*Pck1*), were found to be altered by glycolytic activity in the heart, with *Fbp2* in a coordinate manner. The fact that these genes are modifiable in an organ thought to have limited gluconeogenic potential is noteworthy. This observation provides the opportunity to test the hypothesis that an increase in myocardial gluconeogenic enzyme activity would be cardioprotective from pathologic insult or promote myocyte proliferation by

increasing the bioavailability of carbons for ancillary biosynthetic flux and through regulation of gene transcription. Similarly, we found that centromere protein F (*Cenpf*), a gene associated with chromosome segregation during mitosis,³³⁰ was found to be coordinately regulated by glycolysis. Interrogation of the metabolic regulation of such cell division genes in cardiomyocytes, thought to be terminally differentiated and relatively incapable of division and proliferation, may provide novel therapeutic approaches to regenerate the myocardium following injury.

In conclusion, we demonstrate a clear role of exercise-induced changes in metabolism in coordinating the growth response of the heart and the importance of metabolic flexibility in maintaining cardiac health. It is my hope that the knowledge derived from these studies will form the basis for the development of novel interventions to improve cardiac health (e.g., in patients with heart failure or diabetic cardiomyopathy).

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291. Mookerjee SA, Gerencser AA, Nicholls DG and Brand MD. Quantifying intracellular rates of glycolytic and oxidative ATP production and consumption using extracellular flux measurements. *J Biol Chem*. 2017;292:7189-7207.
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APPENDIX

The contents of Chapters 2, 3, and 4 are derived from published manuscripts. Copyright clearance from the publishers has been granted and the manuscripts have been properly cited by the inclusion of footnotes and proper citations within the reference section of this dissertation.

CURRICULUM VITAE

Andrew A. Gibb, M.S.
2733 Field Ave Apt 1
Louisville, KY 40206
Phone: 216-409-1353 (cell); 502-852-1029 (work)
Email: Andrew.gibb@louisville.edu

EDUCATION

06/2009 B.S. in Exercise Physiology, Ohio University, Athens, OH
05/2011 M.S. in Exercise Physiology, University of Louisville, Louisville, KY
12/2015 M.S. in Physiology, University of Louisville, Louisville, KY
12/2017 *Ph.D. in Physiology, University of Louisville, Louisville, KY*

ACADEMIC APPOINTMENTS

08/2009 – 05/2011 Graduate Teaching Assistant
 Department of Health and Sport Science
 Exercise Physiology Program
 University of Louisville
 Louisville, KY

08/2011 – 12/2012 Adjunct Instructor
 Department of Health and Sport Science
 Exercise Physiology Program
 University of Louisville
 Louisville, KY

08/2012 – present Doctoral Candidate
 Department of Physiology
 University of Louisville
 Louisville, KY

OTHER POSITION AND EMPLOYMENT

01/2010 – 09/2011 Clinical Exercise Physiologist – Kentucky One Healthcare,
 Cardiac Rehabilitation, Louisville, KY

09/2011 – 08/2012	Fitness Coordinator – Corporate Fitness Works, Humana Corporate Headquarters, Louisville KY
02/2016 – present	Research Associate - Department of Medicine, University of Louisville, Louisville, KY

PROFESSIONAL DEVELOPMENT

Nov 2014	Isotope Tracers in Metabolic Research: Principles and practices of kinetic analysis; Mouse Metabolic Phenotyping Center (MMPC) Consortium, Training Conference/Workshop. Nov. 10-14, Cleveland, OH.
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PROFESSIONAL MEMBERSHIPS AND ACTIVITIES

2008 – present	American College of Sports Medicine (ACSM), Student Member
2014 – present	American Heart Association (AHA), Student Member
2015 – present	American Physiological Society (APS), Student Member
2016 – present	American Association for the Advancement of Science (AAAS).

HONORS AND AWARDS

2005 – 2009	Gateway Scholarship, Ohio University
2009 – 2011	Graduate Assistantship, Department of Exercise Physiology, University of Louisville
2012 – 2014	Integrated Programs in Biomedical Sciences (IPIBS) Fellowship Award, University of Louisville
2016	NIKE, Inc. Loren G. Myhre Environmental and Exercise Physiology Predoctoral Research Award, EEP Section of the American Physiological Society (APS)
2016	Physiology Departmental Graduate Student Travel Award
2016 – present	American Heart Association Predoctoral Fellowship Award
2016 – 2019	Nominated for a 3-year sponsored membership to AAAS by the associate dean of graduate and postdoctoral studies, University of Louisville
2017	Abstract Travel Award, APS Physiological Bioenergetics Conference

SERVICE

2015 – 2017	School of Medicine Graduate Council Student Representative
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PUBLICATIONS

Peer-Reviewed Publications

1. Salabei JK, **Gibb AA**, Hill BG. Comprehensive measurement of respiratory activity in permeabilized cells using extracellular flux analysis. *Nat Protoc* 9(2):421-38, 2014.
2. Cummins TD, Holden CR, Sansbury BE, **Gibb AA**, Shah J, Zafar N, Tang Y, Hellmann J, Rai SN, Spite M, Bhatnagar A, Hill BG. Metabolic remodeling of white adipose tissue in obesity. *Am J Physiol Endocrinol Metab* 307(3):E262-77, 2014.
3. Salabei JK, Lorkiewicz PK, Mehra P, **Gibb AA**, Haberzettl P, Hong KU, Wei X, Zhang X, Li Q, Wysoczynski M, Bolli R, Bhatnagar A, Hill BG. Type 2 diabetes dysregulates glucose metabolism in cardiac progenitor cells. *J Biol Chem* 291:13634-48, 2016.
4. **Gibb AA**, Gilbert LA, Riggs DW, Conklin DJ, Bhatnagar A, Hill BG. FVB/NJ mice are a useful model for examining cardiometabolic adaptations to treadmill exercise. *Front Physiol* 7:636, 2016.
5. **Gibb AA**, Lorkiewicz PK, Zheng YT, Zhang X, Bhatnagar A, Jones SP, Hill BG. Integration of flux measurements to resolve changes in anabolic and catabolic metabolism in cardiac myocytes. *Biochem J*, 474: 2785-2801, 2017.
6. **Gibb AA**, Epstein PN, Uchida S, Zheng YT, McNally LA, Obal D, Katragadda K, Trainor P, Conklin DJ, Brittian KR, Tseng MT, Wang J, Jones SP, Bhatnagar A, Hill BG. Exercise-induced changes in glucose metabolism promote physiologic cardiac growth. *Circulation*, 2017. (In Press)

Manuscripts in Review

1. Osuma E, Riggs D, **Gibb AA**, Hill BG. High throughput measurement of metabolism in planarians reveals activation of glycolysis during regeneration. *Regeneration*, 2017.
2. Liu M, Ding C, Luo F, Albeituni S, Hu X, Hamada M, Takahashi S, **Gibb AA**, Kloecker G, Zhang H, Buosamra II M, Fan TW, Lane A, Hill BG, Yan J. Transcription factor c-Maf controls anti-tumor immunity by programming immunosuppressive macrophages in lung cancer. *Sci Transl Med*, 2017.

Manuscripts in Preparation

1. **Gibb AA** & Hill BG. Metabolic regulation of myocardial remodeling. (Invited Review, *Circ Res*)
2. Pena Claderin E, McNally L, **Gibb AA**, Hill BG, Hellmann J. A diet high in fat inhibits exercise-induced biosynthesis of pro-resolving lipid mediators. Manuscript in preparation.

INVITED TALKS

1. Center for Metabolic Origins of Disease
Sanford Burnham Prebys Medical Discovery Institute
Title: Metabolic regulation of cardiac adaptation to exercise.
Host: Doug Lewandowski, Ph.D. April, 2017.
2. Center for Translational Medicine
Lewis Katz School of Medicine, Temple University
Title: Metabolic regulation of cardiac adaptation to exercise.
Host: John Elrod, PhD. August, 2017.
3. Abstract Selection for Oral Presentation
Session Title: Symposium – Energy School II
Title: Phosphofructokinase coordinates mitochondrial and ancillary biosynthetic pathway activities in the cardiomyocyte
APS Bioenergetics, August, 2017

ABSTRACTS

1. Salabei JK, **Gibb AA**, Jones SP, Bhatnagar A, Hill BG. Regulation of the hyperproliferative vascular smooth muscle cell phenotype by mitochondrial fission. *Free Radic Biol Med* 65: S2, 2013.
2. Holden CR, Wysoczynski M, Sansbury B, Hellmann J, Zafar N, **Gibb A**, Bhatnagar A, and Hill BG. Nutrient excess promotes accumulation of bone marrow-derived progenitor cells in adipose tissue. *FASEB J* 28(S1): 641.12, 2014.
3. **Gibb AA**, Sansbury BE, DeMartino AM, Harbeson MA, Jones SP, Bhatnagar A, Hill BG. Regulation of glucose metabolism in the failing heart. *2nd Annual Cardiovascular Forum for Promoting Centers of Excellence and Young Investigators*, 2014.

4. **Gibb AA**, Sansbury BE, DeMartino AM, Harbeson MA, Jones SP, Bhatnagar A, Hill BG. Regulation of glucose metabolism in the failing heart. *Research Louisville*, 2014.
5. **Gibb AA**, Bhatnagar A, Hill BG. Role of cardiac glycolysis in adaptations to exercise. *Circ Res* 117: A387, 2015.
6. **Gibb AA**, Bhatnagar A, Hill BG. Myocardial glycolytic rate regulates exercise capacity and cardiac hypertrophy. *Southeast Regional IDeA Conference*, 2015.
7. **Gibb AA**, Gilbert LA, Brittian KR, Jones SP, Bhatnagar A, Hill BG. Myocardial glycolytic rate regulates exercise-induced physiologic cardiac growth. *FASEB J* 30(S1):1239.7, 2016.
8. **Gibb AA**, Gilbert LA, Lorkiewicz P, Trainor P, Tseng MT, Epstein PN, Bhatnagar A, Hill BG. Altered cardiomyocyte glycolysis causes cardiac hypertrophy and mitochondrial dysfunction. *Circulation* 134:A19448, 2016.
9. **Gibb AA**, Lorkiewicz PK, Zheng YT, Zhang X, Bhatnagar A, Jones SP, Hill BG. Phosphofructokinase coordinates mitochondrial and ancillary biosynthetic pathway activities in the cardiomyocyte. *ABP Bioenergetics*, 2017.
10. **Gibb AA**, Epstein PN, Uchida S, Zheng YT, McNally LA, Trainor P, Conklin DJ, Brittian KR, Tseng MT, Jones SP, Bhatnagar A, Hill BG. Metabolic regulation of exercise-induced cardiac growth. *APS Bioenergetics*, 2017.
11. Zheng YT, **Gibb AA**, McNally LA, Liu S, Merchant M, Hill BG. Multimeric metabolic enzyme complexes in the adult mammalian heart. *APS Bioenergetics*, 2017.

GRANTS AND CONTRACTS

16PRE31010022

Gibb (PI)

American Heart Association Predoctoral Fellowship, Great Rives Affiliate

Title: *Metabolic Regulation of Myocardial Adaptations to Exercise*

The goal of this project is to understand the role of metabolism, specifically glucose metabolism, in the adaptive responses associated with exercise training. \$52,000, 2016-2018.

Priority Score: 1.42; Application Percentile Rank: 0.33%.

Intramural Grant

College of Education Internal Funding Award (University of Louisville)

Equipment procurement for teaching aid technology (CleveMed Software)

Spring 2011

Direct Costs: \$5,000 – Role: Lead

INVITED JOURNAL REVIEWER

1. Toxicological Sciences (1 Review)

ASSISTED JOURNAL REVIEWER

1. Circulation Research (2 Reviews)
2. The American Journal of Physiology – Heart and Circulatory Physiology (2 Reviews)

ACADEMIC INSTRUCTION

Fall 2009	Instructor, University of Louisville, Louisville KY Weight Training HSS 109 Fitness Walking HSS 114
Spring 2010	Instructor, University of Louisville, Louisville KY Indoor Soccer HSS 150
Summer 2010	Instructor, University of Louisville, Louisville KY Outdoor Soccer HSS 136 Weight Training HSS 109
Fall 2010	Instructor, University of Louisville, Louisville KY Physical Fitness and Conditioning HSS 110 Fitness Walking HSS 114 Volleyball HSS 137
Spring 2011	Instructor, University of Louisville, Louisville KY Weight Training HSS 109-06 Weight Training HSS 109-07 Physical Fitness and Conditioning HSS 110-01 Physical Fitness and Conditioning HSS 110-02
Fall 2011	Adjunct Instructor, University of Louisville, Louisville KY Principles in Electrocardiography EXP 611 Mean course evaluation – 4.63/5.0
Fall 2012	Adjunct Instructor, University of Louisville, Louisville KY Principles in Electrocardiography EXP 611 Mean course evaluation – 4.88/5.0 ***Highest semester mean within the department