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Metabolic foundations of exercise-induced cardiac growth.

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METABOLIC FOUNDATIONS OF EXERCISE-INDUCED CARDIAC GROWTH

By

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B.S., Evangel University, 2013
M.S., Missouri State University, 2015
M.S., University of Louisville, 2020

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A Dissertation Approved on

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ABSTRACT

METABOLIC FOUNDATIONS OF EXERCISE-INDUCED CARDIAC GROWTH

Kyle Levi Fulghum

November 9, 2022

Regular aerobic exercise promotes physiological cardiac growth, which is an adaptive response thought to enable the heart to meet higher physical demands. Cardiac growth involves coordination of catabolic and anabolic activities to support ATP generation, macromolecule biosynthesis, and myocyte hypertrophy. Although previous studies suggest that exercise-induced reductions in cardiac glycolysis are critical for physiological myocyte hypertrophy, it remains unclear how exercise influences the many interlinked pathways of metabolism that support adaptive remodeling of the heart. In this thesis project, we tested the general hypothesis that aerobic exercise promotes physiological cardiac growth by coordinating myocardial metabolism to promote glucose-supported anabolic pathway activity. Because little is known about how cardiac mitochondria adapt to exercise, we first characterized exercise-induced changes in murine cardiac mitochondrial metabolism and found that treadmill exercise has minimal effects on respiration and does not influence ADP sensitivity in the isolated organelle (Chapter II). These findings indicate that increases in cardiac mitochondrial respiration during exercise likely occur via changes in mitochondrial substrate abundance or via allosteric regulation of metabolic enzymes. To better describe how exercise influences

cardiac metabolism *in vivo*, we examined changes in cardiac metabolite abundance via untargeted metabolomics. Although exercise altered metabolite abundances in female hearts more than male hearts, physiological cardiac growth was evident only in male hearts. Nevertheless, in both male and female hearts, exercise increased circulating and intracardiac ketone bodies and branched-chain amino acids (BCAAs). The idea that exercise-induced elevations in BCAAs are critical for exercise-induced cardiac growth is suggested by data showing that a diet deficient in BCAAs prevents cardiac growth following a treadmill exercise training program (Chapter III). We next standardized a noninvasive method for delivering $^{13}C_6$-labeled glucose to mice via liquid diet. Paired with resolution mass spectrometry, this method enables insight into relationships between anabolic and catabolic pathways in the heart. We found that low cardiac phosphofructokinase (PFK) activity, which occurs transiently during a bout of intense treadmill exercise, increases glycogen storage and promotes biosynthesis of 5-aminoimidazole-4-carboxyamide ribonucleotide (AICAR). *In vivo* stable isotope tracing paired with native protein complex separation suggest that elevated levels of AICAR that occur with low PFK activity occur via formation of a multimeric complex containing several metabolic enzymes that appear to promote metabolic channeling (Chapter IV). We then performed deep network tracing following various durations of exercise training and found that cardiac glucose oxidation, amino acid synthesis, Krebs cycle activity, and glycogen synthesis increase in the early phases of an exercise training program, but progressively return to levels observed in non-exercised hearts following 4 weeks of training (Chapter V). Collectively, the

findings of this thesis project provide a new working model of exercise-induced cardiac growth. Our data suggest that glucose-derived carbon is a major source of both energy and building material for the remodeling heart that integrates with BCAA metabolism to promote physiological cardiac growth.

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CHAPTER I

GENERAL INTRODUCTION

INTRODUCTION

Exercise promotes general metabolic wellness,[1-3] improves mental health,[4,5] builds and preserves musculoskeletal function,[6] and increases lifespan.[7-10] These beneficial effects of exercise are related, in part, to enhanced function and health of cardiovascular tissues as well as to increased resistance of the heart to injury.[11,12] The magnitude of risk reduction for cardiovascular disease and survival afforded by exercise parallels that of not smoking.[10,13] Moreover, exercise is a core component of cardiac rehabilitation regimens, and, in patients with heart disease, it reduces cardiovascular morbidity and mortality.[14-18] Nevertheless, the molecular mechanisms by which exercise improves cardiovascular health and prevents tissue injury remain unclear.

The recurrent deviations in whole body homeostasis caused by exercise drive adaptations in several organs, including brain, liver, adipose tissue, skeletal muscle, and, the topic of this dissertation—the heart.[6,19] The idea that metabolic perturbations are important for attaining exercise-induced health benefits is consistent with a paradigm suggested first by Galen (*c* 129–210 CE), who recognized that not all movement is exercise and that exercise is most beneficial when vigorous, with “the criterion for vigorousness [defined by a] change in respiration…those movements which do not alter respiration are not called

exercise".[20] Hence, with Galen, a definition of exercise and the overarching tenet that the salutary effects of exercise require significant deviations in metabolism first became apparent. Although several reviews cover the known mechanisms by which exercise regulates the health and adaptation of the heart and vasculature [e.g., [12,21-25]], we highlight in this introduction the knowledge of how cardiac metabolism changes with exercise as well as recent findings of how exercise-induced changes in metabolism may drive cardiac remodeling.

Cardiovascular Effects of Exercise

Cardiac adaptations associated with exercise were first documented in 1899. Physical examination using auscultation and percussion revealed that Nordic skiers[26] and university rowers[27] had increased cardiac dimensions. The latter study highlighted that "the period of greatest enlargement corresponded to the period of the most arduous work,"[27] which provided an early indication that relatively high workloads correspond with exercise-induced cardiac growth. Later studies using electrocardiography and chest radiography identified functional and structural cardiac changes caused by exercise.[28-31] Subsequent echocardiographic studies further described the degree and proportional features of the exercise-remodeled heart [reviewed in [32]]. Collectively, these studies laid the groundwork for understanding how repetitive bouts of exercise stimulate adaptive changes in the heart.

Acute cardiac responses to exercise: Increases in physical activity require changes in the distribution of oxygen and nutrients throughout the body. The increased work and ATP turnover of skeletal muscle[6] are facilitated by several integrated changes including physiological adjustments in ventilation and cardiac output as well as markedly decreased vascular resistance in skeletal muscle.[19] During aerobic exercise, changes in cardiac function occur immediately and are typically associated with several phases. Heart rate and stroke volume increase upon heightened levels of physical activity, and together they augment cardiac output in a relationship defined by the Fick equation.[32,33] After a prolonged period of moderate to high intensity aerobic exercise (e.g., >20 min), cardiac output is maintained; however, heart rate tends to increase further and stroke volume begins to drop due to cardiovascular drift, a phenomenon thought to be associated with vasodilation, hyperthermia, increased blood flow to the skin, decreased filling time, and decreased plasma volume.[34-37] Coordinated changes in vascular function combined with sustained augmentation of cardiac function integrate to increase blood flow to skeletal muscle, with cardiac output distribution to working muscle tracking with exercise intensity[38] (**Fig. 1**).

Whereas the cardiac responses to endurance exercise are directly associated with the use of oxygen for ATP production in skeletal muscle, resistance exercises are more anaerobic in nature. In addition, resistance exercise generally increases blood pressure, which is due in part to mechanical restriction of blood flow during static contraction. These features of resistance exercise result in markedly different cardiac responses as compared with aerobic exercise. The

Figure 1

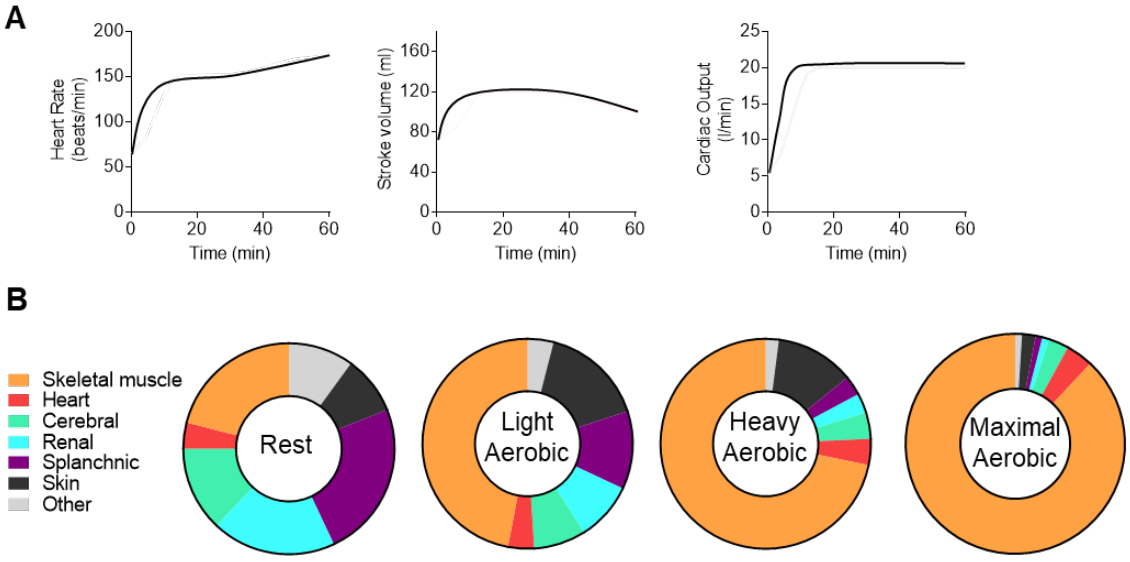


Figure 1: Exercise-mediated changes in cardiac function and in the tissue distribution of cardiac output. (**A**) Generalized schematic of cardiac responses to a moderate to intense, 1 h session of aerobic exercise. (**B**) Distribution of cardiac output at rest and with increasingly intense levels of exercise. Data are adapted from Plowman and Smith [38].

modest increase in cardiac output initiated by resistance exercise is predominantly due to increases in heart rate, with virtually no change in stroke volume.[39,40] A higher number of repetitions increases heart rate and thus leads to larger increases in cardiac output.[41] With heavy weightlifting, the heart must also deal with spikes in blood pressure, which can transiently reach levels of 320/250 mmHg[42] or higher. The degree to which blood pressure changes during resistance exercise appears to be a function of the degree of effort, muscle mass, and the breathing patterns commonly performed during strength training (i.e., the Valsalva maneuver).[41,43]

Chronic effects of exercise on the heart: Repetitive bouts of strenuous exercise can promote mild cardiac hypertrophy and/or chamber enlargement,[32,44,45] which is typically reversible upon prolonged cessation of exercise[46-48] (**Fig. 2**). The type and intensity of exercise determines the nature and degree of exercise-induced cardiac remodeling, with hemodynamic changes during exercise providing a stimulus for growth and chamber adaptation. Isometric or static exercises—commonly grouped as strength training (e.g., weightlifting, wrestling)—involve brief, intense periods of increased peripheral vascular resistance with little to no change in cardiac output and are associated with mild concentric hypertrophy and a normal to mildly enlarged left atrium. The increase in cardiac wall thickness appears largely caused by the parallel addition of sarcomeres within cardiomyocytes. In contrast, prolonged isotonic or dynamic aerobic exercise—generally termed endurance exercise (e.g., long distance running, cycling, rowing, or swimming)—requires sustained elevations in cardiac

Figure 2

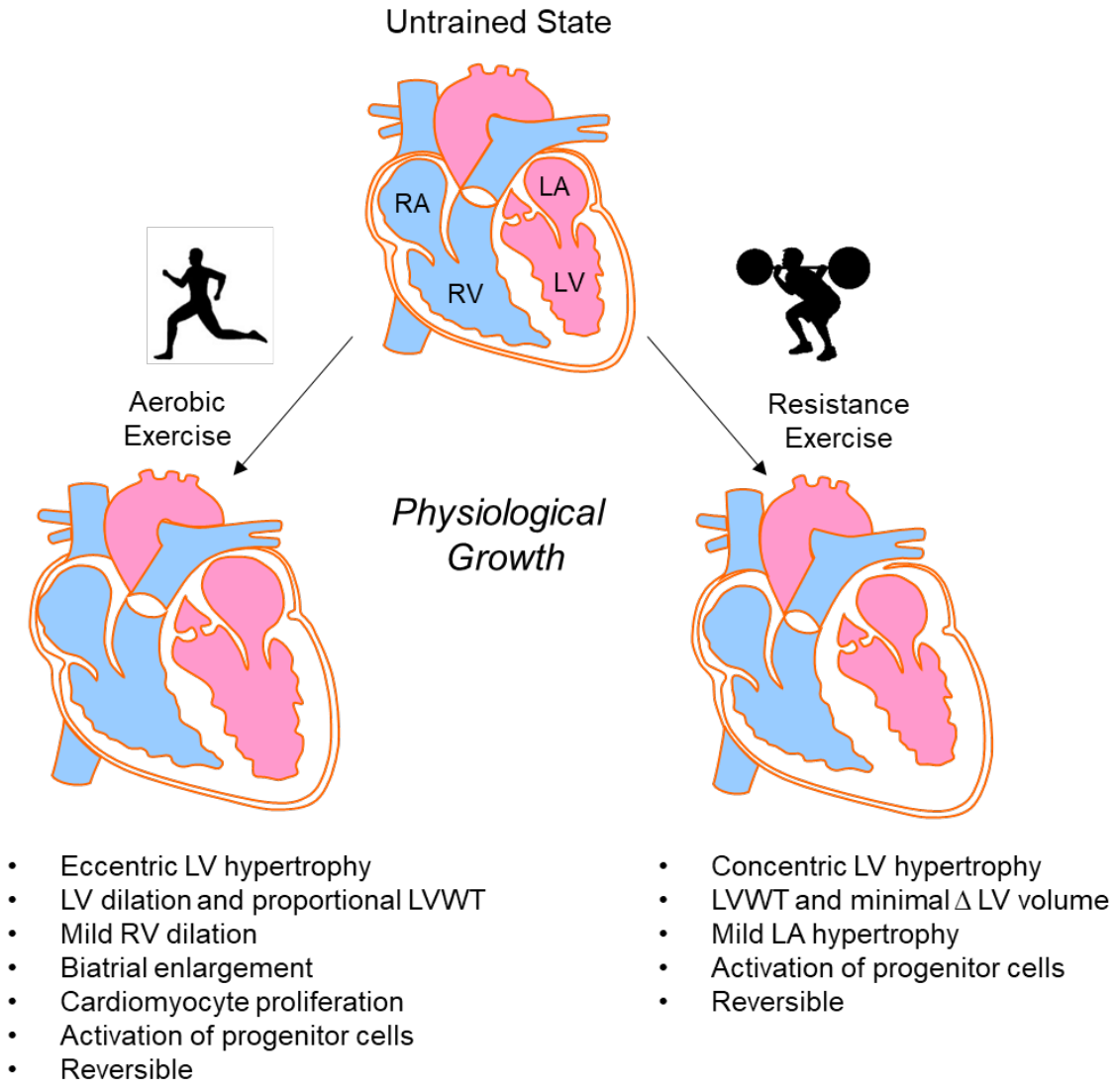


Figure 2: Exercise-induced cardiac growth. Aerobic and resistance exercise elicit different forms of physiological cardiac remodeling. Hypertrophic responses are primarily eccentric in nature for aerobic exercise and concentric in nature for resistance exercise. LA, left atrium; LV = left ventricle; LVWT, left ventricular wall thickness; RA, right atrium; RV, right ventricle.

output and is typically associated with normal or diminished peripheral vascular resistance. Endurance exercise promotes eccentric left ventricular hypertrophy, right ventricular dilation, and biatrial enlargement [[49,50] and reviewed in [32] and [51]]. Addition of cardiomyocyte sarcomeres in series predominates in this form of hypertrophy. Nevertheless, exercise-induced cardiac remodeling caused by endurance training has been suggested to be phasic in nature, with one study showing an initial concentric LV hypertrophy giving way to later eccentric LV hypertrophy[52] and another suggesting early increases in chamber size followed by later increases in wall thickness.[53]

Although regular, intensive endurance exercise can decrease resting and submaximal heart rates [e.g., [44,54]], the effects of exercise on other indices of cardiac function are less conspicuous. A meta-analysis of athletes participating in endurance, strength, or combined dynamic and static sports showed no major changes in systolic or diastolic function between sport type or when compared with control subjects.[55] However, several studies have identified changes in diastolic function in exercise-adapted subjects. For example, endurance exercise appears to enhance diastolic function modestly.[54,56-61] In contrast, strength training may diminish diastolic function, as evinced by studies showing impairment of LV relaxation in American football players.[61] In general, in the rested state, individuals that engage in regular exercise do not show remarkably different ejection fractions or fractional shortening values, compared to individuals who do not exercise, when measured by conventional echocardiography under resting conditions[54,62-65]; however, more subtle changes captured by tissue Doppler and speckle-tracking

echocardiography suggest modestly enhanced systolic function in exercise-adapted subjects.[66-68]

Cardiac remodeling in response to exercise appears to also involve processes beyond cardiomyocyte hypertrophy. For example, exercise increases levels of circulating progenitor cells[69-75] and cardiac-resident stem/progenitor cells,[76-79] which have been implicated in augmentation of vascular density and cardiac repair.[80-82] It appears that both resistance and endurance exercises activate progenitor cells [e.g., [83,84]] and that exercise duration and/or intensity are important in the amplitude and kinetics of their activation.[85-88] While the extent to which progenitor/stem cell subtypes regulate physiological cardiac growth remains unclear, their exercise-mediated activation is consistent with the angiogenesis and coronary vascular remodeling[25,89,90] and the improved responses to injury[91,92] associated with exercise-induced cardiac remodeling. In addition, exercise promotes modest cardiomyocyte proliferation,[78,93] which may be important for physiological cardiac adaptation and for understanding the mechanisms that trigger cardiomyogenesis in the adult, mammalian heart.

Potential deleterious effects of exercise on the heart: Although too little exercise is currently a much more serious health problem than too much exercise,[94] the popularity of intense exercise (e.g., ultramarathon, CrossFit) has increased remarkably over the past 30 years.[95-98] High levels of exercise can transiently increase the risk of acute cardiovascular events such as sudden cardiac death, and it can acutely diminish cardiac function, cause atrial fibrillation, trigger arrhythmias, and lead to pathological remodeling of the heart and vasculature

[reviewed in [95]]. Exercise may also change right ventricular morphology and function, contributing to arrhythmogenesis.[99] Although young individuals that die during exercise commonly bear inherited or conditional abnormalities such as hypertrophic cardiomyopathy,[100] older individuals more commonly die during exercise as a consequence of acute coronary thrombosis and myocardial infarction.[101] Nevertheless, sudden cardiac death during exercise is relatively rare and has been estimated to occur in 1 per 15,000–18,000 formerly asymptomatic adults per year.[102,103]

Prolonged endurance exercise can promote “cardiac fatigue,” characterized by decreased cardiac output and ejection fraction,[104,105] although changes in cardiac function typically recover within two days after exercise.[106] Acute decreases in cardiac function could be due to multiple factors including decreased sensitivity to catecholamines, blood volume redistribution leading to decreased venous return, and cardiomyocyte damage.[95] With respect to the last possibility, mild cardiac injury during intense exercise (e.g., marathons, triathlons) is suggested by elevated levels of circulating cardiac troponins [reviewed in [95]], which are typically used to diagnose acute myocardial infarction,[107] and exercise intensity is a strong predictor of elevated circulating cardiac troponin levels.[108] Other biochemical indicators of cardiac dysfunction, such as B-type natriuretic peptide (BNP) and its cleaved N-terminal fragment (NT-proBNP) may be elevated up to 10-fold after endurance exercise events, but typically return to baseline levels within a few days [reviewed in [95]]. It is hypothesized that exercise-induced BNP/NT-proBNP indicates mild myocardial injury[109] or may be a physiological

phenomenon important for cardiac adaptation.[110] Endurance exercise may also promote myocardial fibrosis and increase coronary artery calcification,[95] although the clinical significance of these effects in athletes remains unclear.

Effects of Exercise on Cardiac Metabolism

The heart has a high energy demand, which requires continuous ATP generation to sustain contractile function, ion homeostasis, anabolic processes, and signaling.[111-114] In normoxia, the heart fuels ATP turnover by generating >95% of its ATP from mitochondrial oxidative phosphorylation, with the remaining 5% derived from substrate level phosphorylation in glycolysis.[113,115] Although the majority of generated ATP supports contractile function, relatively large quantities of ATP are also necessary to maintain ionic homeostasis through ion pumps.[116,117] Below, we review some fundamental aspects of cardiac metabolism, followed by the acute metabolic changes in the heart caused by exercise (**Fig. 3**).

Some fundamental aspects of cardiac metabolism: Oxidation of fatty acids is the primary contributor to ATP production in the heart, with catabolism of lactate, glucose, ketones, and amino acids fulfilling the remaining energy demand.[118-120] This ability of the heart to use a myriad of substrates has led to classification of the heart as a metabolic "omnivore" capable of modulating substrate utilization in a manner dependent on numerous factors, including substrate availability, hormonal stimuli, and myocardial demand. Isotopic labeling studies in humans indicate that 84% of the FFAs entering myocytes are oxidized, with ~16% entering the triacylglycerol (TAG) pool[121]. This TAG pool may contribute to ~10% of cardiac

Figure 3

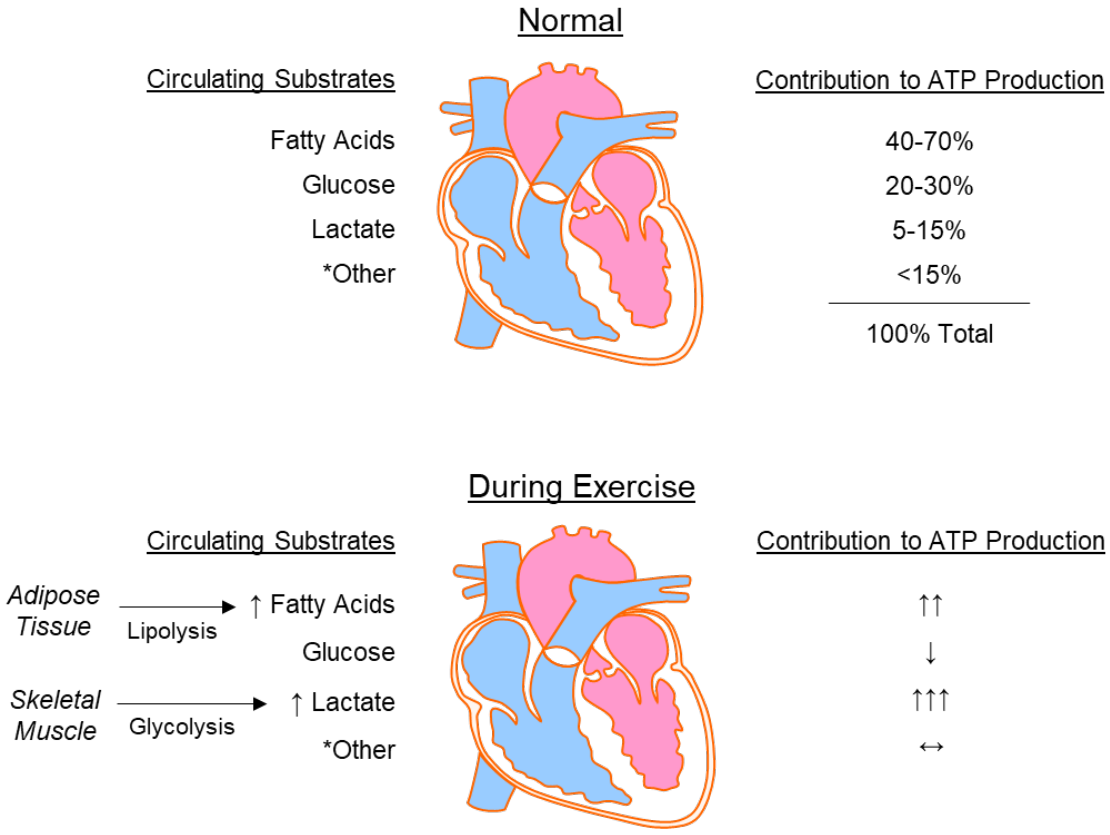


Figure 3: Cardiac metabolism at rest and during exercise. The heart uses numerous substrates for energy provision, with the predominant sources for ATP production being fatty acids, glucose, and lactate. During exercise, lipolysis in adipose tissue and glycolysis in working skeletal muscle increase the circulating levels of fatty acids and lactate, respectively, which are used by the heart to fuel increased energy demands. *Other = ketone bodies, pyruvate, acetate, and branched chain amino acids

ATP production[122,123] and also plays a central role in signaling and gene expression.[124] Although fat oxidation supplies 40–70% cardiac ATP,[125-131] it is also less efficient, which is due in part to fatty acid-induced uncoupling of oxidative phosphorylation.[123,132] The relative ATP yield of fats appears dependent on chain length, with long chain fatty acids yielding ~4 mol ATP/mol acetyl CoA and the shortest chain fatty acid, i.e., acetate, costing 2 mol of ATP/mol of acetyl CoA.[133] Acetate is usually low in circulation (i.e., below 0.2 mM) and is unlikely to contribute meaningfully to metabolism in the normal heart; however, high alcohol consumption can increase circulating acetate levels to low millimolar concentrations[134,135] and may under extreme circumstances contribute to cardiac energy deficits.[136-139]

Substrates such as glucose, lactate, and pyruvate are generally more efficient energy sources for the heart. In the normal mammalian heart, glucose metabolism via glycolysis supplies approximately 2–8% and glucose oxidation contributes up to 30% to the ATP yield.[125] Interestingly, carbon deriving from nearly half of the glucose extracted by the heart is allocated to ancillary pathways of glucose metabolism, which are important for energy storage (glycogen) or biosynthesis of cellular building blocks (e.g., nucleotides, phospholipids, amino acids).[140-145] Lactate is also a major fuel source for the heart, contributing up to 15% of ATP production.[125] Lactate tracer studies indicate that the heart is a net lactate consumer[140,146-148] and that only ~13% of glucose extracted by the heart is converted to lactate.[140] Moreover, arterial lactate concentration correlates positively with myocardial lactate uptake and oxidation.[141,149,150] In humans, lactate

is a significant contributor to cardiac ATP production,[141,146] and, in dogs, it can account for up to 87% of cardiac substrate oxidation.[151] In rat heart, high lactate levels contribute to nearly 40% of ATP production.[150] The myocardium can also use pyruvate readily when extracellular levels are in the millimolar range; however, circulating concentrations of pyruvate are typically less than 150 μM,[118] which make it an unlikely source of myocardial energy *in vivo*.

Ketone bodies such as acetoacetate and β-hydroxybutyrate have received recent attention due to their potential importance in heart failure[152-154]; however, should circulating ketone bodies become highly abundant, the normal heart would be expected to increase ketone body oxidation as well. Early studies showed that high concentrations of ketone bodies (e.g., 1–10 mM) can account for nearly 80% of cardiac oxygen consumption,[155] and that ketone body provision has a pronounced inhibitory effect on glucose[143,156,157] and fat catabolism.[158,159] Interestingly, when provided alone, ketone bodies appear to cause contractile failure[160-162]; however, their availability in the presence of other substrates such as glucose may increase efficiency of the working heart.[163] Such findings have advanced the idea that ketone bodies are a “superfuel” that enable efficient ATP production.[164,165] Although it has been suggested that 5–15% of ATP production in normal heart is via ketone body oxidation,[125] this would depend on the levels of circulating ketones, which in the healthy, fed state are typically less than 500 μM. Although it remains to be clarified whether constitutively high levels of ketone bodies or their oxidation are healthy for the heart,[166] ketone diets and ketone body

supplements may improve exercise performance and augment cardiac energy provision.[167,168]

Amino acids have a relatively minor role in ATP production in the heart; however, they are essential for processes such as protein synthesis and cell signaling. In particular, branched chain amino acids (BCAAs; comprising leucine, isoleucine, and valine) are major amino acids taken up by the heart, with uptake dependent primarily on circulating BCAA concentration.[169] Because they are essential amino acids, their intracellular levels are largely dependent on import, with the L-type amino acid transporters and bidirectional amino acid transporters likely contributing to their abundance in the heart.[170-172] BCAA catabolism contributes to less than 5% of myocardial oxygen consumption,[173] in part because the heart expresses relatively low levels the branched chain aminotransferase enzyme and the branched chain α-keto acid dehydrogenase complex.[174,175] Nevertheless, BCAAs are important regulators of mTOR, which coordinates anabolism and processes such as proliferation, survival, and autophagy.[176] Indeed, high intramyocardial levels of BCAAs are associated with cardiac hypertrophy and heart failure,[177-179] and recent findings indicate that intracellular accumulation of BCAAs, via a glucose-KLF15-BCAA degradation axis, is required for mTOR activation and cardiomyocyte hypertrophy.[180] High intracellular levels of BCAAs may negatively influence cardiac health by inhibiting mitochondrial metabolism.[179,181-183]

Glutamine, a “conditionally essential” amino acid, also appears to regulate the metabolism and health of the heart. In particular, it can activate mTOR in

cardiomyocytes,[184] and it can protect the heart from injury.[185-188] Although many proliferating cells use glutamine as an oxidative fuel,[189-191] the normal heart appears to produce glutamine by amidation of glutamate rather than oxidize it for energy provision.[169,192] Nevertheless, glutamine can augment myocardial oleate oxidation and triglyceride formation[193] as well as activate the hexosamine biosynthetic pathway (HBP).[194,195]

Cardiac intermediary metabolism in exercise: An acute increase in workload during exercise has robust effects on the metabolism of striated muscle.[196] In the heart, exercise increases contractile power and oxygen consumption up to 10-fold above resting rates.[24,123] Changes in substrate utilization and ATP production during exercise are a product of the integrated effects of physiologic cues that occur with changes in circulating hormones, metabolic substrates, and hemodynamics.

An increase in myocardial workload is accompanied by increases in the catabolism of multiple substrates, in particular, fatty acids and lactate.[141,149,197-200] During exercise, hormone-activated lipolysis in adipose tissue increases circulating FFA to levels up to 2.4 mM,[201] which enhances FFA uptake and utilization.[121,147,202] However, heightened levels of circulating FFAs are only partially responsible for increasing fatty acid oxidation because higher cardiac workloads appear sufficient to increase fat oxidation in the heart.[203] Cardiac TAG utilization rates also increase considerably with exercise[198] and appear to be further stimulated by lactate availability, suggesting that lactate may stimulate TAG turnover.[204] Furthermore, after exercise adaptation, genes responsible for fatty

acid transport and catabolism are elevated, which may help optimize fat utilization in the heart.[205-207]

Similar to free fatty acids, plasma lactate levels increase during exercise. The increase in lactate is dependent on the type of exercise, with intense exercise (e.g., 60–80% of VO_2max) resulting in large increases in arterial lactate levels.[208] During intense exercise, circulating lactate levels can increase 5–10-fold (to nearly 10 mM), which is primarily due to lactate extrusion by skeletal muscle. Under these conditions, the contribution of lactate to total oxidative metabolism may account for 60–90% of substrate utilization.[149,151,209,210] Although low to moderate intensity exercise (e.g., 40% of VO_2 max) does not increase circulating lactate levels remarkably,[141] the contribution of lactate oxidation to overall myocardial oxidative metabolism is higher than that compared with the sedentary state.[141] Lactate may also enhance fat oxidation in the heart,[199] which would increase the capacity of the heart to generate ATP under high workloads.

Although circulating levels of glucose are fairly stable compared with levels of lactate and FFAs, weightlifting and prolonged endurance exercise can decrease arterial glucose concentrations,[201,211] whereas high-intensity aerobic exercise may increase blood glucose levels.[197] Hemodynamic changes and increases in local and circulating catecholamines can increase the oxidation of stored glucose (glycogen).[212] Although moderate intensity exercise and increases in cardiac workload have been associated with elevations in myocardial glucose uptake and oxidation,[141,197,199,200] elevations in circulating concentrations of competing substrates such as lactate and FFAs may decrease glucose catabolism.[197-199,213]

Moreover, studies in both humans and animal models suggest that exercise can lower oxygen extraction ratios for glucose and decrease glucose uptake and utilization.[198,213] Recent findings suggest that relatively prolonged, intense endurance exercise can decrease glucose catabolism in the heart by diminishing the activity of phosphofructokinase.[214,215] Collectively, these findings suggest that exercise can acutely increase or decrease both circulating glucose levels and myocardial use in a manner dependent on the type, intensity, or duration of exercise.

Regular exercise also promotes metabolic remodeling in the heart. Perfused mouse heart studies suggest that adaptation to exercise increases the rates of basal glycolysis,[214] glucose oxidation, and fat oxidation[216]; however, compared with hearts from sedentary controls, basal cardiac glycolysis may be diminished in exercise-adapted rats, despite increases in myocardial glucose and palmitate oxidation.[91] That exercise-induced changes in cardiac metabolic remodeling are dependent on exercise intensity is suggested by studies in mice in which a moderate-intensity treadmill regimen showed no effect on basal glucose oxidation, palmitate oxidation, or myocardial oxygen consumption; yet, a high-intensity, interval-style regimen increased glucose oxidation, diminished palmitate oxidation, and led to a net decrease in resting myocardial oxygen consumption.[217] The reasons for discrepancies between studies could be due to model-specific factors (e.g., rodent strain, type of exercise) or differences in cardiac perfusion protocols (e.g., substrate levels, addition of hormones). Circadian rhythm may also account for disparate findings because it influences cardiac metabolism,[218] stress

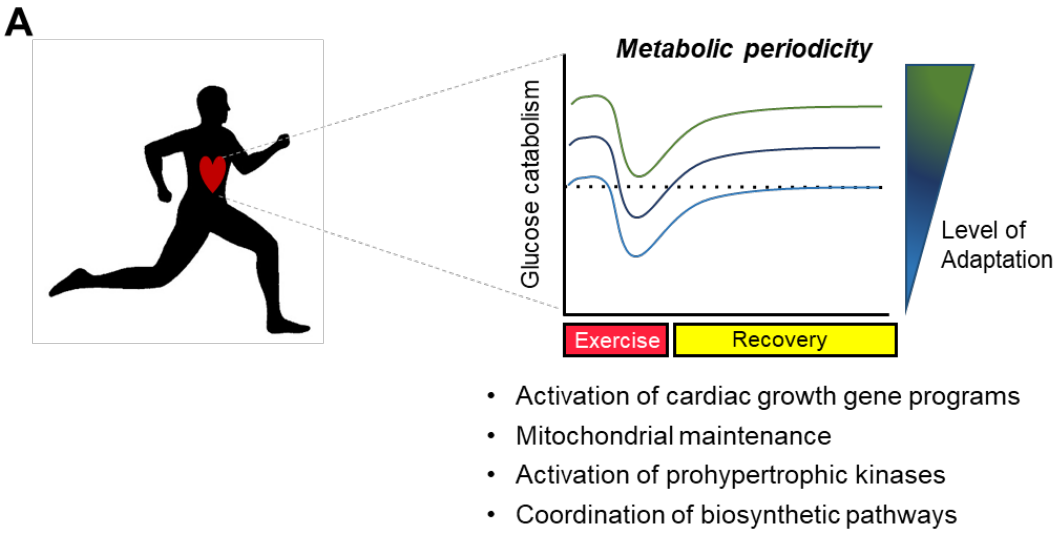
responses and protein turnover,[219] and inflammatory processes.[220] Chronobiology remains an important consideration for understanding how exercise influences cardiac biochemistry and physiology.[221,222]

Metabolic Mechanisms of Exercise-Induced Cardiac Growth

Understanding how changes in metabolism regulate cardiac adaptation to exercise presents a challenge. Metabolic pathways coordinate not only ATP production and biosynthesis, but also modulate cell signaling and redox state.[118] Nevertheless, it is clear that repetitive bouts of exercise elicit changes in metabolism that are important for coordinating gene expression in other tissues such as skeletal muscle.[6] The mechanisms by which exercise-induced metabolic changes may promote cardiac adaptation are reviewed below and are summarized in **Fig. 4**.

Importance of metabolic periodicity in cardiac adaptation: Although episodic changes in metabolism that occur with exercise play an important role in skeletal muscle adaptation,[6] relatively less is known about how exercise-induced metabolic periodicity affects adaptive responses in the heart. Nevertheless, it is clear that periodic bouts of exercise stimulate metabolic processes in both cardiac mitochondria and the cytosol. For example, in mice, exercise acutely promotes fission of cardiac mitochondria, which enhances mitochondrial function; these mitochondrial changes were shown to occur in a manner dependent on adrenergic

Figure 4


A
Metabolic periodicity
Glucose catabolism
Level of Adaptation
Exercise
Recovery
• Activation of cardiac growth gene programs
• Mitochondrial maintenance
• Activation of prohypertrophic kinases
• Coordination of biosynthetic pathways

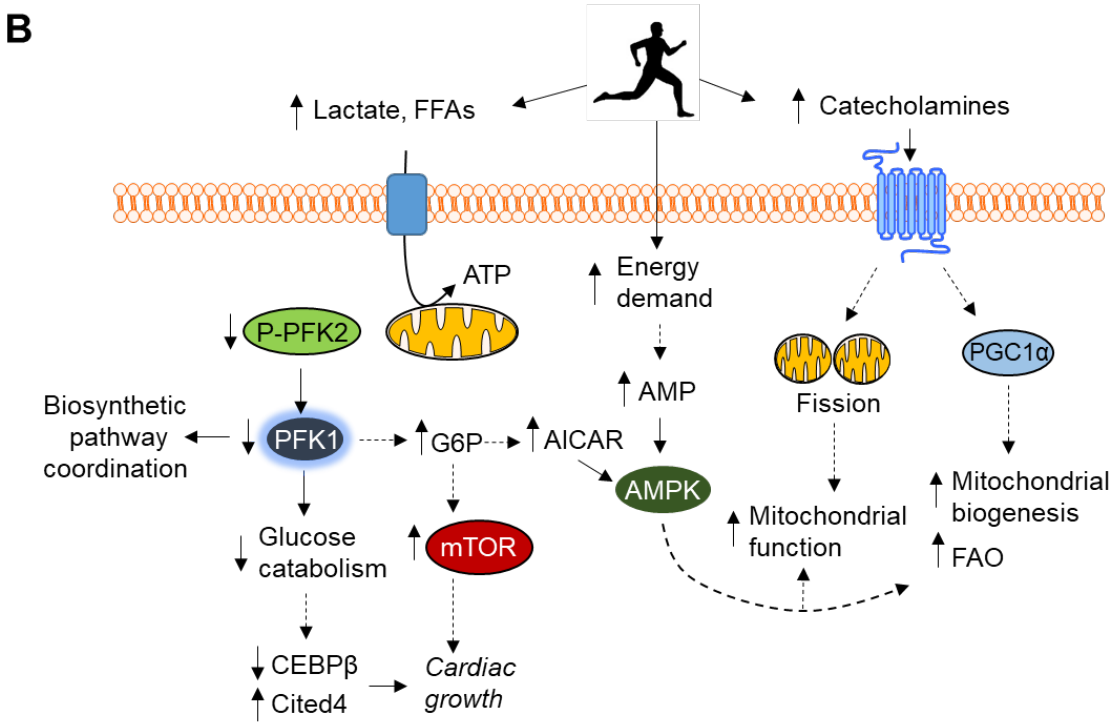

B
Lactate, FFAs
Catecholamines
ATP
Energy demand
P-PFK2
AMP
Fission
PGC1α
Biosynthetic pathway coordination
PFK1
G6P
AICAR
AMPK
Mitochondrial biogenesis
Mitochondrial function
FAO
Glucose catabolism
mTOR
CEBPβ
Cited4
Cardiac growth

Figure 4: Working model of the metabolic mechanisms of exercise-induced cardiac growth. (**A**) Periodic changes in glucose metabolism and mitochondrial activity (i.e., metabolic periodicity) occurring with regular exercise promote activation of gene programs responsible for cardiac growth, regulate mitochondrial quality control and function, activate prohypertrophic kinases, and coordinate biosynthetic pathways, all of which integrate to promote cardiac growth. (**B**) Exercise increases levels of circulating cardiac substrates and catecholamines, which orchestrate changes in cardiomyocyte metabolism. Decreases in the phosphorylation of phosphofructokinase 2 (PFK2) lower phosphofructokinase 1 (PFK1) activity, which decreases glucose catabolism, coordinates ancillary biosynthetic pathways, and increases the levels of upstream glycolytic intermediates (e.g., glucose 6-phosphate, G6P) as well as increases products in the pentose phosphate pathway (e.g., AICAR). Decreases in PFK activity and glucose catabolism appear sufficient to decrease expression of CEBPβ and upregulate Cited4, which promote cardiac growth. In addition, elevated levels of G6P, AMP, and AICAR could activate the prohypertrophic signaling kinase mTOR and AMPK. Catecholamine-triggered signaling cascades promote mitochondrial fission and upregulate PGC1α, which acutely increase mitochondrial function and chronically elevate mitochondrial abundance and fatty acid oxidation (FAO) capacity.

Signaling.[223] A relatively intense bout of exercise also decreases the activity of phosphofructokinase in mouse heart[214]; however, upon adaptation to the exercise regimen and in the rested state (i.e., 24 h after the last exercise bout), apparent myocardial phosphofructokinase activity and glycolytic rate were found to be higher compared with sedentary controls.[214] The acute, exercise-induced decreases in myocardial glycolytic rate appear important for cardiac growth because low phosphofructokinase activity brought forth by expression of a cardiac-specific, kinase-deficient 6-phosphofructokinase/fructose-2,6-bisphosphatase transgene in mice ($Glyco^{Lo}$ mice) appears sufficient to partially phenocopy the exercise-adapted heart and regulate genes [e.g., *Cebpb*, *Cited4* [224,225]] required for exercise-induced cardiac growth.[214] Moreover, activation of the exercise gene program in $Glyco^{Lo}$ mice occurred in the absence of Akt activation, which is thought to be required for regulating physiologic cardiac growth.[21,22,45] These findings suggest that exercise-induced decreases in glycolysis are a proximal regulator of the cardiac growth program. Collectively, these findings indicate that exercise induces metabolic periodicity in the mitochondrial and cytosolic compartments, which regulate exercise capacity and myocardial growth.

It is likely that periodicity in mitochondrial fission and in intermediary metabolism are interconnected phenomena. In other cell systems, mitochondrial fission is important for regulating glucose and lipid metabolism.[226,227] Moreover, mitochondrial fission is important for regulating mitochondrial quality control by facilitating distribution of mitochondrial components to daughter organelles and by culling defective mitochondria via autophagy,[228-230] which is increased the heart

during and early after a bout of exercise.[231,232] Exercise-induced periodicity in glucose metabolism appears important for maintaining mitochondrial health because loss of periodicity, either by constitutively increasing or decreasing glucose catabolism, leads to mitochondrial dysfunction.[214] Nevertheless, some mechanisms underlying mitochondrial adaptations to exercise appear to diverge from those required for cardiac growth,[216,233] which suggest the presence of distinct circuits by which metabolic changes activate the exercise gene program versus how they modulate mitochondrial health.

Metabolic changes as a material cause of adaptation: Insight gleaned from bacteria suggest that cells coordinate growth and function via interconversion of glycolytic metabolites to biomass,[234] which highlights the obvious role of metabolism as a material cause for structural maintenance and modification. It is likely that changes in ancillary biosynthetic pathway activity are also important for coupling the synthesis of structural materials to the activation of cardiac gene programs responsible for exercise-induced cardiac adaptation.

Rate-limiting steps of glycolysis, e.g., the hexokinase, phosphofructokinase and pyruvate kinase steps, are likely important for modulating biosynthetic pathways in the heart.[118] These enzymes are regulated at multiple levels, with allosterism being important for acute changes in activity.[235] In several cell types, the phosphofructokinase step of glycolysis regulates the pentose phosphate pathway (PPP), which is important for nucleotide synthesis and redox regulation.[236-239] Modeling studies in the adult heart demonstrate that phosphofructokinase activity is particularly important for modulating the activities

of the PPP and the polyol pathway.[240] In cardiac myocytes, phosphofructokinase activity modulates several ancillary biosynthetic pathways, such as the PPP, the HBP, and the glycerophospholipid synthesis pathway (GLP) by directly modulating glucose carbon entry into the pathways and by indirectly regulating mitochondria-derived molecules important for building block synthesis (e.g., aspartate).[145] Furthermore, metabolomic studies indicate that phosphofructokinase activity also regulates the abundance of several amino acid and lipid metabolites in the heart.[214] Much less is known about how exercise affects the hexokinase and pyruvate kinase steps of glycolysis; however, pyruvate kinase activity has been shown to be elevated in the exercise-adapted rat [241] and dog [242] heart.

There is relatively little direct knowledge of how other biosynthetic pathways change with exercise. Transient changes in readouts of HBP activity, i.e., UDP-N-acetylhexosamines or O-GlcNAcylated proteins, occur with exercise.[243-246] Changes in the HBP appear important because it may regulate the function and survival of cardiomyocytes[247,248] as well as reparative cardiac cells.[249] To our knowledge, nothing is known regarding how the PPP, GLP, and SBP are influenced in the heart by exercise. While the PPP and the GLP would regulate redox state, nucleotide biosynthesis, and phospholipid biosynthesis, the SBP modulates the levels of methyl donors required for DNA methylation reactions and could represent a critical link between metabolism, epigenetic programming, and changes in cardiac structure and function.[250]

Signaling pathways influencing cardiac adaptation: Several signaling pathways integrate to modulate cardiac metabolism and adaptive responses to

exercise. Exercise-mediated increases in catecholamines promote upregulation of peroxisome proliferator-activated receptor γ coactivator 1 α (PGC1α) via β-adrenergic signaling and activation of endothelial nitric oxide synthase [reviewed in [21]]. The actions of PGC1α may be mediated via activation of nuclear receptors such as peroxisome proliferator activated receptor α (PPARα) and estrogen-related receptor (ERR) as well as nuclear receptor factor 1 (NRF1), which are known to integrate to increase fatty acid oxidation and to promote mitochondrial biogenesis. Moreover, the metabolic, structural, and functional cardiac changes that occur with exercise are influenced by receptor signaling triggered by insulin-like growth factor-1 (IGF-1)[251,252] and neuregulin-1,[78,253] which activate the phosphoinositide 3-kinase (PI3K)/Akt pathway to promote physiologic cardiac growth[254-257] or activate a cardiomyocyte proliferative response.[78,258-260] Interestingly, cardiac glucose metabolism is influenced by catecholamines,[130,261,262] IGF-1,[263-265] and Nrg-1,[265] which suggests that these hormones may provide additional regulation to acute or chronic metabolic changes induced by exercise.

Metabolite signaling: Metabolite signaling is another mechanism that connects exercise-induced changes in metabolism to cardiac adaptation. In particular, glucose-derived metabolites regulate the activities of the prohypertrophic kinases mammalian target of rapamycin (mTOR) and AMP-activated kinase (AMPK).[45] The intracellular levels of glucose 6-phosphate (G6P) regulate mTOR activity in the heart,[266-268] and 5-amino-4-imidazolecarboxamide ribonucleotide (AICAR), which is an intermediate of the PPP,[269] stimulates

AMPK.[270] It is anticipated that G6P, AMP, and AICAR increase in the heart with exercise. Predictions from crossover theorem[271-274] and modeling studies[240] suggest that acute decreases in phosphofructokinase activity, such as occurs during exercise,[118] would increase G6P as well as augment PPP activity, which could increase AICAR levels. In addition, the large increase in myocardial ATP demand would be thought to increase intracellular AMP levels.

Circulating metabolites are also important regulators of exercise-induced cardiac growth. Hormone-mediated adipose tissue lipolysis during exercise liberates palmitoleate (C16:1n7), which promotes cardiac growth potentially by activating G-protein-coupled receptors (GPCRs), Akt, or nuclear receptors.[275] The cardiac growth-stimulating effect of palmitoleate is similar to the fatty acid-induced cardiac hypertrophy that occurs in the python heart after a large meal.[276] Interestingly, FFAs not only increase acutely with exercise,[201] but they appear to remain elevated in the exercise-adapted state as well[277,278]; hence, they could stimulate the signaling required to sustain cardiac adaptations. Given that numerous metabolites have cognate GPCRs,[279] it is likely that other metabolites elevated during or after exercise have important roles to play in tissue adaptation. Understanding how circulating metabolites trigger structural and functional changes in the heart could lead to the development of novel therapies to improve cardiac health.

Current Limitations and Gaps in Knowledge

Metabolic changes caused by exercise are important for cardiac remodeling and adaptation. The integrative metabolic changes brought forth by exercise combine with changes in cardiac workload to regulate cardiac metabolism. In particular, exercise alters levels of competing substrates, and it changes the abundance of circulating hormones, which cue metabolic pathways that are critical for transcriptional changes and cardiac growth. In addition, changes in circulating and endogenous metabolites can trigger physiologic growth by activating prohypertrophic signaling pathways. Nevertheless, numerous questions remain, including questions of how to optimize the amount of exercise in males and females to produce beneficial, as opposed to deleterious, effects on cardiovascular health[280] as well as mechanistic questions of how exercise-induced changes in metabolism couple the synthesis of structural materials to activation of the physiological cardiac growth program.[118] While this knowledge is acquired, it appears that we would be best served by sticking to the advice of the ancient Greeks—"Exercise till the mind feels delight in reposing from the fatigue." – Socrates.

This chapter previously appeared as an article in the journal entitled Frontiers in Cardiovascular Medicine and has been adapted to fit this dissertation. The original citation is as follows: Fulghum K and Hill BG. Metabolic Mechanisms of Exercise-Induced Cardiac Remodeling. *Front Cardiovasc Med* 5:127. 2018.

Dissertation Hypothesis and Specific Aims

Aerobic exercise dynamically regulates cardiac substrate availability and utilization, and exercise training specifically influences cardiac glucose metabolism by modulating phosphofructokinase (PFK) activity.[214] Because PFK has been shown to impact biosynthetic pathway activity in cardiomyocytes,[281] we hypothesize the following:

Hypothesis: Aerobic exercise promotes physiological growth of the heart by regulating cardiac phosphofructokinase activity and coordinating anabolic pathway activity in the heart. To test this hypothesis, we will:

Aim 1: Characterize metabolic changes in the heart in response to exercise.

Aim 2: Determine the control exerted by cardiac phosphofructokinase activity on amphibolic metabolite abundances and ancillary biosynthetic pathway activity in the heart.

Aim 3: Confirm the necessity of exercise-induced changes in substrate availability in promoting physiological growth of the heart.

The data obtained from these studies will provide fundamental knowledge and mechanistic insights regarding the influence of exercise training on cardiac metabolism and physiological growth. Furthermore, these studies could identify actionable targets for the optimization of cardiovascular benefits associated with exercise, the promotion of physiological remodeling in the heart, and the prevention of deleterious remodeling in the heart.

CHAPTER II

AEROBIC EXERCISE DOES NOT INFLUENCE CARDIAC MITOCHONDRIAL RESPIRATION OR ADP SENSITIVITY IN THE ISOLATED ORGANELLE

INTRODUCTION

Aerobic exercise training improves cardiovascular function, with the physiological response largely dependent upon training frequency and duration.[282-284] Some studies have shown that exercise training leads to adaptations that enhance mitochondrial respiration in the heart[216,285,286]; however, other studies suggest unchanged or diminished cardiac mitochondrial respiration.[287-289] Because of these conflicting data, the cardiac mitochondrial response to exercise remains uncertain. When considering mitochondrial function, it is still unclear which events contribute to metabolic control[290] and substrate utilization,[291,292] and how each could be influenced by exercise training.

Exercise increases circulating levels of fatty acids and lactate, which are readily taken up by the heart to maintain ATP synthesis. Lactate levels increase most remarkably during exercise, with blood levels reaching 5–10 mM.[293] During exercise, higher circulating levels of lactate, derived from skeletal muscle glycolysis, provide the heart with increased levels of oxidizable substrate.[6,291,294] Higher blood lactate levels augment its utilization by the heart and diminish myocardial glucose catabolism.[150,214,291] Nevertheless, the mechanisms by which

lactate and other substrates regulate cardiac energetics and function remain unclear.

Lactate oxidation is thought to occur via cytosolic lactate dehydrogenase (LDH), which converts lactate to pyruvate in an NAD^+-dependent manner; the pyruvate can then be oxidized in mitochondria. Interestingly, several studies suggest that LDH exists within mitochondria,[295-298] yet other studies indicate that LDH is not within mitochondria and that the contribution of mitochondrial LDH (mLDH) to energetics is negligible.[299-301] Intramitochondrial LDH could be advantageous to the heart, especially during exercise, because the additional NADH generated in the LDH reaction would be directly available for respiration, without the need for the malate-aspartate shuttle system to transport reducing equivalents across the inner mitochondrial membrane.

In addition to substrate oxidation, mitochondrial respiration might also be limited by ADP/ATP transport through the adenine nucleotide translocase (ANT) in the inner mitochondrial membrane.[302] Higher sensitivity of the ANT to ADP could increase ATP synthesis, thereby providing more useable energy for muscular work. While substrate delivery and electron capacity have been well-studied, cardiac mitochondrial ADP sensitivity is not well-understood in response to exercise training. Previous studies suggest that $ANT^{-\backslash-}$ mice exhibit severe exercise intolerance,[303] but how exercise influences ADP sensitivity in the heart has not been well-studied.

In this chapter, we hypothesized that the duration of exercise training is positively correlated with mitochondrial respiration on common substrates in

circulation and that the positive correlation is mediated by enhanced sensitivity of ADP import through ANT channels. Since lactate levels increase remarkably during intense exercise, we also wanted to highlight the effects of exercise training on mitochondrial lactate oxidation, determine whether mLDH exists in the heart, and delineate whether exercise influences LDH localization or intramitochondrial lactate oxidation.

We found that exercise training does not significantly influence cardiac mitochondrial respiration or ADP sensitivity within the isolated organelle. Furthermore, cardiac mitochondria from mice do not contain LDH and exercise does not influence mLDH abundance. Lastly, our results indicate that cytosolic LDH is the primary vehicle for cardiac lactate oxidation.

EXPERIMENTAL METHODS

Experimental animals: All procedures were approved by the Institutional Animal Care and Use Committee at the University of Louisville. Adult, male and female FVB/NJ mice (15–20 weeks of age) on a 12 h:12 h light: dark cycle were used in all experiments. Chow and water were provided *ad libitum*. Upon completion of each experiment, mice were anesthetized with sodium pentobarbital (150 mg/kg, i.p.) prior to tissue harvest.

Exercise capacity testing and treadmill training: Exercise familiarization, capacity testing, and training were performed as described previously[293] for up to four weeks. We acclimated the mice to treadmill running for two days before the first

exercise capacity test (ECT). For all exercise studies, mice were exercised at 75% of their initial exercise capacity, at a 10° incline for 40 min (Week 1, 5 d/wk), 50 min (Week 2, 5 d/wk), and 60 min (Week 3–4, 5 d/wk). On the final day of training, mice ran a second ECT and were immediately euthanized alongside sedentary controls.

To test the effects of exercise training on mitochondrial lactate oxidation, we performed additional ECTs at the end of each week and continued the training regimen until work determined during the ECT increased by at least an average of 1.5-fold above pretraining values, then we trained the mice one additional week prior to euthanasia. Work was calculated as the product of body weight (kg) and vertical distance (m), where vertical distance = distance run (m) × sin θ (θ = inclination angle).[293,304] To examine the acute effects of exercise on mitochondrial lactate oxidation, mice were either subjected to an ECT or mice ran at a 10° incline at 10 m/min for the first 10 min, then the speed was adjusted to 19.5 m/min for the next 40 min. In this protocol, we confirmed compliance if >90% of the 19.5 m/min portion of the exercise bout was completed (i.e. >36 min at 19.5 m/min). We euthanized mice immediately following the exercise protocol and excised cardiac and skeletal muscle tissue for mitochondrial isolation and extracellular flux analyses.

Circulating glucose and lactate measurements: Blood samples for circulating glucose and lactate were obtained via tail clip and measured using the Accu-Check Aviva meter (Roche) and the Lactate Plus meter (Nova Biomedical), respectively.

Mitochondrial Isolation: Hearts and/or gastrocnemius muscles from sedentary and exercised mice were isolated and homogenized in 1 ml of isolation buffer (Buffer A: 220 mM mannitol, 70 mM sucrose, 5 mM MOPS, 1 mM EGTA, 0.2% fatty acid-free BSA, pH 7.2) using a Potter Elvehjem tube and a Teflon pestle. The homogenate was centrifuged at 800*g* for 10 min at 4°C. The supernatant was then centrifuged at 10,000*g* for 15 min at 4°C to obtain the mitochondrial fraction. The mitochondrial pellets were washed twice in 500 µL of isolation buffer and then resuspended in 400 µL of respiration buffer (120 mM KCl, 25 mM sucrose, 10 mM HEPES, 1 mM $MgCl_2$, 5 mM KH_2PO_4, pH 7.2) for extracellular flux analysis and biochemical assays. Protein concentration was assessed using the Lowry DC Protein Assay kit (Biorad).

Extracellular Flux Analysis: For acute, two-week, and four-week exercise-trained mice, we measured respiration using a Seahorse XF96e analyzer (Agilent). Briefly, 2.5 µg cardiac mitochondrial protein from each group were suspended in 20 µl respiration buffer and loaded into a 96-well XF culture plate. The plate was centrifuged for 3 min at 500*g* and 4°C, then 160 µl of warm (37°C) respiration buffer was added just before loading the plate into the XF96e analyzer. State 3 respiration was stimulated using the following substrate combinations: 5 mM pyruvate + 2.5 mM malate + 1 mM ADP, 5 mM succinate + 1 µM rotenone + 1 mM ADP, 5 mM glutamate + 2.5 mM malate + 1 mM ADP, or 100 µM octanoyl-carnitine + 2.5 mM malate + 1 mM ADP. State 4 respiration was then measured by addition

of 1 μM oligomycin and non-mitochondrial respiration was measured using 10 μM antimycin A + 1 μM rotenone. For ADP sensitivity studies, we measured state 2 respiration with 5 mM pyruvate + 2.5 mM malate and measured oxygen consumption rates (OCR). Then, state 3 respiration was stimulated by the addition of variable concentrations of ADP (2.5 μM–5.0 mM). Finally, 1.0 μM oligomycin was provided to mitochondria to measure state 4 respiration.

To test mitochondrial lactate oxidation, respiration was assessed using a Seahorse XF24 analyzer (Agilent), as described previously.[214,305,306] Briefly, 10 μg of mitochondrial protein was suspended in 50 μL of respiration buffer and loaded into 24-well XF culture microplates. The microplates were centrifuged at 500*g* for 3 min at 4°C, followed by addition of 625 μL of warm (37°C) respiration buffer. We used the following substrates to stimulate state 3 respiration: for cardiac mitochondria, we provided 5 mM pyruvate, 2.5 mM malate, and 1 mM ADP; for skeletal muscle mitochondria, we provided 5 mM glutamate, 2.5 mM malate, and 1 mM ADP; and for both cardiac and skeletal muscle mitochondria, we provided 5 mM lactate, 2.5 mM malate, and 1 mM ADP. We next provided 1 mM NAD^+ (final concentration), which enabled understanding of how LDH localized to the intermembrane space or outer mitochondrial membrane might contribute to mitochondrial energetics.[307] The rate of oxygen consumption after delivery of oligomycin (4 μM oligomycin; final concentration) was used to calculate state 4 respiration rates. Last, 10 μM antimycin A was added to ensure that all oxygen consumption was due to mitochondrial respiration.

Protease Protection Assay: To examine mitochondrial LDH localization, freshly isolated mitochondria (100 µg cardiac mitochondrial protein) were suspended in Buffer A in the presence or absence of 0.25 mg/ml trypsin (Becton, Dickinson and Company 215240). Mitochondria solubilized with 1% (v/v) Triton X-100 in the presence of 0.25 mg/ml trypsin served as an additional control. Reaction volumes (100 µl) were incubated at room temperature for 15 min, and the reaction was stopped by addition 2 µl of 100× protease inhibitor cocktail (Sigma P8340). We then collected mitochondria by centrifugation at 10,000*g* for 10 min at 4°C. The supernatants were discarded from non-solubilized treatment groups. The mitochondrial pellets were then solubilized in Buffer A containing 1% Triton X-100 and 2% SDS (v/v). Protein amount was determined via Lowry DC Protein Assay Kit (BioRad) and samples were prepared for protein separation via SDS-PAGE.

Immunoblotting: Mitochondrial proteins were separated by SDS-PAGE (12% resolving gel) and transferred to PVDF membranes. After blocking in 5% milk, the membranes were incubated with antibodies against LDHB (1:2,000, Abcam), GAPDH with HRP-conjugated (1:4000, Cell Signaling), ALDH2 (1:4,000, Invitrogen), or HSP60 (1:2000, Cell Signaling) overnight at 4˚C. After incubation with HRP-linked secondary antibodies (anti-mouse, 1:2,500, Cell Signaling; anti-rabbit, 1:2,500, Cell Signaling; and anti-goat, 1:2,500, Invitrogen), the membranes were developed using Pierce™ ECL Western Blotting Substrate (ThermoFisher). Immunoreactive proteins were imaged using a BioRad™ ChemiDoc™ MP Imager.

Statistical Analyses: Data are mean ± S.D. Statistical analyses were performed using two-tailed, paired or unpaired Student's t-tests, two-way or three-way ANOVA, where appropriate. Linear mixed-effects models were conducted to estimate associations between lactate and distance. These models used the difference between pre-ECT and post-ECT lactate levels (Δ lactate) as the outcome. An interaction term was included to test whether this association was modified by weeks of training. The null hypothesis was rejected if $p<0.05$. All statistical analyses were performed using SAS, version 9.4 (SAS Institute, Inc., Cary, North Carolina) and GraphPad Prism, version 7 (GraphPad Software, La Jolla, California).

RESULTS

Aerobic exercise training leads to cardiovascular adaptation. Compared with previously untrained mice (Acute Exe), two weeks of exercise training significantly increased distance run to exhaustion in both male and female mice. Mice that exercised for 4 weeks showed no further improvement in exercise capacity or heart weight than mice exercised for 2 weeks (**Fig. 5A**). Consistent with previous studies, we observed a significant increase in heart weight of male mice exercised for 2 and 4 weeks; however, we did not observe changes in heart weight of female mice (**Fig. 5B**). Circulating lactate levels were robustly elevated at the conclusion

Figure 5

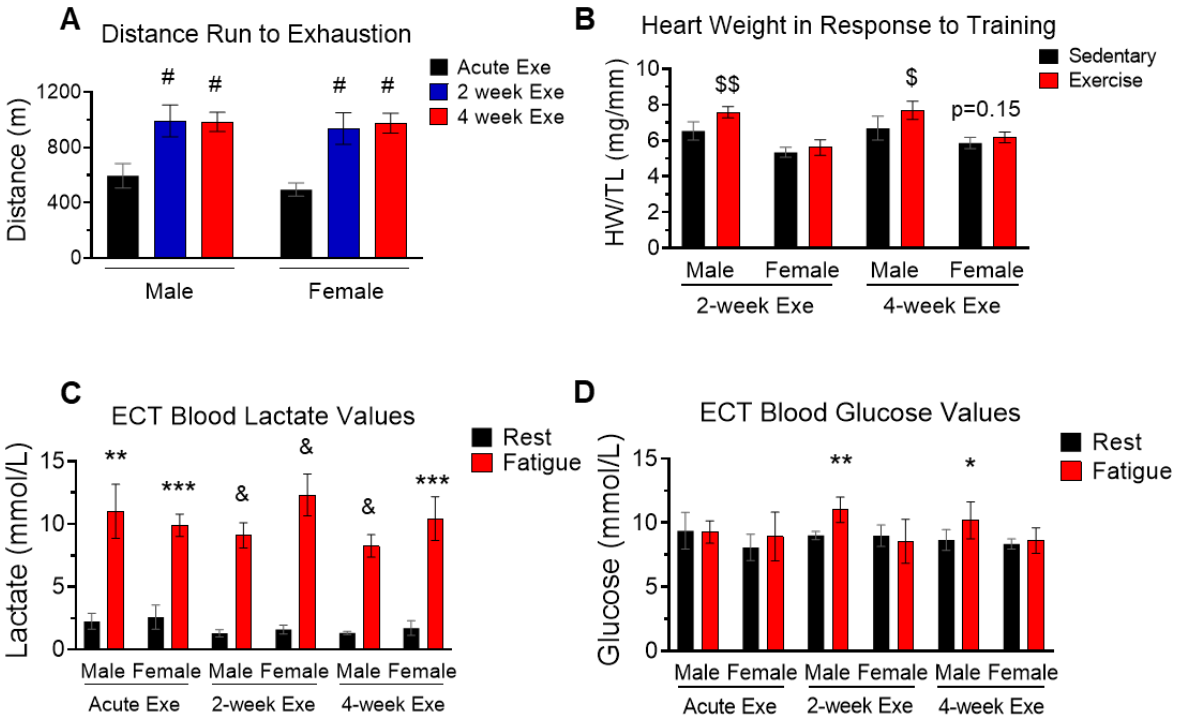


Figure 5: Cardiovascular adaptations to exercise training. Effect of training duration on endurance run to exhaustion (**A**), heart mass (**B**), circulating lactate (**C**), and circulating blood glucose (**D**). *n*=5/gp, #p<0.05, Kruskal-Wallis test with Dunn's correction vs acute exercise; $p<0.05, $$p<0.01, Mann-Whitney test sedentary vs exe; *p<0.05, **p<0.01, ***p<0.001, &p<0.0001, paired t-test rest vs fatigue. ECT = exercise capacity test, HW/TL = heart weight normalized to tibia length

of each ECT, confirming that mice ran to exhaustion (**Fig. 5C**). Blood glucose levels, also measured at the conclusion of each ECT, were slightly higher in male mice exercised for 2 and 4 weeks, but were not different in female mice (**Fig. 5D**). Collectively, these data suggest that in FVB/NJ mice, 2 weeks of exercise training could be sufficient to provide maximal increases in exercise capacity and that female FVB/NJ mice might have a different response to exercise training than male mice.

Exercise training elicits cardiac growth and shifts associations between lactate and running distance: Following two weeks of exercise training, the mice in our lactate groups met our *a priori* criteria for exercise adaptation, i.e., at least 50% increase in work during exercise capacity testing. Mice were then exercised one additional week before euthanasia. Blood lactate levels increased significantly after each exercise capacity test, indicating that the mice ran to exhaustion (**Fig. 6A**). Initial exercise capacity tests in untrained mice suggested a negative relationship (β = -0.015) between running distance and circulating blood lactate levels at fatigue. One week of training led to a minimally positive relationship (β = +0.004) and two weeks of training led to a positive relationship between lactate and distance (β = +0.010) (**Fig. 6B**). The distance × week interaction term showed that the relationship between lactate and distance was modified by the duration of training (p = 0.015). Similar to previous findings,[214,293] exercise-adapted mice showed a 15% increase in heart weight to body weight ratio (**Fig. 6C**). There were no differences in body weight after completion of the training regimen (**Fig. 6D**). These

Figure 6

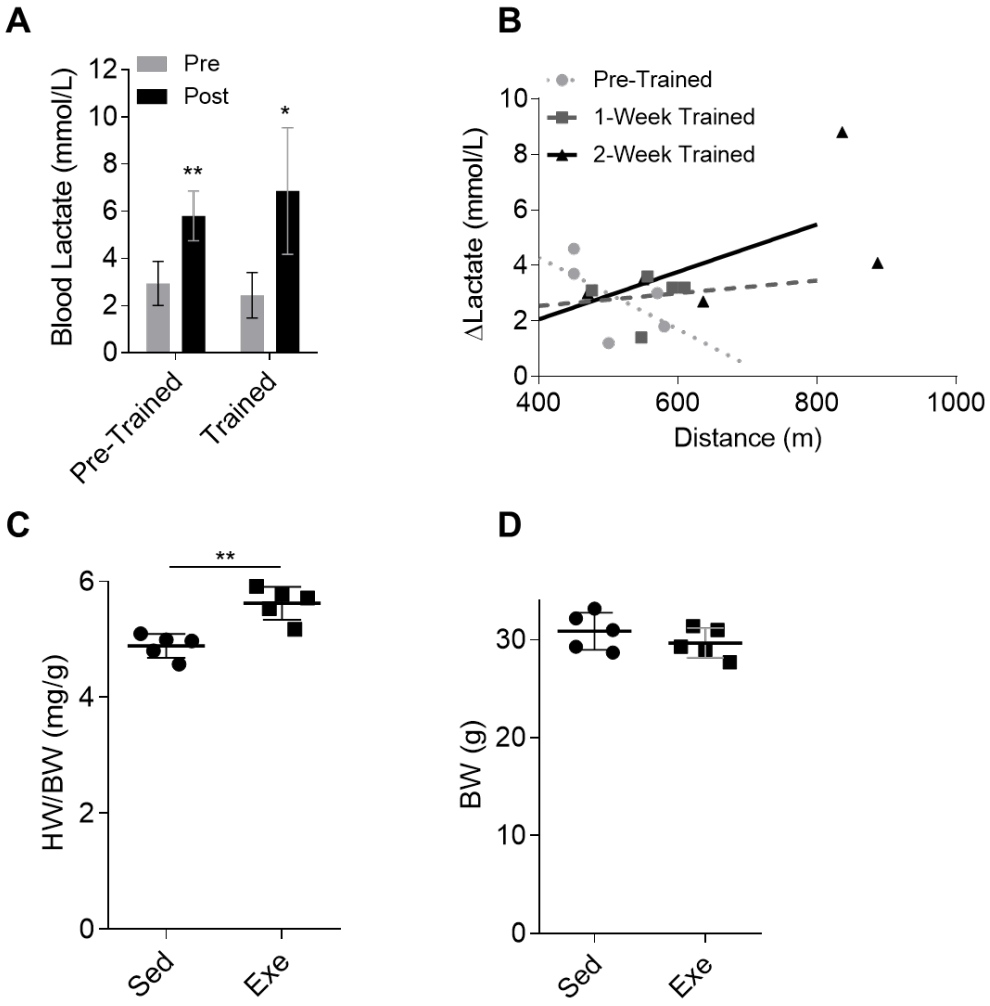


Figure 6: Altered lactate handling in exercise-induced cardiac growth. (**A**) Change in blood lactate level before (Pre) and after (Post) exercise capacity tests in pre-trained mice and in mice subjected to treadmill training for two weeks; (**B**) Linear regression models comparing the distance run and pre-vs. post-lactate levels (ΔLactate) measured during each exercise capacity test; (**C**) Myocardial growth as indicated by heart weight (HW) normalized to body weight (BW) in sedentary (Sed) and exercise-trained (Exe) mice; and (**D**) BW in sedentary and exercise-trained mice. Statistical tests included: paired *t*-test (panel A), linear mixed effects models (panel B) and unpaired t-tests (panels C and D). n = 5 per group, *p < 0.05, **p < 0.01.

findings could suggest adaptations that improve the ability of organs to use lactate as an energy source.

Aerobic exercise training has minimal effects on cardiac mitochondrial respiration. Following the acute, 2-week, and 4-week exercise regimens, we isolated cardiac mitochondria and examined respiration under conditions of saturating substrate and ADP concentrations. We found little evidence of mitochondrial biogenesis; mitochondrial yield was not different between the groups (**Fig. 7A**). State 3 respiration supported by glutamate (**Fig. 7B**), pyruvate (**Fig. 7C**), succinate (**Fig. 7D**), and octanoylcarnitine (**Fig. 7E**) was largely unchanged by exercise training, with the exception of a small, but significant increase in octanoylcarnitine-supported respiration in acutely exercised female mice (**Fig. 7E**). However, when further investigated, there were no differences in p/t ACC (**Fig. 7F**), which suggests that fatty-acid transport was not a reason for enhanced oxidation in acutely exercised mice. These data indicate that aerobic exercise does not significantly affect respiration in isolated cardiac mitochondria provided with saturating concentrations of substrate and ADP.

Aerobic exercise has minimal effects on cardiac mitochondrial ADP sensitivity. Although several studies in the literature have addressed cardiac mitochondrial respiration in response to exercise,[216,285,308] whether ADP sensitivity is affected by exercise has not been addressed. To test whether ADP sensitivity is altered by

Figure 7

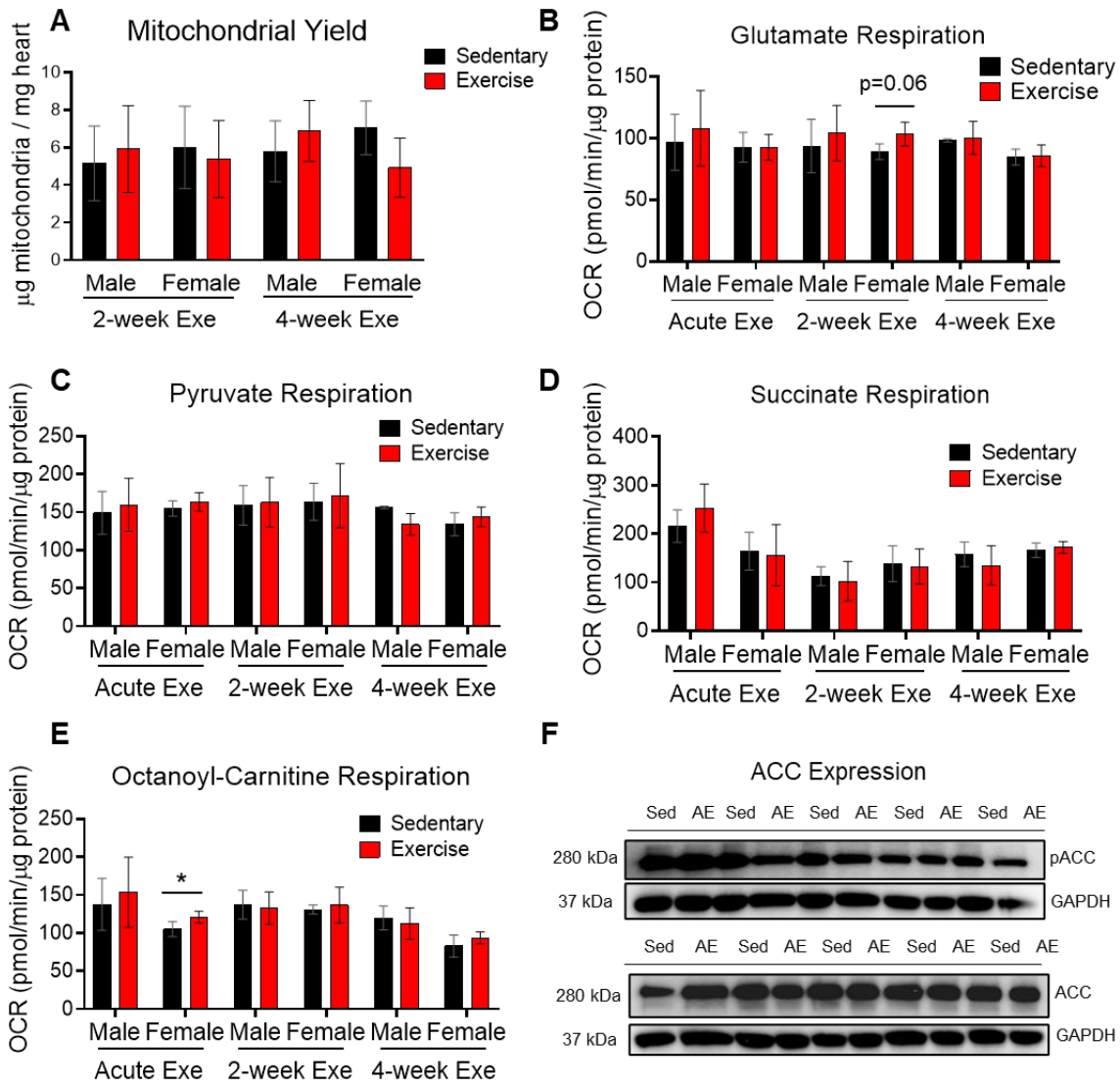


Figure 7: Exercise training does not influence cardiac mitochondrial yield or respiration in the isolated organelle. Training-induced effects on crude mitochondrial yield (**A**) as well as state 3 cardiac mitochondrial respiration on glutamate (**B**), pyruvate (**C**), succinate (**D**), and octanoyl-carnitine (**E**). Immunoblot of p/t ACC in sedentary (Sed) and acutely exercised (AE) female mice (**F**). *n*=5/gp, *p<0.05 two-way ANOVA with Tukey's post-hoc test.

exercise, we measured mitochondrial respiration using pyruvate as a substrate and performed the measurements in the presence of different ADP concentrations. Results from pilot studies using 1–10,000 μM ADP concentrations demonstrate that ADP-dependent increases in respiration in our respirometry system become apparent at ~50 μM ADP and saturate at ~500 μM ADP (**Fig. 8A**). Therefore, to determine how exercise affects ADP sensitivity, we used a concentration range of 25–500 μM. Overall, exercise training did not significantly affect ADP sensitivity in exercised mice. Although we found a significant difference in ADP sensitivity between male and female mice in the acute exercise group (**Fig. 8B**), these findings were not recapitulated in the 2-week and 4-week exercise groups or their corresponding sedentary controls (**Figs. 8C-D**). To further investigate a potential sex-dependent effect on ADP sensitivity, we performed an additional ADP titration experiment in sedentary male and female mice but found no significant differences in cardiac mitochondrial ADP sensitivity (**Fig. 8E**). Overall, these results suggest that exercise is unlikely to affect ADP sensitivity in isolated cardiac mitochondria.

Lactate does not drive substantial respiration in isolated mitochondria: Because previous studies suggest that mitochondria may harbor LDH,[295-297] which could provide an energetic advantage to the heart when circulating lactate levels are high, we next assessed whether isolated cardiac mitochondria from sedentary or exercise-adapted mice respire on lactate. As shown in **Fig. 9A**, mitochondria isolated from hearts of sedentary and exercise-adapted mice showed >20-fold higher state 3 respiration when provided with pyruvate and malate as a substrate

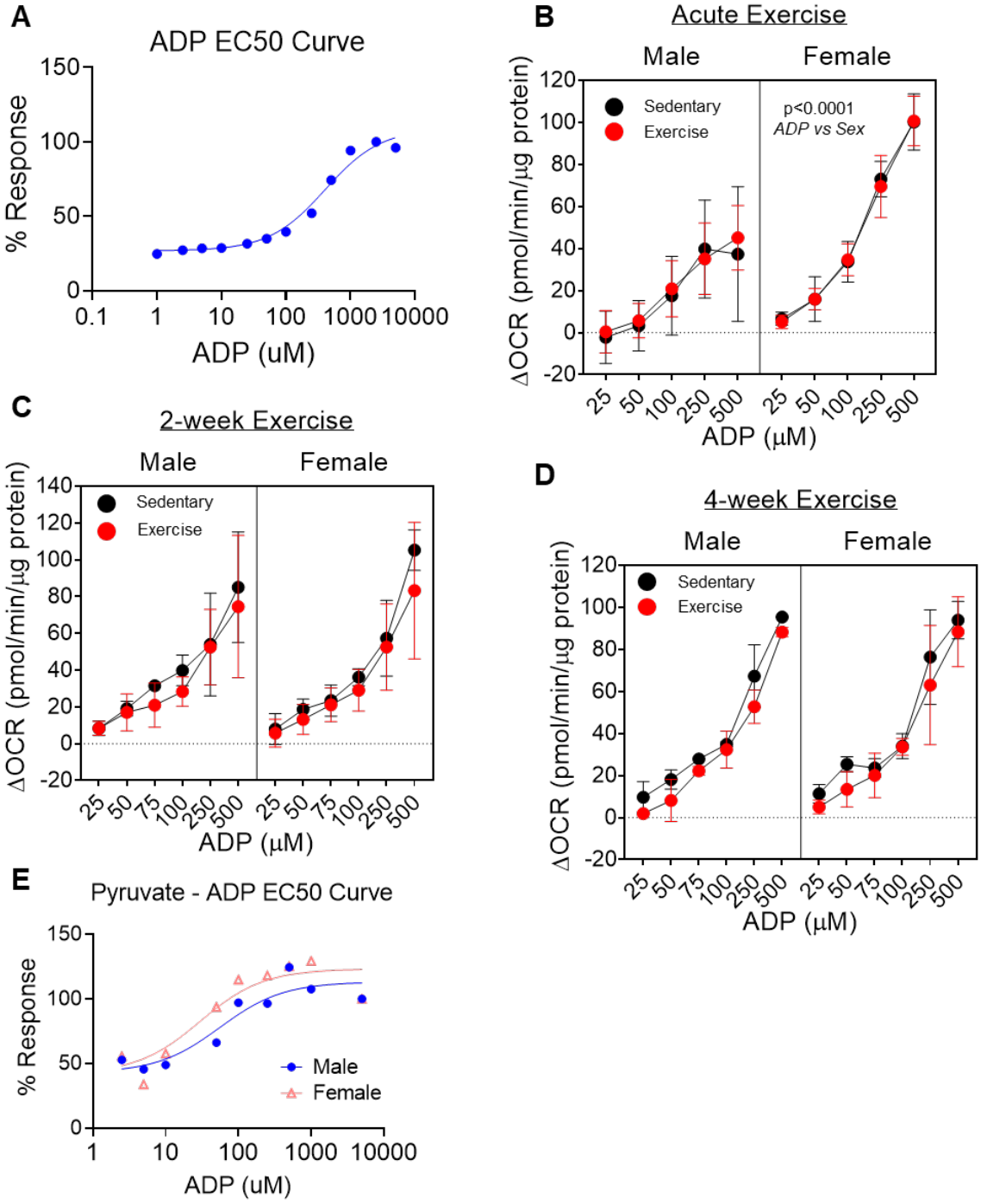


Figure 8: ADP Sensitivity in response to exercise training. ADP titration curve in isolated cardiac mitochondria (**A**). Effect of training duration on ADP sensitivity in hearts of male and female mice exercised acutely (**B**), for two weeks (**C**), and for four weeks (**D**). ADP titration curve in mitochondria isolated from hearts of male and female FVB/NJ mice respiring on pyruvate (**E**). ADP sensitivity curves three-way ANOVA. Panels A *n*=1, panels B-D *n*=5/gp, panel E *n*=3/gp.

Figure 9

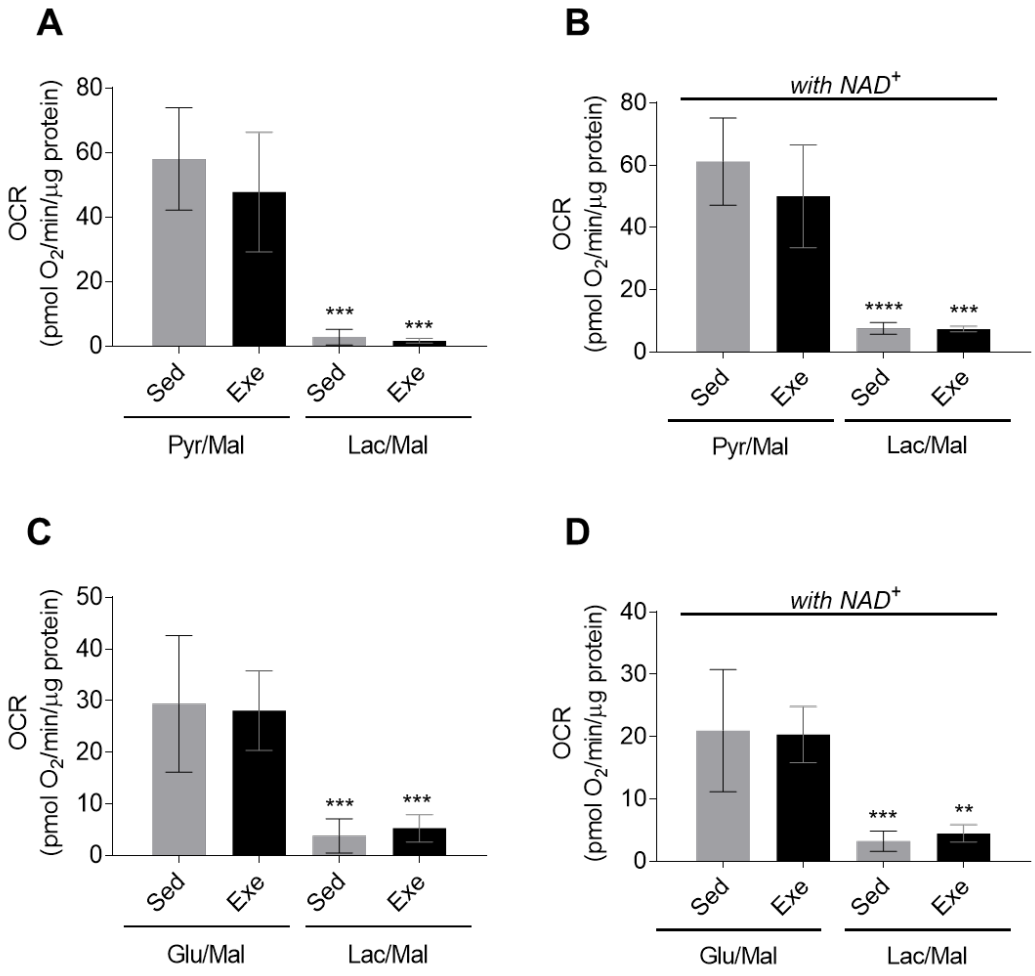


Figure 9: Lactate contributes minimally to respiration in isolated mitochondria from striated muscle. State 3 respiration of mitochondria isolated from sedentary (Sed) and exercise-adapted (Exe) mice: (**A**) Oxygen consumption rate (OCR) of cardiac mitochondria provided with 5 mM pyruvate, 2.5 mM malate, and 1 mM ADP (Pyr/Mal) or 5 mM lactate, 2.5 mM malate, and 1 mM ADP (Lac/Mal); (**B**) OCR of cardiac mitochondria provided with Pyr/Mal or Lac/Mal in the presence of 1 mM NAD^+; (**C**) OCR of skeletal muscle mitochondria provided with Pyr/Mal or Lac/Mal; and (**D**) OCR of skeletal muscle mitochondria provided with Pyr/Mal or Lac/Mal in the presence of 1 mM NAD^+. The statistical test used was two-way ANOVA with Tukey's test for multiple comparisons. n = 5 per group, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs. corresponding Sed or Exe group.

compared with lactate and malate. Because studies suggest that mLDH is in the intermembrane space of mitochondria and thus requires extramitochondrial NAD^+ for activity,[307] we also tested whether NAD^+ would stimulate lactate oxidation to appreciable levels. As shown in **Fig. 9B**, NAD^+ did not provide mitochondria with a significant ability to respire on lactate. Similar results were obtained for skeletal muscle mitochondria, which lacked the ability to respire on lactate compared with typical substrates that support Complex I-driven respiration (i.e., glutamate+malate) in the absence (**Fig. 9C**) or presence (**Fig. 9D**) of exogenous NAD^+. Exercise did not significantly affect cardiac or skeletal muscle mitochondrial respiration.

Acute exercise does not influence mitochondrial lactate utilization: Because a recent study indicated that exercise acutely augments respiratory capacity of cardiac mitochondria,[223] we next tested whether an acute bout of exercise affects lactate oxidation in isolated mitochondria. For this, we subjected mice to an intense bout (60 min) of treadmill running and immediately isolated mitochondria for respirometry. Mitochondria supplied with pyruvate-driven respiration showed a trend toward increased state 3 respiration in acutely exercised mice compared with sedentary mice); however, this did not reach statistical significance ($p=0.136$). Lactate did not drive appreciable levels of respiration in either the sedentary or acutely exercised groups (**Fig. 10A**), and exogenous NAD^+ did not affect respiration (**Fig. 10B**). Skeletal muscle mitochondrial respiration was not affected

Figure 10

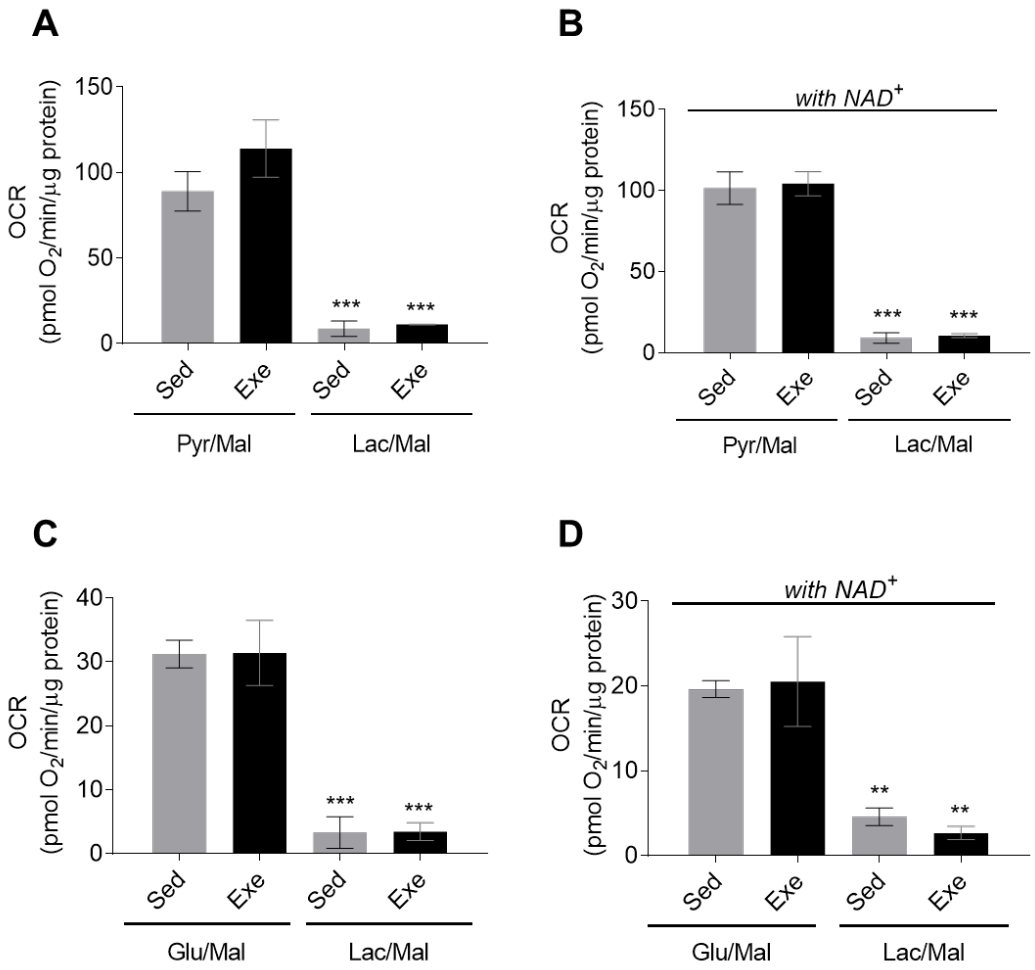


Figure 10: Acute exercise does not promote mitochondrial lactate utilization. State 3 respiration in mitochondria isolated immediately after one bout of exercise (Exe) or from sedentary (Sed) mice: (**A**) Oxygen consumption rate (OCR) of cardiac mitochondria provided with 5 mM pyruvate, 2.5 mM malate, and 1 mM ADP (Pyr/Mal) or 5 mM lactate, 2.5 mM malate, and 1 mM ADP (Lac/Mal); (**B**) OCR of cardiac mitochondria provided with Pyr/Mal or Lac/Mal in the presence of 1 mM NAD^+; (**C**) OCR of skeletal muscle mitochondria provided with Pyr/Mal or Lac/Mal; and (**D**) OCR of skeletal muscle mitochondria provided with Pyr/Mal or Lac/Mal in the presence of 1 mM NAD^+. The statistical test used was two-way ANOVA with Tukey's test for multiple comparisons. n = 3 per group, **p < 0.01, ***p < 0.001 vs. corresponding Sed or Exe group.

by acute bouts of exercise, in the absence (**Fig. 10C**) or presence (**Fig. 10D**) of NAD^+.

LDH is not located in the matrix of cardiac mitochondria: The respirometry results suggest that intramitochondrial LDH is not a significant source of lactate oxidation in striated muscle, especially in cardiac muscle, which consumes lactate for energy. To confirm that LDH is not an intramitochondrial protein in the heart, we performed protease protection assays of isolated mitochondria. For this, isolated mitochondria were incubated with trypsin, which results in proteolysis of extramitochondrial proteins that persist as either contaminants or outer mitochondrial membrane-associated proteins. As shown in **Fig. 11**, treatment of isolated mitochondria with trypsin resulted in loss of GAPDH, and LDHB immunoreactivity; however, the matrix-residing proteins ALDH2 and HSP60 were equally detected in mitochondrial fractions in the absence or presence of trypsin. Solubilization of mitochondria with non-ionic detergent (Triton X-100) enabled trypsin-mediated degradation of ALDH2 and HSP60 (**Fig 11**). These findings show that LDH is not an intramitochondrial protein in murine heart.

DISCUSSION

In this study, we subjected male and female mice to forced treadmill running and measured both heart mass and cardiac mitochondrial respiration following various durations of training. We found that maximal adaptation to exercise occurs

Figure 11

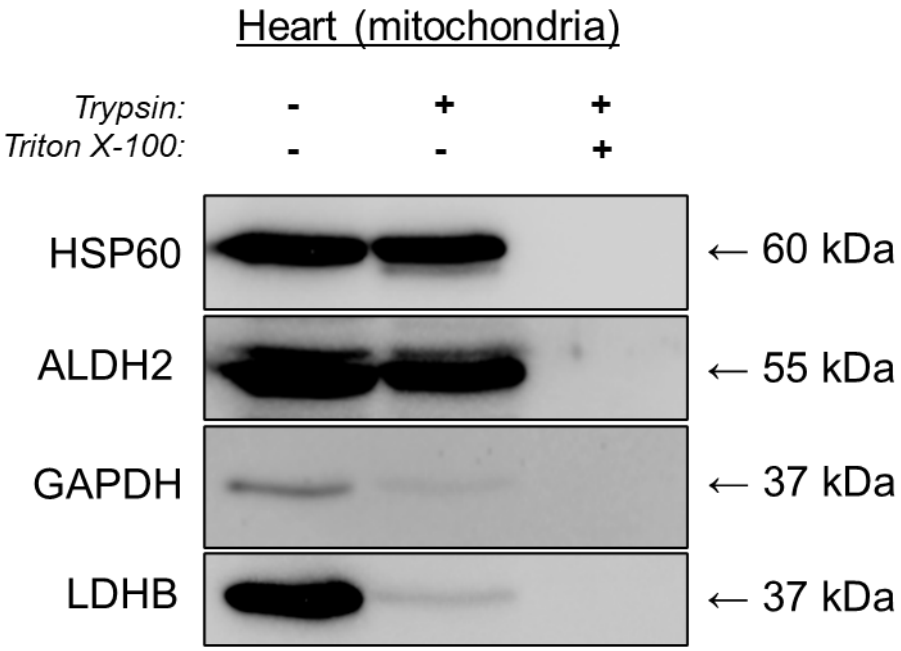


Figure 11: LDHB is not localized within cardiac mitochondria of mice. Protease protection assay: Isolated cardiac mitochondria were treated with or without trypsin (0.25 mg/ml) for 15 min in the absence or presence of Triton X-100 (1% v/v). Mitochondrial lysates were examined by Western blotting for the presence of: lactate dehydrogenase B (LDHB); the known intramitochondrial proteins, heat shock protein 60 (HSP60) and aldehyde dehydrogenase 2 (ALDH2); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which served as a control for cytosolic contaminants. Data are representative of two independent experiments.

following two weeks of training and that hearts of male, but not female, FVB/NJ mice hypertrophy in response to exercise. Although there was no exercise-induced growth observed in female hearts, the hearts of female mice showed greater ADP sensitivity than hearts of male mice. Importantly, we also found that exercise training has minimal effects on mitochondrial respiration in the isolated organelle from cardiac and skeletal muscle. Furthermore, we found that the presence of exogenous NAD^+ does not augment lactate oxidation in isolated, striated muscle mitochondria and that LDH does not exist within cardiac mitochondria. Collectively, these findings indicate there are biological sex-dependent differences in the murine cardiovascular response to exercise and that myocardial lactate oxidation in murine hearts occurs after LDH-mediated extramitochondrial conversion of lactate to pyruvate.

We found few differences in mitochondrial respiration resulting from exercise duration in hearts of male and female mice, and the only differences observed in ADP sensitivity resulted when comparing hearts of acutely exercised male and female mice. Exercise-induced cardiac remodeling appeared to be sex-dependent, where male mice showed significant hypertrophic responses with no changes in mitochondrial respiration, whereas female mice showed no hypertrophic responses but showed evidence of slight changes in mitochondrial respiration (**Fig. 7**). However, more studies are needed to confirm different remodeling phenotypes in male and female hearts.

Previous reports suggest that exercise influences mitochondrial dynamics in the heart,[286] but our findings suggest that respiration in isolated mitochondria is

likely not influenced by exercise. Interestingly, another group recently reported similar findings to ours regarding exercise and ADP sensitivity, but showed that ADP affinity might be substrate-dependent.[309] We only measured ADP sensitivity when cardiac mitochondria were supplied pyruvate with malate. Currently, we understand that ANT is important in exercise-induced responses to exercise, as $ANT^{-/-}$ mice show severe exercise intolerance.[303] Cardiomyocytes maintain low, intracellular levels of ADP, which do not change significantly during exercise.[310] Small changes are thought to be immediately buffered by the phosphocreatine:ATP buffering system.[311] One study demonstrated that resistance exercise training was sufficient to reverse the reduction in ADP sensitivity of the adenine nucleotide translocase observed in aging skeletal muscle,[312] while another study showed a slight increase in ADP sensitivity immediately following an acute bout of aerobic exercise.[311] The discrepancies in our findings could be due to differences in methods or model, but seem to be in support of results by others.[309]

Several studies indicate that LDH may be localized to mammalian mitochondria.[295-298] The presence of LDH within mitochondria could be particularly important to the heart, which has a basally high energetic requirement that only increases with exercise. The rationale for examining mLDH is strengthened by the fact that circulating lactate concentration correlates positively with myocardial lactate uptake and oxidation[141,149,150] and can contribute remarkably to cardiac ATP production in mammals.[291] Importantly, any NADH generated within mitochondria during the LDH reaction would be directly available to the respiratory

chain, bypassing the need to transport reducing equivalents across the inner mitochondrial membrane via the malate-aspartate shuttle. Therefore, we examined whether striated muscle mitochondria have the capacity to oxidize lactate.

Our findings indicate that LDH is in neither the matrix nor the intermembrane space of murine cardiac mitochondria. Evidence for this conclusion is provided by our protease protection assays. The protease protection assay is based on the concept that intramitochondrial proteins are protected from protease-mediated degradation: the defined exclusion limit of the outer mitochondrial membrane is 3–5 kDa[313,314] and trypsin is approximately 23 kDa. Therefore, only proteins that contaminate the mitochondrial preparation or that are associated with the outer leaflet of the outer mitochondrial membrane can be degraded by trypsin. In our mitochondrial preparations, the intramitochondrial proteins HSP60 and ALDH2 remained unaffected by trypsin; however, trypsin treatment proteolyzed LDHB and GAPDH, a cytosolic contaminant that served as a control. LDHB is the predominant LDH isozyme expressed in mammalian heart, and, unlike LDHA, it has a higher affinity for lactate than pyruvate.[315] Thus, although LDHB is likely to contribute most to lactate oxidation by the heart, its catalytic activity appears confined to extramitochondrial locales in myocytes.

Our respiration studies complement the conclusion that LDH is not localized in significant amounts within striated muscle mitochondria. In both cardiac and skeletal muscle mitochondria, lactate failed to support significant levels of respiration. Furthermore, provision of extramitrochondrial NAD^+ did not

significantly affect lactate oxidation by isolated mitochondria. These data, along with findings of the protease protection assay, appear to contradict previous findings that suggest LDH is within mitochondria[295-298] and supports the idea that LDH catalysis occurs primarily in the cytosol.[299-301] Although it remains possible that discrepant results could be due to model-specific or technical factors (e.g., species, mouse strain, technical differences), our studies suggest that LDH catalysis is not an intramitochondrial phenomenon.

Interestingly, we found that lactate abundance negatively correlated with running distance only in untrained mice, which suggested potential adaptations to circulating lactate. Because the heart strongly adapts to exercise,[291] is a net lactate consumer,[291] and is a primary contributor to exercise capacity,[316] we tested whether exercise influences lactate-supported respiration in isolated heart mitochondria. As expected, chronic treadmill exercise promoted cardiac growth; however, neither acute nor chronic exercise promoted lactate oxidation by mitochondrial isolates.

In summary, we find that aerobic exercise training leads to cardiac growth in male FVB/NJ mice but has minimal effects on mitochondrial respiration and ADP sensitivity. Furthermore, we find that LDHB—the primary LDH isozyme involved in lactate oxidation—is not present within murine cardiac mitochondria. Mitochondria isolated from neither skeletal muscle nor cardiac mitochondria respired substantially on lactate. These data suggest that mLDH is not influential in cardiac bioenergetics and that cytosolic LDH is the primary contributor to cardiac lactate oxidation.

Part of this chapter previously appeared as an article in the journal Redox Biology and another part of this chapter appeared as an article in the Journal of Sport and Health Science. The original citations are as follows: Fulghum KL et al. Mitochondria-associated lactate dehydrogenase is not a biologically significant contributor to bioenergetic function in murine striated muscle. *Redox Biology* Vol 24, 2019; and Fulghum K et al. Influence of biological sex and exercise on murine cardiac metabolism. *Journal of Sport and Health Science* 11(4):479-494, 2022.

CHAPTER III

INVESTIGATING THE EFFECTS OF BIOLOGICAL SEX AND EXERCISE ON MURINE CARDIAC METABOLISM EXPOSE ROLE OF BCAAS IN PHYSIOLOGICAL CARDIAC GROWTH

INTRODUCTION

Exercise presents a major challenge to systemic metabolic homeostasis.[317] Moderate- to high-intensity exercise is particularly demanding because maintenance of high levels of physical and cardiac work requires higher oxygen and substrate utilization in skeletal muscle and the heart. Although it is known that the metabolic requirements of tissues vary as a product of exercise intensity and duration,[6,317,318] it remains unclear how exercise affects the many different pathways of metabolism and how these stimulate adaptive changes in the heart. This is important to understand because recent evidence suggests that transient changes in metabolism are critical for adaptive responses to exercise.[6,291] In particular, little is understood about how cardiac metabolism changes during and after exercise, and even less is understood about how these changes could influence hypertrophic responses in the heart.

During exercise, cardiac contractile power and oxygen consumption can increase by up to 10-fold above resting rates.[24,123] This increase in myocardial workload is accompanied by increased catabolism of several circulating

substrates, including glucose, lactate, fatty acids, and branched-chain amino acids.[291,319] Although moderate-intensity exercise has been associated with elevations in myocardial glucose uptake and oxidation, elevations in circulating concentrations of competing substrates such as lactate and fatty acids may decrease glucose catabolism.[118,291] Regular exercise also promotes adaptive metabolic remodeling in the heart. Perfused heart studies indicate that acclimation to an exercise regimen is associated with increases in the rates of basal glycolysis,[214] glucose oxidation, and fat oxidation[216] in mice; however, cardiac glycolysis has been suggested to be lower in exercise-adapted rats, despite higher myocardial palmitate and glucose oxidation.[91]

Because adaptive changes are triggered early in an exercise training program,[6] we examined the changes in metabolism that occur with a single bout of exercise. Using metabolomics, we assessed how exercise influences the cardiac metabolome at different times following an exercise session in male and female mice. Our data suggest that the metabolic response to exercise is different in male versus female murine hearts and that, even basally, female hearts have remarkably different metabolite profiles than male hearts. Importantly, we found that exercise increased the abundance of circulating and myocardial BCAAs, which could be a stimulus for exercise-induced cardiac growth. Indeed, we measured exercise-induced cardiac growth in mice fed a high-BCAA diet, but not in mice fed a low-BCAA diet. Collectively, these data could expose BCAAs as an important exercise-induced trigger for physiological growth.

EXPERIMENTAL METHODS

Experimental animals

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Louisville. Adult male and female FVB/NJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained on 12 h: 12 h (light:dark) cycle, with both chow and water provided *ad libitum* unless fasted (6 h) for untargeted metabolomics experiments. All mice were 13 weeks of age at the time of exercise experimentation, and all mice were both exercised and euthanized at the same time of day. For metabolomics studies, all mice were fasted for 6 h prior to euthanasia. Upon completion of each experiment, mice were anesthetized with sodium pentobarbital (Sigma-Aldrich, St. Louis, MO, USA; 150 mg/kg, i.p.), and mice were euthanized via excision of the heart, which was freeze-clamped *in situ* for metabolomics studies. Additional tissues were harvested following euthanasia. These procedures are consistent with the American Veterinary Medical Association *Guidelines on Euthanasia.*[320]

Exercise training protocol

Mice were acclimated to forced treadmill running and exercised as previously described.[293] Briefly, mice were exercised to exhaustion to determine initial exercise capacity. Training intensity was then determined from this initial capacity test: low-intensity exercise was carried out at 12 m/min for 40 min with 10° incline (55–60% of the initial exercise capacity); moderate-intensity exercise was carried out at 19.1 m/min for 40 min with 10° incline (75% of the initial exercise

capacity); and the exercise capacity test (ECT) served as a high-intensity, exhaustive bout of exercise. In this ECT, mice run to exhaustion with increasing treadmill speed and incline, as described previously.[214,293] For studies investigating the role of BCAAs in exercise-induced cardiac growth, mice were exercised via forced treadmill running for two weeks at 75% initial exercise capacity and 10° incline for 40 min/day (5 d/wk) for the first week and 50 min/day (5 d/wk) for week 2. A second ECT was performed on the final day of exercise and mice were euthanized 24 hours following final ECT. Blood glucose, lactate, and 3-hydroxybutyrate measurements were acquired from tail blood before and after the exercise bout using an Accu-Check Aviva meter (Roche, San Francisco, CA, USA), a Lactate Plus meter (Nova Biomedical, Waltham, MA, USA), and a Keto-Mojo meter (Keto-Mojo, Amsterdam Duivendrecht, Netherlands), respectively. Sedentary control mice were subjected to the same conditions as exercised mice but were sat on a treadmill with speed = 0 m/s. For metabolomics studies, all mice were fasted for 6 h prior to euthanasia.

Low and High BCAA Diet

To determine the effect of BCAAs on exercise-induced cardiac growth, we performed an initial ECT on male, FVB/NJ mice as described above and then provided mice with either a low-BCAA (Teklad TD.150662) or high-BCAA (Teklad TD.170323) custom diet for the duration of exercise training. On the last day of exercise training, mice performed a final ECT and were euthanized 24 h afterward, as described above.

Metabolomics

Hearts were freeze-clamped *in situ* using liquid nitrogen-cooled Wollenberger Tongs and powdered under liquid nitrogen. The samples were then prepared by Metabolon using an automated MicroLab STAR® system (Hamilton Company, Reno, NV, USA). First, tissue homogenates were made in water at a ratio of 5 µl 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 precipitated with methanol (VWR, Radnor, PA, USA; final concentration 80% v/v) under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000, Clifton, NJ, USA) 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)/ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) with positive ion mode electrospray ionization (ESI), by RP/UPLC-MS/MS with negative ion mode ESI, and by hydrophilic interaction chromatography (HILIC)/UPLC-MS/MS with negative ion mode ESI. Samples were placed briefly

on a TurboVap® (Zymark, Clackamas, OR, USA) to remove the organic solvent. All methods utilized a Waters ACQUITY UPLC (Milford, MA, USA) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer (Bethlehem, KY, USA) 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 C18-2.1×100 mm, 1.7 µm) 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 2.1×150 mm, 1.7 µm) 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 MS^n scans using dynamic exclusion. The scan range covered 70–1000 *m/z* (mass to charge ratio).

Raw data were extracted, peak-identified, and processed using Metabolon's proprietary hardware and software (Metabolon Inc., Research Triangle Park, NC, USA). Compounds were identified by comparison to library entries of purified, authenticated standards or recurrent unknown entities, with known retention times/indices (RI), *m/z*, and chromatographic signatures (including MS/MS spectral data). Biochemical identifications were based on 3 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., Research Triangle Park, NC, USA) 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. The area under the curve was used for peak quantification.

Statistical analyses

Original scale data (raw area counts) were analyzed using Metaboanalyst 5.0 software (http://www.metaboanalyst.ca/).[321] Metabolites with missing values were omitted and the data were filtered by interquartile range, followed by log-transformation. For multiple comparison testing, *q* values were calculated using a method embedded within the Metaboanalyst software that controlled for the false discovery rate (FDR).[322] An FDR cutoff of $p < 0.10$ was implemented to assume significance. We used two-way analysis of variance (ANOVA) where appropriate

and confirmed significance with Bonferroni's post-hoc test as indicated. Statistical significance was assumed where $p < 0.05$.

RESULTS

Acute exercise alters circulating substrate availability: Because exercise intensity could influence circulating substrate levels, we first examined the effects of low-, moderate-, and high-intensity exercise bouts on blood lactate, glucose, and ketone body levels. To accomplish this, we measured circulating substrate levels before (at rest) and after the session of exercise. For low- and moderate-intensity exercise, mice were run for 40 min at 12 m/min or 19.1 m/min (both at a 10° incline), respectively; for high-intensity exercise, the mice were subjected to an exercise capacity test, where belt speed and incline were increased (up to 30 m/min and 15° incline) until exhaustion (**Fig. 12A**). As shown in **Fig. 12B** and **12C**, distance and work were higher in the moderate-intensity group than the low-intensity group. Due to their relatively early exhaustion during the exercise bout, the high-intensity exercise group ran a shorter distance and performed less work than the moderate-intensity group.

Circulating lactate levels immediately after the exercise bout were not influenced by low- or moderate-intensity exercise, but were significantly increased in both male and female mice by high-intensity exercise (**Fig. 12D**). Although circulating glucose was relatively stable regardless of exercise intensity, we found modestly higher blood glucose levels in male mice subjected to moderate-intensity exercise (**Fig. 12E**). Interestingly, circulating ketone body levels changed in a manner dependent on exercise intensity: 3-hydroxybutrate was higher immediately

Figure 12

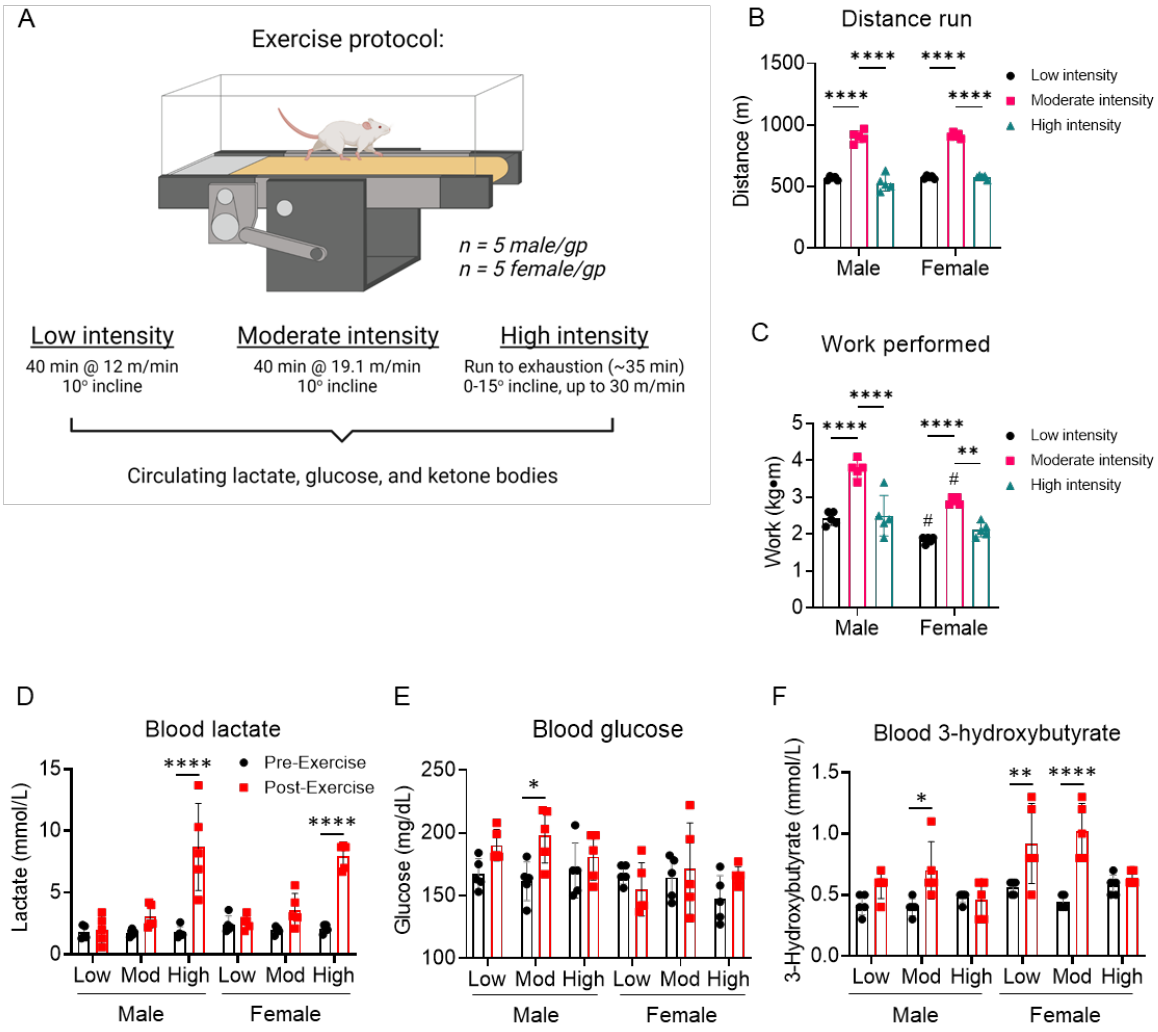


Figure 12: Exercise intensity affects circulating substrate levels. Mice were subjected to low-, moderate-, and high-intensity exercise followed by measurement of circulating substrates immediately after the exercise bout. (A) Schematic of study design; (B) distance run to exhaustion; (C) work performed during the exercise session; measurements of (D) circulating lactate, (E) glucose, and (F) 3-hydroxybutyrate at the end of exercise bout. *n* = 5 mice per group, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, #p < 0.05 female *vs*. male, two-way ANOVA with Bonferroni's *post hoc* test (B–F).

Abbreviations: ANOVA = analysis of variance; gp = group.

after moderate-intensity exercise in male mice and both low- and moderate-intensity exercise in female mice, but it was not changed immediately following high-intensity exercise (**Fig. 12F**). Collectively, these data indicate that circulating lactate and 3-hydroxybutyrate levels respond in a dissimilar fashion to different exercise intensities and that biological sex could influence this response.

We next examined how circulating substrates change with time after a bout of high-intensity, exhaustive exercise. Blood lactate, glucose, and 3-hydroxybutyrate levels were measured immediately after exercise, 1 h following exercise, or 24 h following exercise, with appropriate sedentary controls (**Fig. 13A**). These timepoints were chosen because in our previous studies in male mice,[214] we found decreased activation of cardiac 6-phosphofructo-2-kinase (Pfkfb2) and elevated levels of cardiac glycogen immediately after exercise, which suggests marked changes in cardiac glucose metabolism. During initial exercise capacity tests, all exercise groups showed similar distance to exhaustion and work performed (**Fig. 13B, 13C**). As expected, circulating levels of lactate increased in both male and female mice during exercise but returned to normal levels within 1 h following the exercise session (**Fig. 13D**). While male mice showed no changes in blood glucose concentration following exercise, we observed in female mice a slight reduction in circulating glucose 1 h following exercise (**Fig. 13E,** $p = 0.05$). At this same time (i.e., 1 h after exercise), female mice had significantly higher circulating 3-hydroxybutyrate levels compared with sedentary controls; this

Figure 13

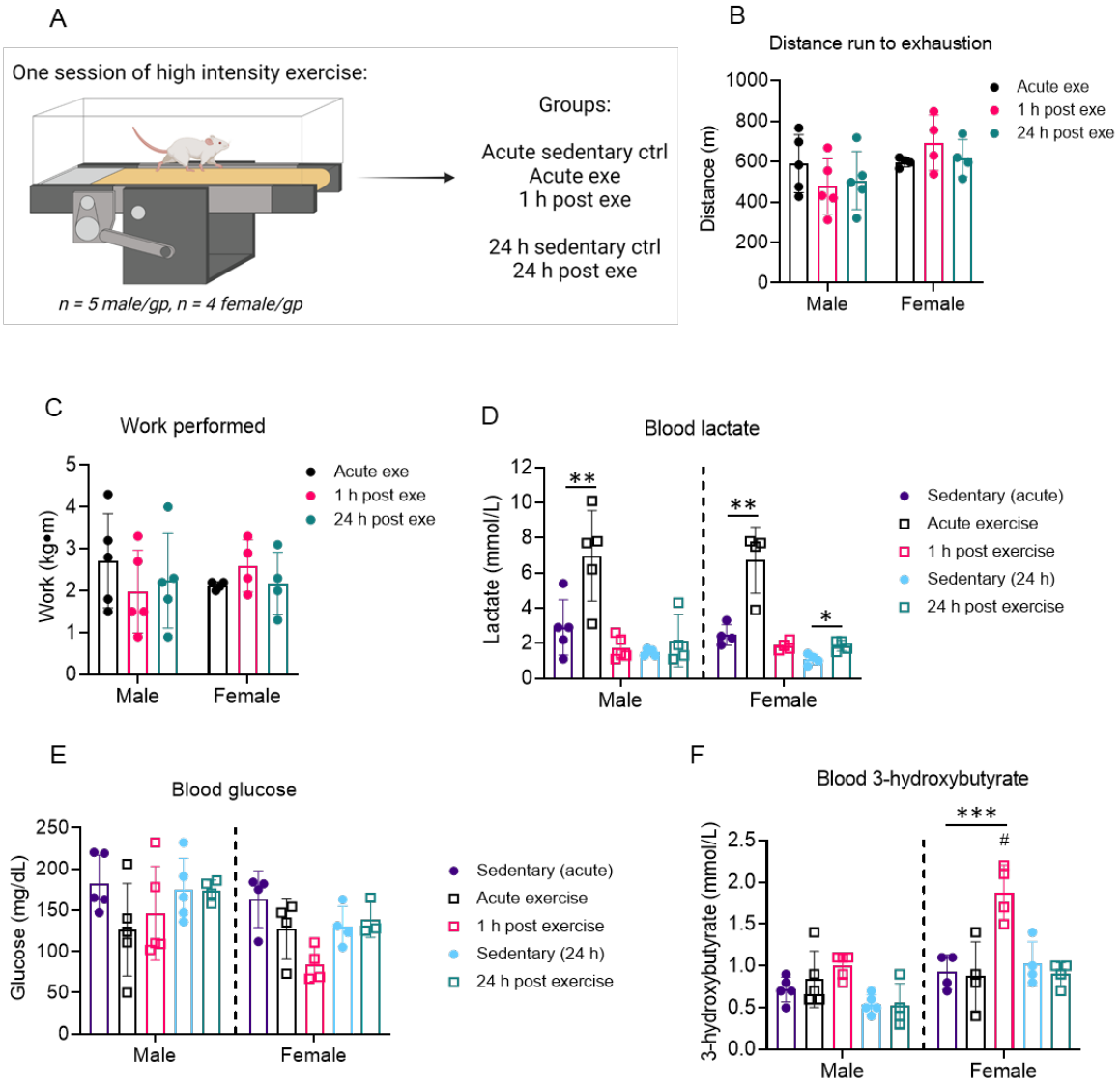


Figure 13: Time-dependent changes in circulating substrates after 1 bout of high-intensity exercise. Mice were subjected to 1 bout of high-intensity exercise (i.e., exercise capacity test) followed by measurement of circulating substrates immediately, 1 h, and 24 h after the exercise bout. (A) schematic of study design; (B) distance run to exhaustion; (C) work performed during the exercise session; and measurements of circulating (D) lactate, (E) glucose, and (F) 3-hydroxybutrate. *n* = 4–5 mice per group, ** $p < 0.01$, ***$p < 0.001$, two-way ANOVA with Bonferroni's post-hoc test (B, C), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, #p<0.05 female *vs*. male, two-way ANOVA with Bonferroni's *post hoc* test for Acute Sedentary Ctrl, Acute Exe, and 1 h post Exe group; student's *t* test for 24 h Sedentary Ctrl and 24 h post Exe group (D–F).

Abbreviations: ANOVA = analysis of variance;ctrl = control; Exe = exercise; gp = group.

indicates a shift in circulating substrate availability, which could affect tissue metabolism. However, there were no significant changes in 3-hydroxybutyrate levels in male mice after a single, exhaustive bout of exercise (**Fig. 13F**).

S*ex-dependent differences in cardiac metabolism:* Because the influence of biological sex on metabolic phenotype is not well characterized, we first examined the metabolomic profiles of the sedentary male and female hearts. We found 69 cardiac metabolites that were significantly different (FDR < 0.10) in abundance between the sexes (**Fig. 14A, Table 1**). Partial least-squares discriminant analysis (PLS-DA) showed distinct group clustering by biological sex (**Fig. 14B**), with several metabolites highlighted in a variable importance in projection (VIP) score plot as being important to the PLS-DA model (**Fig. 14C**). Compared with male hearts, these analyses in female hearts revealed higher levels of heme, pantothenate, triethanolamine, and phospholipid species but lower levels of α-hydroxyvalerate, xenobiotics, carnitinylated and glycinated species, carnosine, gulonate, maltotriose, and acetyl CoA, all of which contributed strongly to group differences.

To visualize further baseline sex differences in cardiac metabolite abundance, the 50 most changed metabolites in male and female sedentary hearts are displayed as a heatmap (**Fig. 15A**). As summarized in the Venn diagram in **Fig. 15B**, female hearts had higher levels of vasodilatory metabolites, such as homoarginine, and of B vitamins, such as pantothenate and pyridoxamine, as well as higher levels of several sphingomyelin and glycerophospholipid species;

Figure 14

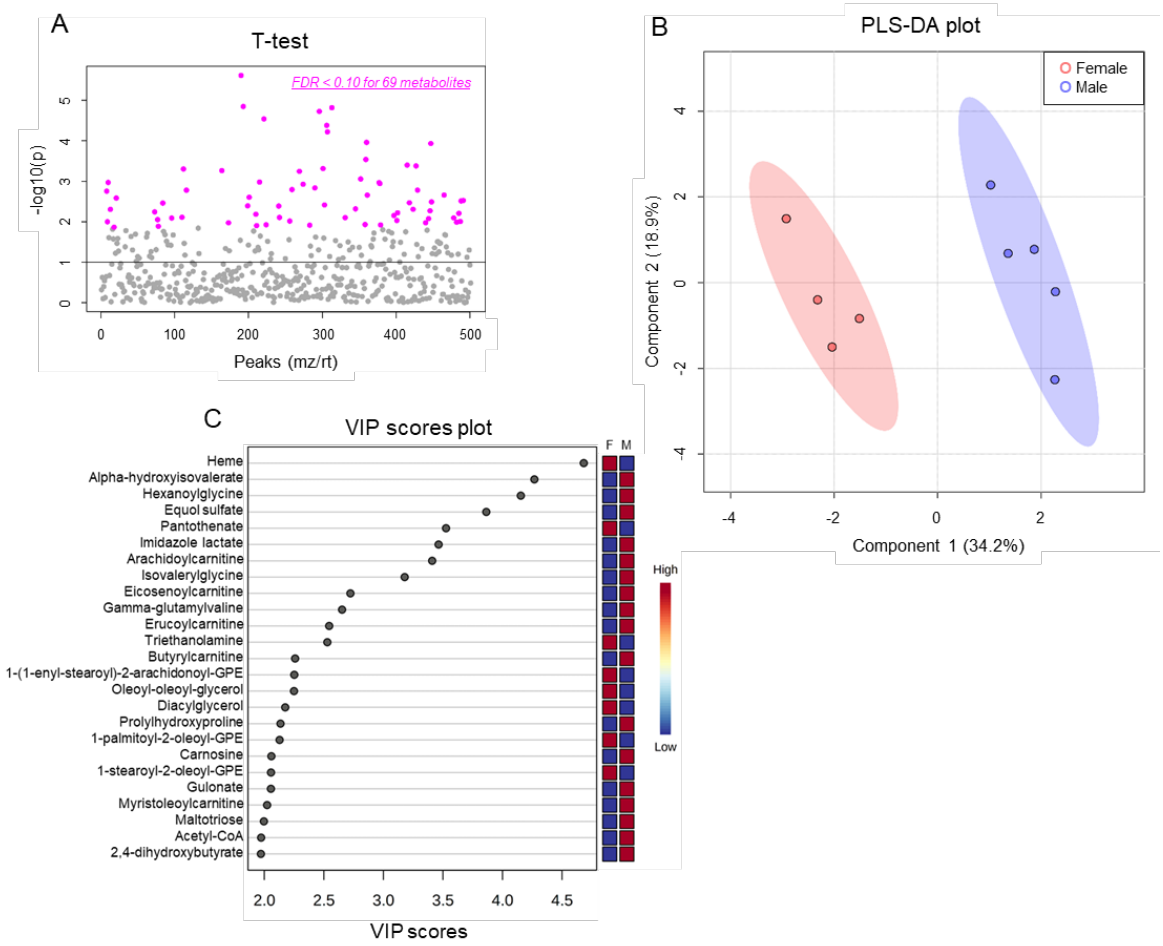


Figure 14: Biological sex influences baseline cardiac metabolite abundances. Unbiased metabolomics of male and female sedentary mice: (A) dot plot showing 69 significantly different (FDR < 0.10) metabolites between male and female hearts; (B) partial least-squares discriminant analysis; and (C) variable importance plot assessing metabolite contribution to the PLS-DA model. *n* = 4–5 mice per group.

Abbreviations: F = female; FDR = false discovery rate; M = male; PLS-DA = partial least-squares discriminant analysis; VIP = variable importance plot. GPE = glycerophosphoethanolamine , mz = mass to charge ratio, rt = retention time

Figure 15

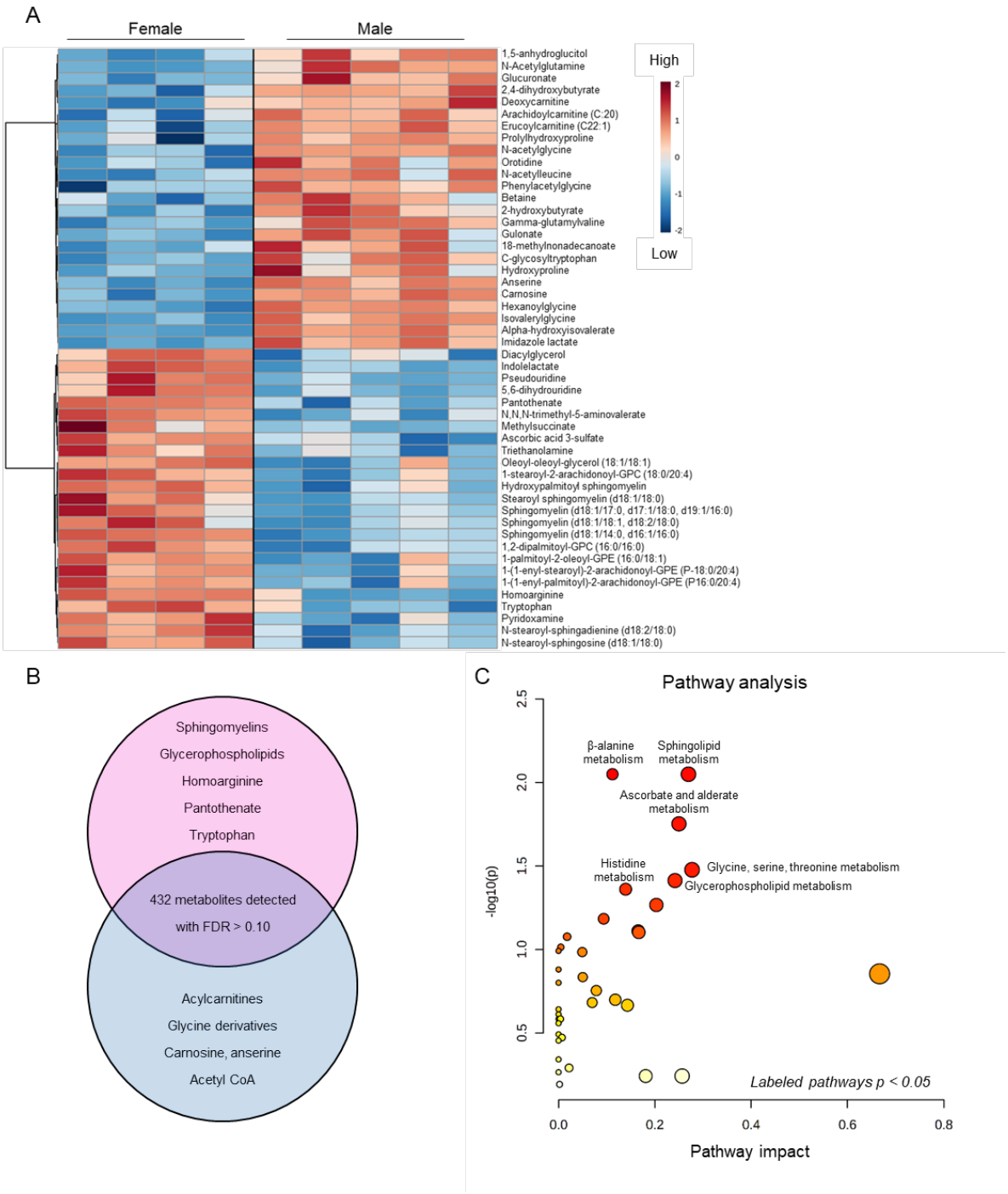


Figure 15: Major metabolomic differences between male and female hearts. Metabolomic analyses highlighting the influence of sex on basal metabolite abundances in the hearts of sedentary mice: (A) heatmap of top 50 significantly different metabolites; (B) Venn diagram displaying major metabolites and metabolite classes that differ based on sex; and (C) pathway impact analysis. *n* = 4–5 mice per group. FDR = false discovery rate; GPC = glycerophosphocholine

however, there were lower levels of some long-chain acylcarnitines (arachidoylcarnitine, erucoylcarnitine) and collagen precursor-breakdown products (hydroxyproline, prolyl-hydroxyproline). Furthermore, glucuronate, gulonate, carnosine, anserine, acetyl CoA, and glycinated metabolites were lower in female hearts. Pathway impact analyses further confirmed the significance of these changes to individual metabolic pathways (**Fig. 15C**). Collectively, these data suggest that biological sex influences the cardiac metabolome in mice.

Acute effects of exercise on the female cardiac metabolome: Because of these sex-dependent differences in baseline cardiac metabolite abundance, we separately analyzed metabolomics data from hearts of exercised male and female mice. In female hearts, 30 metabolites changed immediately after exercise or upon 1 h of recovery (FDR < 0.10) from an acute bout of high-intensity exercise. Heatmap and relative abundance analyses suggest exercise-induced increases in a cluster of metabolites, including corticosterone, N-acetylleucine, indolelactate, allantoin, and amino acids such as tyrosine and tryptophan, all of which return to near sedentary levels 1 h following exercise (**Fig. 16A, 16B**). Abundances of alanine and serine decreased immediately following exercise and were even lower in the 1 h recovery period. Interestingly, the majority of metabolite changes occurred 1 h following high-intensity exercise, with prominent increases in 3-hydroxybutyrate and isoleucine as well as lipid pathway metabolites, such as the major intermediate of phospholipid biosynthesis, CDP-choline. Several long chain fatty acids and glycerolipid species were also increased 1 h after exercise in the

Figure 16

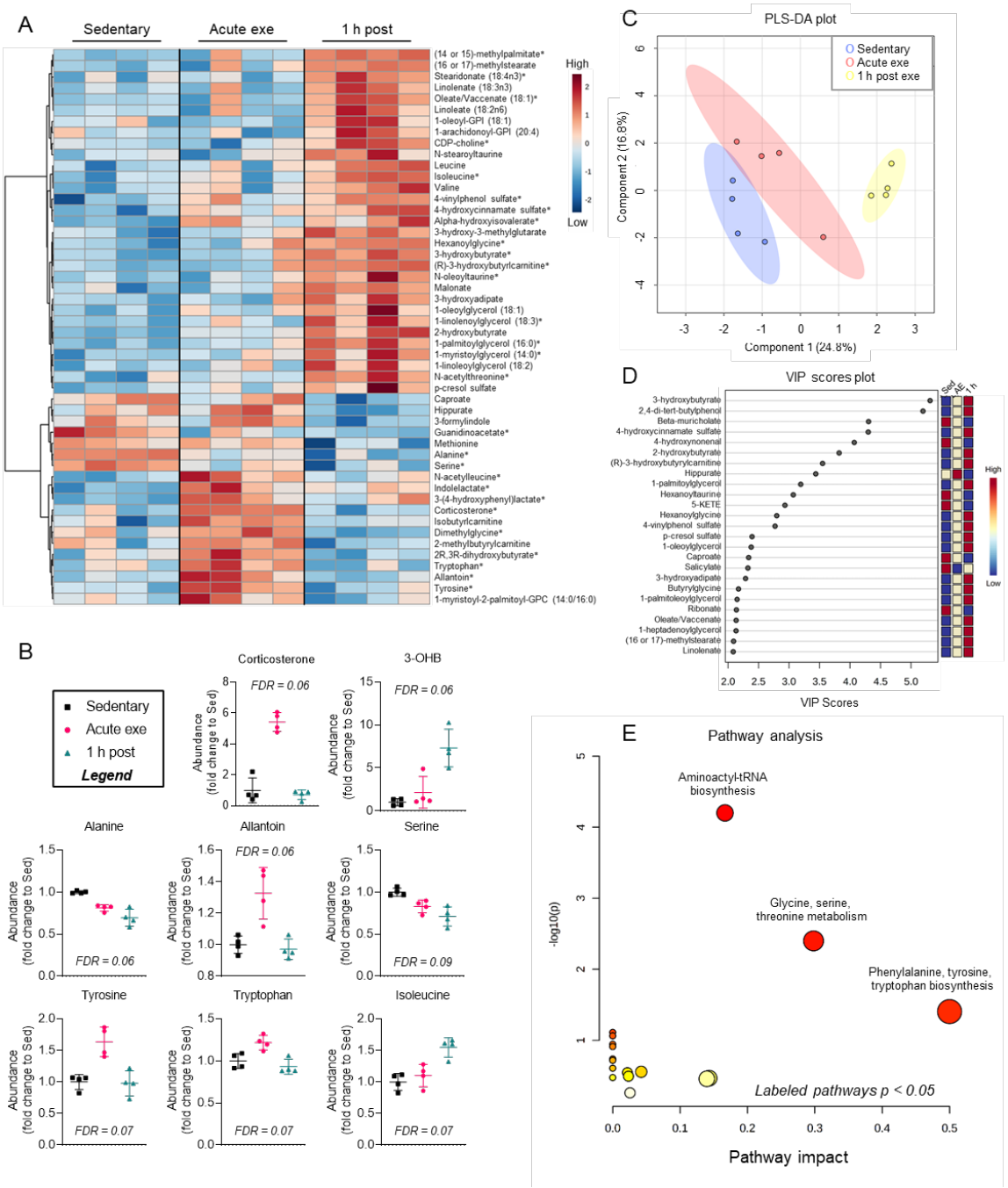


Figure 16: Changes in cardiac metabolite abundances following 1 session of exercise in female murine hearts. Female mice were subjected to 1 bout of high-intensity exercise and hearts were freeze-clamped immediately or 1 h after the exercise bout for unbiased metabolomic analyses: (A) heatmap of the top 50 most changed metabolites in hearts from exercised versus sedentary female mice; bolded metabolites indicate FDR < 0.10 following one-way ANOVA; (B) graphs of individual metabolites that showed the most prominent changes caused by

exercise; (C) PLS-DA plot and (D) corresponding VIP plot; and (E) pathway impact analysis derived from most significantly changed metabolites. *n* = 4 female mice per group.

Abbreviations: AE = acute exercise; ANOVA = analysis of variance; FDR = false discovery rate; PLS-DA = partial least-squares discriminant analysis; Sed = sedentary; VIP = variable importance plot. GPI = glycosylphosphatidylinositol; GPC = glycerophosphocholine; 3-OHB = 3-hydroxybutyrate

female hearts (**Fig. 16A**). PLS-DA and VIP score plots further support a prominent response in the female cardiac metabolome 1 h after exercise, with 3-hydroxybutyrate contributing most to group separation (**Fig. 16C, 16D**). Pathway impact analysis suggest that amino acid metabolism and biosynthesis are acute responses to exercise (**Fig. 16E**), which could highlight the importance of amino acid mobilization or utilization in the female heart during exercise.

Acute effects of exercise on the male cardiac metabolome: In hearts from male mice subjected to an acute bout of high-intensity exercise, we observed few significant changes in metabolite abundances following exercise (**Fig. 17A**); corticosterone was the only significantly changed metabolite (FDR < 0.10) following exercise in male hearts. Furthermore, PLS-DA plots suggest modest overlap of group clustering (**Fig. 17B**) and no indication of substantial divergence. Nevertheless, three metabolites contributed substantially (VIP score > 5) to group differences (**Fig. 17C**), including the bile acid tauro-β-muricholate, the ketone body 3-hydroxybutrate, and the phenylsulfate 4-vinylphenol sulfate. Collectively, these findings suggest that a single bout of high-intensity exercise increases corticosterone levels acutely in the murine heart and that female hearts have more pronounced metabolic responses to exercise compared with male hearts.

The murine heart metabolome 24 h after an exercise bout: To determine how the cardiac metabolome responds to a longer period of recovery following a bout of exercise, we performed separate analyses on samples collected 24 h after

Figure 17

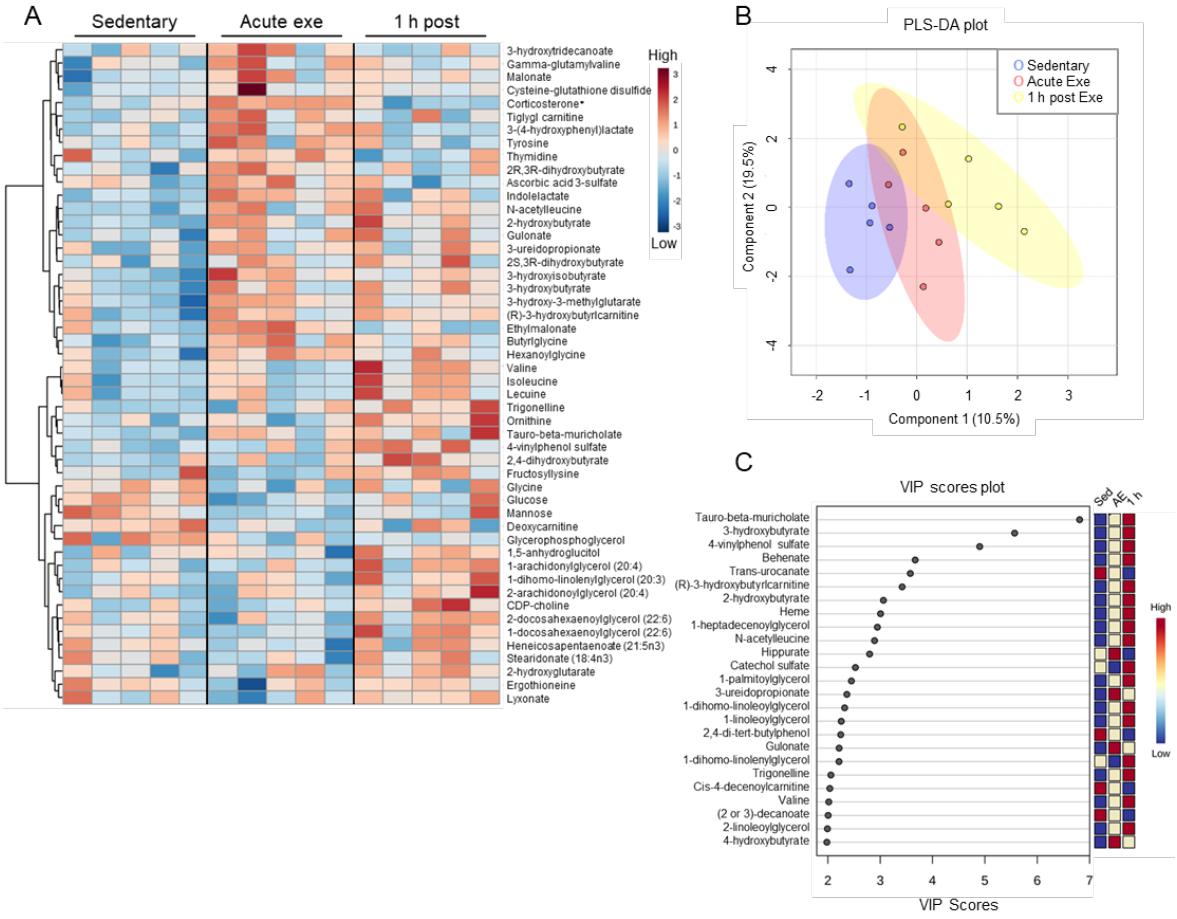


Figure 17: Male hearts demonstrate few significantly changed metabolites after a bout of high-intensity exercise. Male mice were subjected to one bout of high-intensity exercise and hearts were freeze-clamped immediately or 1 h after the exercise bout for unbiased metabolomic analyses: (A) heatmap of the top 50 most changed metabolites in hearts from exercised and sedentary male mice; bolded metabolites indicate FDR<0.10 following one-way ANOVA; (B) PLS-DA plot and (C) corresponding VIP plot. *n* = 5 male mice per group.

Abbreviations: AE = acute exercise; ANOVA = analysis of variance; PLS-DA = partial least-squares discriminant analysis; Sed = sedentary; VIP = variable importance plot. Exe = exercise; CDP = Cytidine diphosphate; FDR = False discovery rate

1 bout of high-intensity exercise. In female hearts, heatmap analysis of the top 50 most significantly changed metabolites indicate distinct clustering of sedentary and exercised mouse hearts; however, only 1,5-anhydroglucitol and 4-chlorobenzoic acid reached an FDR < 0.10 (**Fig. 18A**). Nevertheless, PLS-DA analysis suggested significant separation of groups (**Fig. 18B**), with heme, α-hydroxyisovalerate, and hexanoylglycine contributing greatest to group separation (data not shown).

Because 1,5-anhydroglucitol is known to be an indicator of glycemic control[323,324] and could suggest differences in intermediary metabolism, we further examined glucose-derived metabolites in the female hearts. We observed no changes in glucose, glucose-6-phosphate, or fructose-1,6-bisphosphate in hearts 24 h following exercise; however, compared with hearts of sedentary mice, the abundances of 3-carbon glycolytic intermediates (3-phosphoglycerate, phosphoenolpyruvate, and pyruvate) appeared lower in the hearts from exercised mice (**Fig. 18C**). The Krebs cycle metabolites citrate and aconitate were 35%–50% higher in exercised female hearts; however, α-ketoglutarate, fumarate, and malate were 10%–25% lower than sedentary controls (**Fig. 18D**). Because exercise has been suggested to be a robust regulator of the antioxidant response,[325] we also examined the effect of exercise on antioxidants [e.g., reduced glutathione (GSH), α-tocopherol, anserine, carnosine] and species derived from oxidative stress [e.g., oxidized glutathione (GSSG), 4-hydroxynonenal (4-HNE)]; although no significant differences were observed, the levels of GSSG and 4-HNE appeared lower in female hearts 24 h after exercise (**Fig. 18E**).

Figure 18

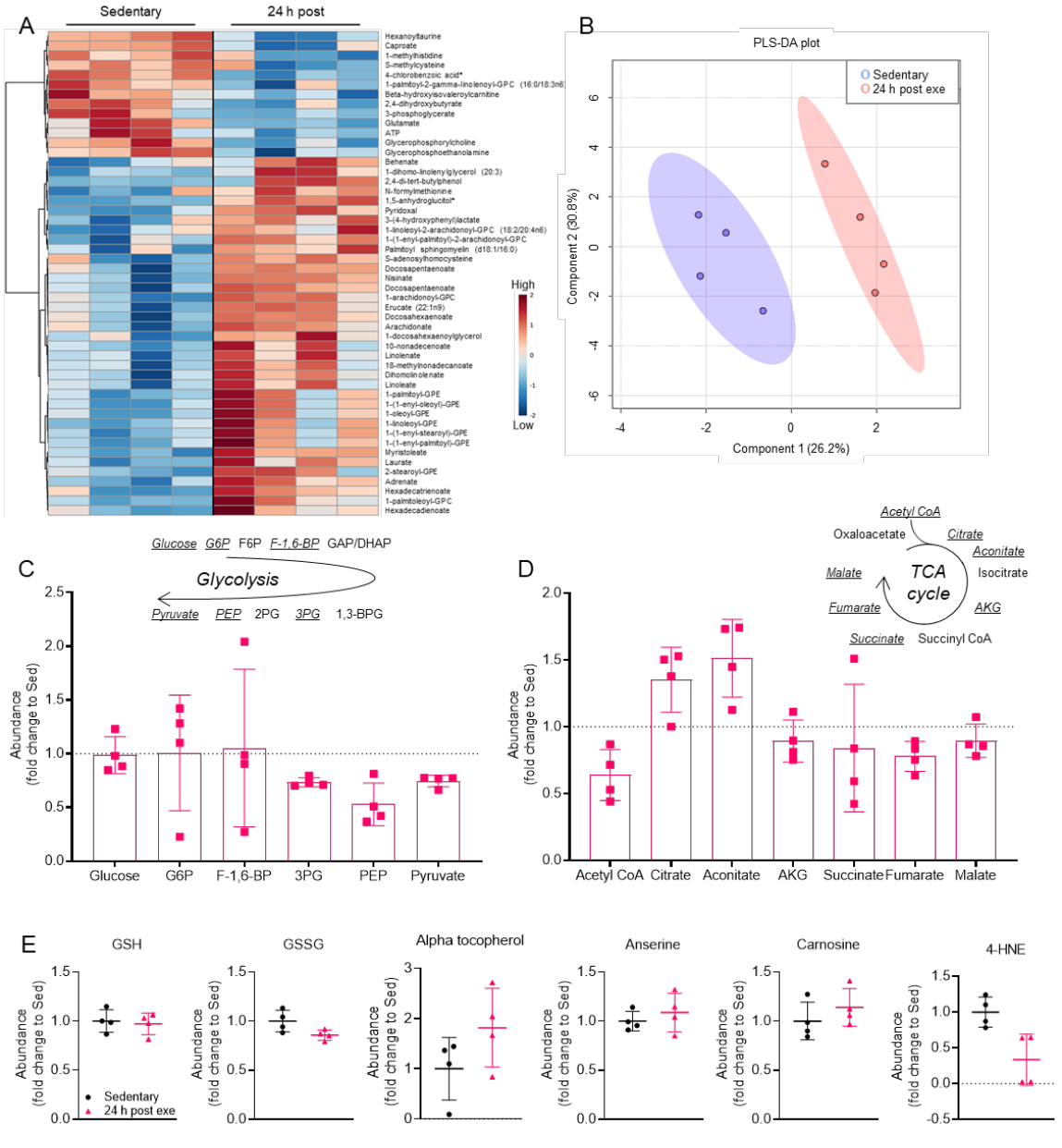


Figure 18: Changes in the murine cardiac metabolome in female mice 24 h following exercise. Female mice were subjected to one bout of high-intensity exercise and hearts were freeze-clamped 24 h after the exercise bout for unbiased metabolomic analyses: (A) heatmap of the top 50 most changed metabolites in hearts from exercised versus sedentary female mice; bolded metabolites indicate FDR < 0.10 following one-way ANOVA; (B) PLS-DA plot; relative abundances of (**C**) glycolytic and (**D**) TCA cycle intermediates shown as fold change relative to sedentary mice; and abundances of antioxidants and oxidation products. *n* = 4 female mice per group.

Abbreviations: ANOVA = analysis of variance; FDR = false discovery rate; TCA = tricarboxylic acid; PEP = phosphoenolpyruvate; 3PG = 3-phosphoglycerate; 1,3-BPG = 1,3-bisphosphoglycerate; G6P = glucose 6-phosphate; AKG = α-ketoglutarate; GSH = reduced glutathione; GSSG = oxidized glutathione; 4-HNE = 4-hydroxynonenal; PLS-DA = partial least-squares discriminant analysis.

In male hearts, there were no significantly changed metabolites 24 h after the exercise bout (**Fig. 19A**); however, PLS-DA analysis suggested modest separation between groups (**Fig. 19B**). In general, glycolytic intermediates seemed higher 24 h after exercise (**Fig. 19C**), and few changes in Krebs cycle metabolites were observed, with only slight reductions in α-ketoglutarate and succinate (15% and 52% reduction, respectively) compared with sedentary hearts (**Fig. 19D**). Distinct patterns in antioxidant and oxidation products were observed in male hearts as compared with female hearts 24 h after an exercise bout, with GSH, anserine, and carnosine appearing lower (**Fig. 19E** male**, Fig. 18E** female). Although these sex-dependent patterns warrant further investigation, these findings indicate that exercise-induced changes in the murine cardiac metabolome generally return to resting levels 24 h after an exercise bout.

Influence of BCAAs in exercise-induced cardiac growth: In this study, we found elevated abundance of BCAAs in male and female hearts following exercise. Interestingly, we also found BCAAs increase in circulation acutely following exercise (**Fig. 20A**). Because BCAAs are known to promote protein synthesis in the heart,[326,327] we next investigated the extent to which exercise-induced elevations in BCAAs could influence physiological cardiac growth. After determining initial exercise capacity, we assigned mice to either a low- or high-BCAA diet and exercised mice for two weeks to stimulate cardiac growth. Two weeks of training resulted in increased exercise capacity (**Fig. 20D**) but lower body weight (**Fig. 20E**) than initial values. Exercise training elicited a significant increase

Figure 19

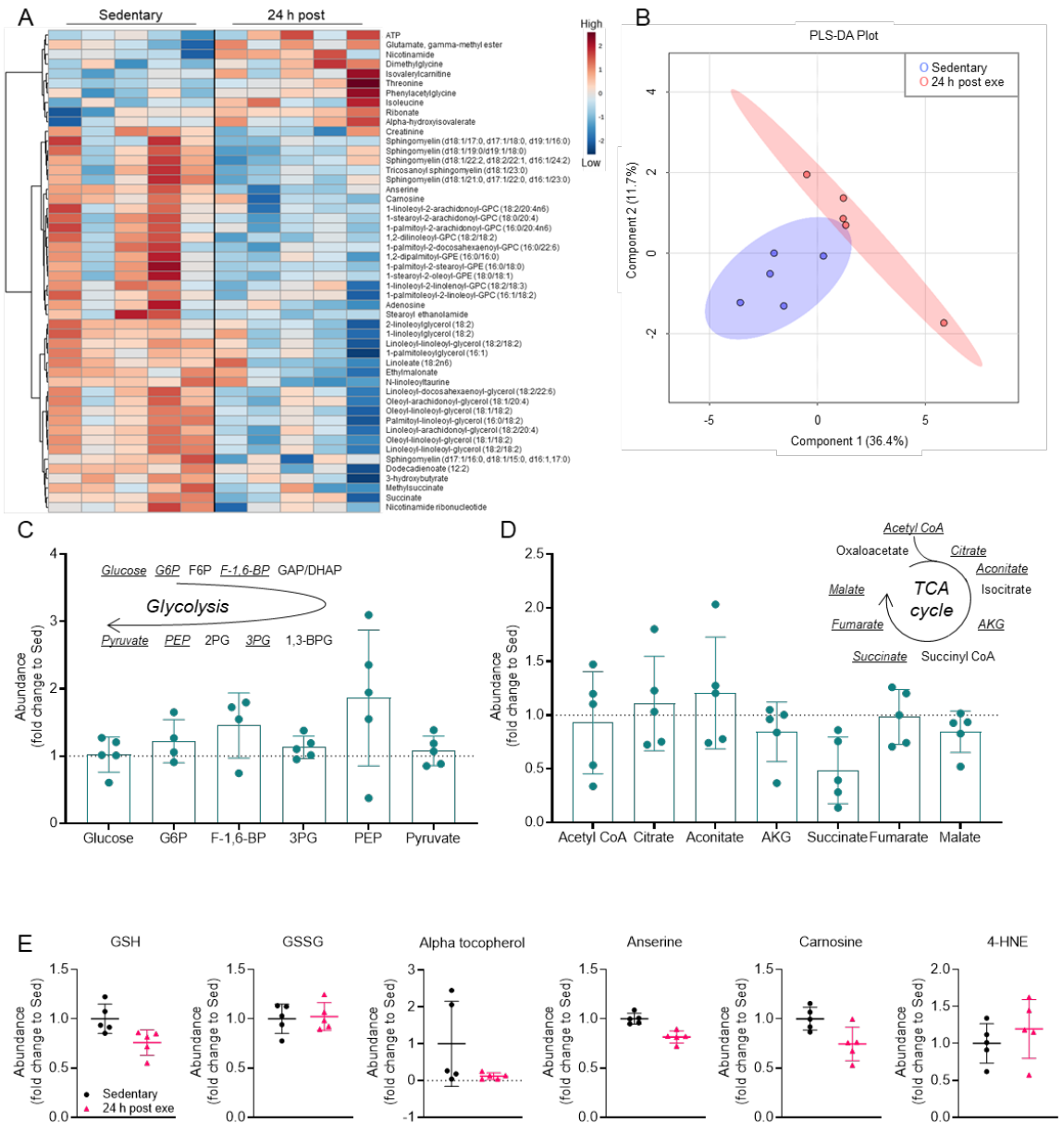


Figure 19: Changes in the murine cardiac metabolome in male mice 24 h following exercise. Male mice were subjected to one bout of high-intensity exercise and hearts were freeze-clamped 24 h after the exercise bout for unbiased metabolomic analyses: (A) heatmap of the top 50 most changed metabolites in hearts from exercised versus sedentary male mice; (B) PLS-DA plot; relative abundances of (C) glycolytic and (D) TCA cycle intermediates shown as fold change relative to sedentary mice; and (E) abundances of antioxidants and oxidation products. *n* = 5 male mice per group.

Abbreviations: TCA = tricarboxylic acid; PEP = phosphoenolpyruvate; 3PG = 3-phosphoglycerate; 1,3-BPG = 1,3-bisphosphoglycerate; G6P = glucose 6-phosphate; AKG = α-ketoglutarate; GSH = reduced glutathione; GSSG = oxidized glutathione; 4-HNE = 4-hydroxynonenal; PLS-DA = partial least-squares discriminant analysis.

Figure 20

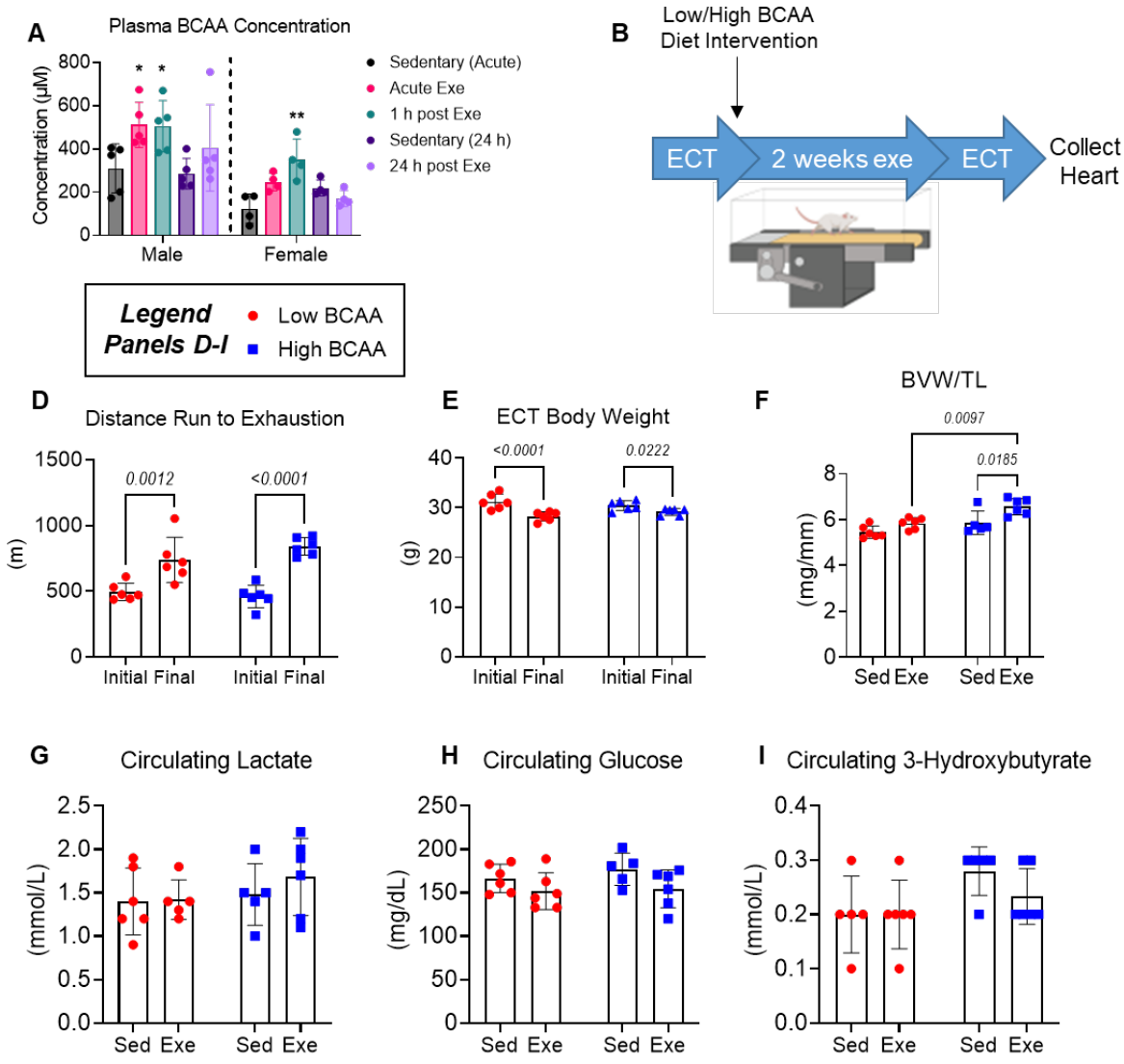


Figure 20: Exercise-induced elevations in BCAAs are necessary for exercise-induced cardiac growth. Plasma BCAA concentrations at various timepoints following exercise (**A**) and schematic of study design to test effects of BCAAs on cardiac response to exercise (**B**). Exercise training increased exercise capacity in low and high BCAA groups (**D**) but decreased body weight in both groups (**E**). Biventricular weight (BVM) normalized to tibia length (TL) in sedentary and exercised mice consuming low and high BCAA diets (**F**). Effect of diet and exercise on resting blood lactate (**G**), blood glucose (**H**), and blood 3-hydroxybutyrate (**I**). *n*=4–6/gp, *p<0.05, **p<0.01 one way ANOVA with Tukey's post-hoc test vs. sedentary. P-values indicated otherwise result from two-way ANOVA with Tukey's post-hoc test. ECT = exercise capacity test, Sed = sedentary, Exe = exercise.

in biventricular weight of mice fed a high-BCAA diet, but not in mice fed a low-BCAA diet (**Fig. 20F**). Therefore, low-BCAA diet prevented exercise-induced cardiac growth. There were no significant changes in circulating lactate, glucose, or 3-hydroxybutyrate because of dietary or exercise intervention (**Fig. 20G–20I**).

DISCUSSION

Guided by the premise that changes in metabolism influence structural and functional adaptations of the heart to exercise, we examined cardiac metabolite profiles after a session of treadmill exercise. We found that exercise intensity differentially affects levels of circulating lactate and 3-hydroxybutyrate in both male and female mice, and that high-intensity exercise alters circulating 3-hydroxybutrate only in female mice 1 h following exercise. Untargeted metabolomics also revealed marked differences in the female and male cardiac metabolomes and distinguished the female heart as having more marked changes in cardiac metabolite profile after exercise compared with male hearts. Notable sex-dependent differences in the basal cardiac metabolome include higher levels of heme, pantothenate, homoarginine, and several sphingolipid and phospholipid species but lower levels of several metabolites, including acetyl CoA, glucuronate, carnosine, anserine, hydroxyproline, prohydroxyproline, and carnitinylated and glycinated species, in female hearts compared with male hearts. Female mice also had more extensive exercise-induced changes in the cardiac metabolome, characterized by significant increases in tyrosine, tryptophan, branched-chain amino acids, and 3-hydroxybutyrate and decreases in serine and alanine. We further tested the importance of exercise-induced elevations in branched-chain

amino acids by exercising mice for two weeks while providing low- or high-BCAA diets and found that exercise-induced cardiac growth did not occur in exercised mice consuming low-BCAA diet. Together, these findings in mice suggest that the cardiac metabolite profile is distinct in male and female mice, that female mice demonstrate more robust changes in cardiac metabolite abundance following a session of high-intensity exercise, and that BCAAs are important in stimulating exercise-induced cardiac growth.

We chose the FVB/NJ mouse strain and treadmill exercise as our model system for several reasons. Compared with other mouse strains, FVB/NJ mice are elite treadmill runners and show clear cardiac adaptation to regular treadmill training regimens.[214,293,304,328,329] Also, this strain is devoid of the known nicotinamide nucleotide transhydrogenase (*Nnt*) mutations present in the commonly used C57BL/6J strain,[330-333] which could influence metabolic responses to exercise. The treadmill exercise modality was chosen because it allows control of work and intensity and because compliance with the treadmill protocol is not an issue with the FVB/NJ strain.[12,293] Using this protocol, we observed apparent sex-based differences in work performed at low and moderate intensities; however, there was no difference in work performed during the high-intensity exercise capacity test. Thus, because we chose to study metabolic changes in the heart after a high-intensity bout of exercise, which showed no difference in work between male and female mice, differences in work do not seem to underlie the disparate responses in the cardiac metabolomes to exercise in male and female mice. Nevertheless, we found that different intensities of exercise training elicit

differential changes in circulating lactate and ketone bodies, which are known to influence cardiac metabolism.[118,291] The finding that the high-intensity protocol increased circulating 3-hydroxybutyrate only in female mice and that this corresponded with lower blood glucose levels suggests different systemic glucose handling compared with male mice. This could be due to sex-dependent differences in hormones, which could affect metabolism and are known to influence critical processes such as liver gluconeogenesis and glycogenolysis.[334] Regardless, the higher blood levels of 3-hydroxybutyrate following exercise in female mice could explain the increase in intracardiac 3-hydroxybutryate, especially since recent studies confirmed a mass-action relationship between circulating 3-hydroxybutyrate and its levels and utilization in tissue.[335] These differences in ketone body metabolism could be important given recent advances in our understanding of the significance of ketone bodies to cardiac biology.[336-338]

One of the most surprising findings of our study was the marked differences in cardiac metabolite profiles between male and female mice, even in the absence of exercise stress. Unfortunately, few comparisons between female and male sexes in cardiac biology and metabolism are available in preclinical research literature; however, numerous recent studies address sex-based differences in cardiac structure, function, metabolism, and responses to stress. For example, gonadal hormones influence cardiac cellularity and modify the levels of mesenchymal cell and leukocyte populations.[339,340] Thus, it remains possible that hormonal differences between male and female mice affect the levels of resident and circulating cells in the heart, which could influence the cardiac metabolome at

the whole-organ level. Furthermore, estrogen hormones such as 17β-estradiol influence mitochondrial dynamics[341] and could influence the steady state levels of metabolites in cardiomyocytes. As the field progresses, we anticipate that our understanding of hormone-mediated, sex-based differences in cardiac metabolism will improve.

Consistent with previous studies showing that lipid metabolism between sexes may be different,[342,343] we found higher levels of sphingomyelin and glycerophospholipid species in female hearts. Sphingomyelins are a common sphingolipid in mammalian tissues and have important structural and signaling roles.[344] The higher levels of several sphingomyelins and sphingomyelin-related species (as well as glycerophospholipids) in the female heart could imply higher synthesis or transport, or lower turnover, of these species than in the male heart; however, the significance of these differences to sex-dependent differences in cardiac biology remain unclear and require further investigation. Similarly, tryptophan, pantothenate, pyridoxamine, and homoarginine were higher basally in female hearts compared to male hearts. Tryptophan is a precursor for several intermediates and end products, including kynurenine, nicotinamide, NAD^+, and acetyl CoA. Uncontrolled catabolism of tryptophan has been demonstrated in conditions of cardiovascular disease,[345] and it remains possible that female mice have generally lower catabolism of tryptophan, which could in part underlie their endogenous cardioprotected phenotype.[346] Pantothenate and pyridoxamine, which were also higher in female hearts, are B vitamins (vitamins B5 and B6, respectively) that could affect energy metabolism. Pantothenate is required for

coenzyme A biosynthesis, and deletion of pantothenate kinase exacerbates ventricular dysfunction in pressure overload and causes marked metabolic changes.[347] Thus, it is possible that the higher basal levels of pantothenate in female hearts could play a role in maintaining CoA levels and bioenergetics. Higher levels of pyridoxamine in female hearts could also underpin the more resilient nature of the female heart[348] because pyridoxamine has been shown to protect against cardiac dysfunction caused by aging[349] or myocardial infarction.[350] Also markedly higher in female hearts was homoarginine. Homoarginine is a non-proteinogenic, vasoactive amino acid that is a candidate cardiovascular risk factor, with low circulating levels associated with cardiovascular disease.[351,352] It also has protective actions in the context of myocardial responses to ischemic injury,[353,354] which may in part underlie the known cardioprotected female phenotype.[348]

Several metabolites critical in intermediary metabolism were lower in non-exercised female hearts than in corresponding male hearts. The generally lower levels of acetyl CoA, anserine, and carnosine suggest potential differences in central carbon metabolism. Acetyl CoA participates in carbohydrate, protein, and lipid metabolism, with a primary function being the delivery of acetyl groups to the tricarboxylic acid (TCA) cycle. Although these data could suggest a limitation in energy metabolism in the female heart, the fact that none of the TCA cycle intermediates were significantly lower suggests that the lower levels of acetyl CoA are likely not related to energy deficits. Metabolomic data further indicated sex-dependent differences in glucose metabolism. Maltotriose, an intermediate in glycogen metabolism, was lower in female hearts, as were carnosine and

anserine, which are small dipeptides that influence glycolytic rate by buffering protons and can scavenge reactive electrophiles generated from oxidative stress.[355] Moreover, glucose-derived intermediates in the ascorbic acid synthesis pathway, i.e., glucuronate and gulonate, were lower in female hearts. Further understanding of basal sex-dependent differences in cardiac glucose metabolism may require carefully designed glucose tracer studies.

After exercise, corticosterone was the only metabolite that was consistently higher in both male and female hearts. While not measured in this study, blood levels of corticosterone increase immediately after exercise in several species,[356-362] which could underlie its higher abundance in the heart after exercise. Corticosterone is required in fetal cardiac development,[363] and corticosterone signaling is essential for maintaining cardiac function in adult mice.[364] Although it remains unclear what the role of corticosterone may be in the post-exercised heart, it has been suggested to influence exercise-induced cardiac hypertrophy.[365] Nevertheless, the extent to which it does so remains unclear. Given that it influences inflammatory responses,[366] known to be important for tissue repair,[366] it could play a role in tissue remodeling by altering immune cell responses. Furthermore, glucocorticoids such as corticosterone can influence the expression of genes such as cyclooxygenase-1 and -2 in cardiomyocytes[367-369] in part through its interaction with C/EBPβ,[370] which is known to be involved in exercise-induced cardiac remodeling.[225] Moreover, glucocorticoid receptor activation in myocytes regulates the expression of numerous genes that contribute to cardiac hypertrophy.[371] Therefore, it is plausible that acute, exercise-induced increases in

corticosterone could influence gene programs important for cardiac adaptation to exercise.

Immediately after high-intensity exercise, female hearts had higher levels of tryptophan, tyrosine, and allantoin but lower levels of alanine and serine. One hour after the exercise bout, several species (in addition to 3-hydroxybutryate) were elevated, including long chain amino acids, branched-chain amino acids, CDP-choline, and glycerophospholipid species. This could suggest alterations in catabolic pathways such as fatty acid and BCAA oxidation as well as phospholipid metabolism. Understanding the significance of elevations in these metabolites to cardiac responses to exercise requires further inquiry.

Because BCAAs promote protein synthesis in the heart,[326,327] investigated the role of exercise-induced elevations in cardiac BCAA abundance in cardiovascular adaptation to exercise by subjecting mice to two weeks of exercise training while consuming low-BCAA or high-BCAA diets. To start, we chose to use only male, FVB/NJ mice because we consistently observe significant growth in the heart following two weeks of forced treadmill running; we see no significant growth in hearts of female mice following any duration of exercise training. Consistent with the role of BCAAs in protein synthesis, we observed growth in hearts of exercised mice consuming high-BCAA diet, but not in exercised mice consuming low-BCAA diet. Conventionally, BCAA-induced hypertrophic responses are mediated by nutrient sensing of leucine by sestrin2 to activate mammalian target of rapamycin.[372] The finding that female FVB/NJ mice had significant elevations in plasma and myocardial BCAAs but historically do not show exercise-induced

cardiac growth could highlight other roles of BCAAs in cardiovascular responses to exercise. Some studies suggest that myocardial BCAA oxidation increases following exercise,[373] and it is worth further study to test whether exercise-induced elevations in BCAAs are fated for oxidation to maintain TCA pool, or if these transient elevations promote hypertrophic signaling through mTOR.

There are some limitations to this study that deserve mention. First, the untargeted metabolomics approach used in this study to measure cardiac metabolite abundances does not deliver confident assessments in flux.[374] Thus, additional studies using stable isotope tracers *in vivo*[375] could provide a more refined view of how cardiac metabolism changes with exercise. Second, although we showed that exercise intensity influences circulating substrate availability, we did not perform metabolomics analyses on hearts of mice exercised at low or moderate intensities; rather, we examined metabolomic changes in the heart after an acute, exhaustive bout of exercise, which is thought to be a stronger stimulus for growth. Future studies will be required to delineate how low- and moderate-intensity exercise affects cardiac metabolism. Because the focus of this study was on acute changes in cardiac metabolism after exercise, we did not include an exercise-adapted group as a comparison. Additionally, in examining how BCAAs influence exercise-induced cardiac growth, we forced chronic elevations in BCAAs through dietary intervention, which likely influences systemic physiology. Because chronic elevations in BCAAs are associated with insulin resistance[376] and cardiac dysfunction,[179] future studies are needed to discriminate between beneficial and deleterious actions of BCAAs. Finally, after a chronic training regimen, we must

consider it possible the metabolic response to exercise may be different than the responses seen in the untrained heart. Additionally, the state of exercise adaptation could exacerbate, or even negate, the biological sex-dependent differences observed following an acute bout of exercise in the untrained state.

CONCLUSIONS

The findings of this study show intrinsic differences in cardiac metabolite profiles between male and female mice and show that, following exercise, the female cardiac metabolome changes to a greater extent than the male cardiac metabolome. Whether these sex-dependent differences are a result of a different hormonal milieu or are due to intrinsically higher sensitivity to metabolic stress requires further study. Exercise-induced elevations in myocardial BCAAs appear important for exercise-induced cardiac growth, but the role is likely sex-dependent since we only observe exercise-induced cardiac growth in hearts of male, FVB/NJ mice. Understanding how these metabolic differences influence exercise-induced cardiac adaptations and contribute to cardiac resilience to insult or injury is an exciting goal for future studies.

Part of this chapter previously appeared as an article in the Journal of Sport and Health Science and was adapted to fit this dissertation. The original citation is as follows: Fulghum K, Collins HE, Jones SP, Hill BG. Influence of biological sex and exercise on murine cardiac metabolism. *J Sport and Health Sci.* 11(4):479-494, 2022.

Table 1

Metabolite	HMDB	FDR	*p* value	Relative fold change (female/male)
1-(1-enyl-palmitoyl)-2-arachidonoyl-GPE (P-16:0/20:4) *	HMDB0011352	0.058985	0.0049448	1.8496
1-(1-enyl-palmitoyl)-2-palmitoyl-GPC (P-16:0/16:0) *	HMDB0011206	0.098967	0.01363	1.5773
1-(1-enyl-stearoyl)-2-arachidonoyl-GPE (P-18:0/20:4)*	HMDB0005779	0.042021	0.0026001	2.4368
1,2-dipalmitoyl-GPC (16:0/16:0)	HMDB0000564	0.032553	0.0017543	1.1384
1,2-dipalmitoyl-GPE (16:0/16:0) *	HMDB0008923	0.085015	0.010012	1.8653
1,5-anhydroglucitol (1,5-AG)	HMDB0002712	0.026958	0.001071	0.74591
18-methylnonadecanoate (i20:0)		0.076639	0.0077645	0.64032
1-palmitoyl-2-oleoyl-GPE (16:0/18:1)	HMDB0005320	0.064962	0.0057053	1.7594
1-palmitoyl-2-stearoyl-GPC (16:0/18:0)	HMDB0007970	0.080764	0.0088663	1.4596
1-palmitoyl-2-stearoyl-GPE (16:0/18:0) *	HMDB08925	0.095363	0.012943	2.2263
1-stearoyl-2-arachidonoyl-GPC (18:0/20:4)	HMDB0008048	0.048158	0.0034605	1.388
1-stearoyl-2-oleoyl-GPE (18:0/18:1)	HMDB0008993	0.076639	0.0081075	2.1764
2,4-dihydroxybutyrate	HMDB0000360	0.017783	0.0004969	0.45552
2-hydroxybutyrate/2-hydroxyisobutyrate	HMDB0000729 HMDB00008	0.032046	0.0016631	0.57854
5,6-dihydrouridine	HMDB0000497	0.017806	0.0005452	2.0412
acetyl-CoA	HMDB0001206	0.085608	0.010594	0.48448
alpha-hydroxyisovalerate	HMDB0000407	0.0012158	2.43E-06	0.17297
anserine	HMDB0000194	0.0023567	1.42E-05	0.45349
arachidoylcarnitine (C20) *	HMDB0006460	0.052613	0.0040478	0.28036
ascorbic acid 3-sulfate*		0.041488	0.0024843	1.8814
betaine	HMDB0000043	0.069631	0.0065322	0.75022
bicine	HMDB0011727	0.093213	0.012466	1.922
carnosine	HMDB0000033	0.0028901	2.88E-05	0.43553
ceramide (d18:2/24:1, d18:1/24:2) *		0.092441	0.011879	1.6207
C-glycosyltryptophan	HMDB0240296	0.026958	0.0010465	0.49009
deoxycarnitine	HMDB0001161	0.052613	0.0040956	0.78712
diacylglycerol (16:1/18:2 [2], 16:0/18:3 [1]) *		0.076639	0.0078431	2.2958

equol sulfate		0.084622	0.0096276	0.20255
erucoylcarnitine (C22:1) *		0.032046	0.0016039	0.37269
gamma-glutamylvaline	HMDB0011172	0.017806	0.0005687	0.33472
glucuronate	HMDB0000127	0.026958	0.0011838	0.52161
glycerophosphoethanolamine	HMDB0000114	0.092559	0.012193	0.64594
gulonate*	HMDB0003290	0.031793	0.0014596	0.42189
hexanoylglycine (C6)	HMDB0000701	0.0023567	1.88E-05	0.18636
homoarginine	HMDB0000670	0.017783	0.0004852	1.7604
hydroxypalmitoyl sphingomyelin (d18:1/16:0(OH))		0.052069	0.0038454	1.3499
hydroxyproline		0.067516	0.0061991	0.60524
imidazole lactate	HMDB0002320	0.0034709	4.16E-05	0.23819
indolelactate	HMDB0000671	0.0043038	6.01E-05	1.9888
isovalerylglycine	HMDB00678	0.0023567	1.52E-05	0.27293
maleate	HMDB0000176	0.076639	0.0079138	0.5487
methylsuccinate	HMDB0001844	0.058985	0.0048017	1.2955
N,N,N-trimethyl-5-aminovalerate		0.025865	0.0008777	2.105
N-acetylglutamate	HMDB0001138	0.092441	0.011732	0.78083
N-acetylglutamine	HMDB0006029	0.014512	0.0002897	0.51854
N-acetylglycine	HMDB0000532	0.0064952	0.0001095	0.52201
N-acetylleucine	HMDB0011756	0.038054	0.0022027	0.59356
N-stearoyl-sphingadienine (d18:2/18:0) *		0.026958	0.0010791	2.0202
N-stearoyl-sphingosine (d18:1/18:0) *	HMDB0004950	0.026958	0.0011398	1.868
N-stearoyltaurine		0.092441	0.011993	0.75217
oleoyl-oleoyl-glycerol (18:1/18:1) [2] *	HMDB0007218	0.073131	0.0070066	2.279
orotate	HMDB0000226	0.083315	0.0093126	0.59776
orotidine	HMDB0000788	0.066986	0.0060167	0.73148
pantothenate (Vitamin B5)	HMDB0000210	0.017429	0.0003993	3.9236
phenylacetylglycine	HMDB0000821	0.048158	0.0033927	0.52079
prolyl-hydroxyproline		0.058985	0.0049184	0.44851
pseudouridine	HMDB0000767	0.017429	0.0004175	1.7891
pyridoxamine	HMDB0001431	0.032046	0.0016585	1.4963
serine	HMDB0000187	0.085608	0.01058	1.3084
sphingomyelin (d18:0/18:0, d19:0/17:0) *	HMDB0012087	0.077799	0.0083855	1.9696
sphingomyelin (d18:1/14:0, d16:1/16:0) *	HMDB0012097	0.062539	0.0053676	1.5269
sphingomyelin (d18:1/17:0, d17:1/18:0, d19:1/16:0)		0.0064952	0.0001167	1.6125
sphingomyelin (d18:1/18:1, d18:2/18:0)	HMDB0012101	0.047581	0.003229	1.4112

stearoyl sphingomyelin (d18:1/18:0)	HMDB0001348	0.038054	0.0021945	1.4065
thiamin diphosphate	HMDB0001372	0.076639	0.0080241	0.72402
threonine	HMDB0000167	0.085608	0.010285	1.2521
tricosanoyl sphingomyelin (d18:1/23:0) *	HMDB0012105	0.085015	0.0098422	1.6406
triethanolamine	HMDB0032538	0.046539	0.0030654	2.7857
tryptophan	HMDB0000929	0.046539	0.0029834	1.4203

Table 1. Significantly different metabolites in male *vs.* female mouse hearts under sedentary conditions.

Notes: Hearts from male and female FVB/NJ wild-type mice were freeze-clamped, and 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 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/MS^2 accurate mass platform. Shown are those metabolites with an FDR value threshold of 0.10 or less. *n* = 4 female hearts and 5 male hearts per group.

Abbreviations: FDR = false discovery rate; HMDB = human metabolome database; LC/MS = liquid chromatography/mass spectrometry; UHPLC/MS/MS = Ultra-high performance liquid chromatography/mass spectrometry/mass spectrometry; GPE = glycerophosphoethanolamine ; GPC = glycerophosphocholine

CHAPTER IV

IN VIVO DEEP NETWORK TRACING REVEALS PHOSPHOFRUCTOKINASE-MEDIATED COORDINATION OF BIOSYNTHETIC PATHWAY ACTIVITY IN THE MYOCARDIUM

INTRODUCTION

Several enzymatic steps in glucose metabolism modulate cardiac structure and function. For example, recent studies indicate that lactate dehydrogenase,[377,378] pyruvate dehydrogenase,[379-381] and the mitochondrial pyruvate carrier[382-384] influence pathological or physiological remodeling of the heart. Phosphofructokinase-1 (PFK1) also influences cardiac remodeling. PFK1 activity is elevated in pressure overloaded hearts,[385] and high PFK1 activity appears sufficient to promote mild dilated cardiomyopathy.[214] Conversely, cardiac PFK1 activity is lower during exercise and instigates physiologic cardiac growth.[214] PFK1 activity is modulated both by post-translational modification and by allosteric effectors, most notably fructose-2,6-bisphosphate (F2,6BP), which is produced by isoforms of phosphofructokinase-2 (PFK2).[214] Nevertheless, it remains unclear how changes in the activity of critical metabolic enzymes such as PFK1 coordinate energy conversion and anabolic activity.

The networks of metabolism provide conduits for directing nutrients toward multiple fates. Particularly important to nutrient fate are metabolic intermediates that convene at branchpoint sites and direct metabolites for catabolism or building block synthesis. For example, metabolites such as glucose 6-phosphate (G6P), fructose 6-phosphate (F6P), dihydroxyacetone phosphate (DHAP), and 3-phosphoglycerate (3PG) can remain in the glycolytic pathway for transformation to pyruvate, or they can enter into ancillary pathways such the pentose phosphate, hexosamine biosynthetic, glycerolipid, and serine synthesis pathways,[294] some of which branch further to direct carbon flow to additional fates (**Fig. 21**). How these branchpoints are regulated in tissues such as the heart *in vivo* remains unclear.

Much of our knowledge of the metabolic phenotype of the heart has arisen from isolated organ, cell, or organelle data. In the intact heart, most approaches to measure metabolism fail to provide detailed information on biosynthetic pathways. Furthermore, most *in vivo* ^{13}C labeling approaches are transient in nature and require bolus injections, gavage, or continuous tracer infusion, which can produce unwanted artifacts or stress responses due to anesthesia or physical restraint.[386] Moreover, these labeling methods are often limited to fast turnover pathways such as glycolysis and the Krebs cycle and may not provide sufficient time for adequate labeling of biosynthetic pathways. Introduction of dietary $^{13}C_6$-glucose, when coupled with mass spectrometry (MS) and nuclear magnetic resonance (NMR) analysis, can be used to trace the fate of glucose-derived carbon in relatively slower biosynthetic pathways such as the nucleotide and glycerophospholipid synthesis pathways.[386,387]

Figure 21

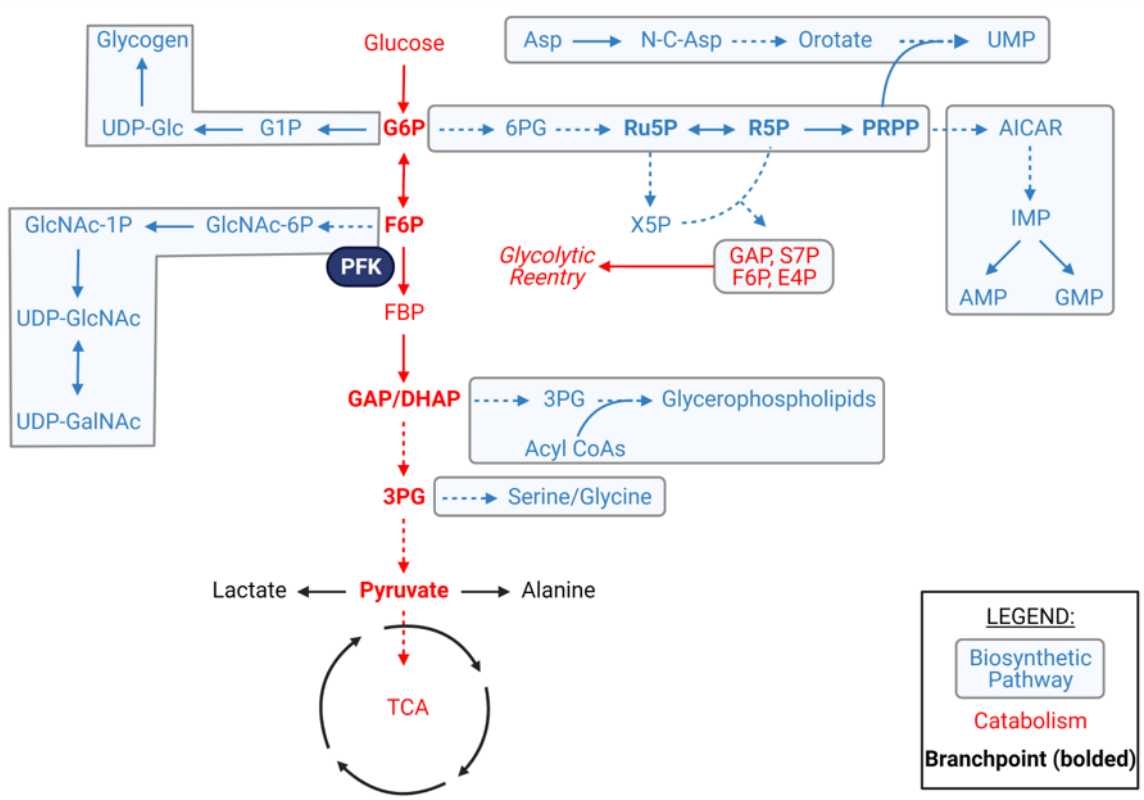


Figure 21: Glucose-derived amphibolic metabolites and biosynthetic pathway enrichment. Metabolic network map highlighting potential modes of glucose utilization and branchpoint intermediates (bolded) which could influence the balance between catabolism and anabolism within a cell. Asp = aspartate, UMP = uridine monophosphate, UDP = uridine diphosphate, Glc = glucose, G1P = glucose-1-phosphate, G6P = glucose-6-phosphate, 6PG = 6-phosphogluconate, Ru5P = ribulose-5-phosphate, R5P = ribose-5-phosphate, PRPP = phosphoribosyl pyrophosphate, AICAR = 5-aminoimidazole-4-caroxamide ribonucleotide, IMP = inosine monophosphate, GMP = guanosine monophosphate, AMP = adenosine monophosphate, F6P = fructose-6-phosphate, FBP = fructose-1,6-bisphosphate, PFK = phosphofructokinase, X5P = xylulose-5-phosphate, GAP = glyceraldehyde-3-phosphate, S7P = seduheptulose-7-phosphate, E4P = erythrose-4-phosphate, DHAP = dihydroxyacetone phosphate, 3PG = 3-phosphoglycerate, TCA = tricarboxylic acid cycle.

Here, we report dietary administration of ^{13}C-labeled glucose to examine ancillary biosynthetic pathways of glucose metabolism in transgenic mice bearing cardiac-specific isoforms of PFK2. These transgenes code for the bifunctional enzyme defective in either the kinase or phosphatase domains; when expressed, they increase or decrease F2,6BP levels, thereby augmenting or lowering PFK1 activity and glycolytic flux.[145,214] We found that phosphorylated sugars accumulate in mouse hearts with constitutively low PFK1 activity and are associated with elevated glycogen levels; however, hexosamine biosynthetic pathway activity is not affected by high levels of sugar phosphates. Interestingly, we found that low PFK1 activity diverts glucose-derived carbon toward synthesis of the purine biosynthetic pathway intermediate 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), which was associated with the presence of large multimeric complexes containing chaperones and metabolic enzymes, including those required for AICAR biosynthesis. Collectively, these findings illustrate the utility of deep network tracing for gaining new insights into the regulation of cardiac metabolism *in vivo* and provide evidence of PFK-regulated metabolic channeling in the heart.

EXPERIMENTAL METHODS

Resource Table

Reagent	Source	Catalog
Rabbit polyclonal Anti-PAICS antibody	Millipore Sigma	AV46049 (1:1000 dilution)

HRP-linked Anti-Rabbit antibody	Cell Signaling Technologies	#7074 (1:1000 dilution)
Basal diet base	Envigo	TD.150344.PWD
$^{12}C_6$-glucose	Sigma Aldrich	#G7528
$^{13}C_6$-glucose	Sigma Aldrich	#389374
Acetonitrile	ThermoFisher	A956-1
Chloroform	Sigma Aldrich	650498
MOPS	Fisher	BP308
Mannitol	VWR	BDH9248
Sucrose	VWR	BDH9308
EGTA	Sigma Aldrich	E4378
Digitonin	Sigma Aldrich	300410
Coomassie Blue G250	Fisher	BP100
Polyacrylamide (acrylamide:bis-acrylamide 37.5:1)	BioRad	#1610158
Tricine	Sigma Aldrich	T0377
Bis-Tris	VWR	#0715
Trypsin	Promega	V511A
DTT	Sigma Aldrich	D0632
TEA-BC	Sigma Aldrich	T7408
Iodoacetamide	Sigma Aldrich	I1149
Formic Acid	Fisher	A117-50
Acetonitrile	Fisher	A955-4
LC-MS grade Water	Fisher	W6-4
Equipment	**Source**	**Catalog**
Free Fatty-Acid Quantitation Kit	Sigma Aldrich	MAK044-1KT
Rat/Mouse Insulin ELISA	Millipore Sigma	EZRMMI-13K
Orbitrap Fusion Tribrid mass spectrometer	ThermoFisher	https://www.thermofisher.com/order/catalog/product/IQLAAEGAAPFADBMBCX#/IQLAAEGAAPFADBMBCX
Dionex ICS 5000	Dionex	https://www.thermofisher.com/us/en/home/industrial/chromatography/ion-chromatography-ic/ion-chromatography-systems.html
Orbitrap Elite - ETD mass spectrometer	ThermoFisher	https://assets.thermofisher.com/TFS-Assets/CMD/brochures/BR-30232-LC-MS-Orbitrap-Elite-BR30232-EN.pdf

Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer	ThermoFisher	https://assets.thermofisher.com/TFS-Assets/CMD/Specification-Sheets/PS-64048-LC-MS-Q-Exactive-HF-Orbitrap-PS64048-EN.pdf
MestReNova v.12.0.0	Mestrelab Research S.L.	https://mestrelab.com/download/mnova/
Agilent Varian DD2 spectrometer	Agilent	https://www.agilent.com/cs/library/slidepresentation/public/2.DD2console.pdf
0.45 µm Regenerated Cellulose Syringe Filter	Fisher	F2504-7
Equipment	**Source**	**Catalog**
MetaboAnalyst 5.0	MetaboAnalyst	https://www.metaboanalyst.ca/
Prism 9.0	GraphPad	https://www.graphpad.com/scientific-software/prism/
Proteome Discoverer v1.4.1.114	ThermoFisher	https://www.thermofisher.com
Xcalibur v2.2	ThermoFisher	https://www.thermofisher.com
TraceFinder 3.3	ThermoFisher	https://assets.thermofisher.com/TFS-Assets/CMD/manuals/Man-XCALI-97728-TraceFinder-33-User-Quan-ManXCALI97728-A-EN.pdf

Mice

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Louisville. For liquid diet standardization, adult, male FVB/6J and C57BL/6J mice (16–18 weeks of age) were used. For studies addressing the influence of PFK on metabolism, adult, male mice on the FVB background (22–24 weeks of age) overexpressing kinase- or phosphatase-deficient 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase transgenes[388,389] in the heart (termed Glyco^{Lo} or Glyco^{Hi} mice, respectively) and wild-type (WT) littermates were used. All mice were kept on a 12 h:12 h light: dark cycle. Normal

chow and water were provided *ad libitum* unless provided liquid diet for deep network tracing experiments. Upon completion of each experiment, mice were anesthetized with sodium pentobarbital (150 mg/kg, i.p.) and mice were euthanized via excision of the heart. Following, tissues were harvested. These procedures are consistent with the AVMA *Guidelines on Euthanasia.*

Stable isotope-resolved metabolomics (SIRM)

Powdered, basal diet (TD.150344.PWD) was purchased from Envigo Teklad Diets (Madison, WI) and dissolved in water, with addition of the glucose source ($^{12}C_6$-glucose and $^{13}C_6$-glucose purchased from Sigma Aldrich, #G7528 and #389374, respectively) to create liquid diet. For isotope tracing, adult, male Glyco^{Lo} and Glyco^{Hi} mice, alongside WT littermates, were provided ~20 g of $^{12}C_6$-glucose-containing liquid diet (0.167 g glucose/g diet) per day over the course of 54 h to acclimate to feeding on liquid diet. Then, diet was replaced with $^{13}C_6$-glucose-containing liquid diet (~20 g; 0.173 g glucose/g diet) for the final 18 h of feeding. Each mouse was singly housed during the liquid diet feeding protocol. Following the feeding protocol, mice were anesthetized with pentobarbital and euthanized. Hearts were freeze-clamped *in situ* using liquid N_2-cooled Wollenberger tongs for analyses while additional tissues were snap-frozen in liquid N_2. Frozen heart tissue was pulverized in liquid N_2 into small particles, and 20 mg of each sample was extracted in acetonitrile/water/chloroform (V/V 2:1.5:1) to separate into polar, lipid, and protein fractions. Polar fractions were lyophilized and reconstituted in 30 µL nanopure water for analysis via Dionex ICS-5000+ion

chromatograph interfaced to Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Peak areas were integrated and exported to Excel via TraceFinder 3.3 (Thermo) software package, and natural abundance was corrected for each isotopologue as described previously.[390,391] Fractional enrichment and total abundance (μmole/g protein) were calculated to quantify ^{13}C enrichment.

Nuclear magnetic resonance

The polar extracts were additionally analyzed via NMR spectroscopy. NMR spectra were recorded at 15°C on 14.1 T on a Varian DD2 spectrometer equipped with a 3 mm inverse triple resonance (HCN) cold probe as previously described.[392] ^{1}H PRESAT spectra were recorded with an acquisition time of 2 s with a relaxation delay of 4 s, during which a weak transmitter pulse was applied to suppress the residual HDO resonance. $^{1}H[^{13}C]$ HSQC spectra were recorded with an acquisition time of 0.25 s and a relaxation delay of 1.75 s, with GARP decoupling during the detection period. Free induction decays were transformed using MNOVA software with zero-filling to 128 k points (PRESAT) or 8 k points (HSQC), anodizing with a cosine-squared function and a 1 Hz (PRESAT) or 4 Hz (HSQC) line broadening exponential function. Spectra were phased, baseline corrected using third order Bernstein polynomials, and referenced to internal DSS-d6 (27.5 nmol) at 0 ppm. Resonances of individual metabolites were integrated using mixed Lorentzian-Gaussian line fitting, and the areas were normalized to the DSS resonance. This normalized value was then corrected for the amount of material in the extract and

further normalized to the tissue protein level. Isotopomer levels, F, were calculated as: $F = A(^{13}C)/[A(^{13}C)+A(^{12}C)]$ where $A(^{13}C)$, $A(^{12}C)$ are the areas of the protons attached to ^{13}C or ^{12}C, respectively.

Circulating substrate measurements

Blood samples for circulating glucose and lactate were obtained via tail clip and measured in about 0.7 µl blood using the Accu-Check Aviva meter (Roche) and the Lactate Plus meter (Nova Biomedical), respectively.

Indirect calorimetry

Respiratory exchange ratio (VCO_2/VO_2) and locomotion, ambulatory and fine movements were measured using a metabolic cage system (TSE PhenoMaster System, Bad Homberg, Germany). Mice were acclimated to metabolic cage system and then monitored for 24 h while consuming either normal chow or liquid diet.

Blue-native PAGE separation of protein complexes

Multimeric enzyme complexes were separated in their native state using Blue Native PAGE (BN-PAGE). Briefly, hearts were homogenized in 5 mM MOPS buffer containing 220 mM mannitol, 70 mM sucrose, and 1 mM EGTA (pH 7.2) using a Teflon-coated Glass-Col homogenizer. The homogenate was then centrifuged at 10,000*g* for 10 min. To the supernatant, we added digitonin to give

a detergent/protein ratio of 8:1 (gram:gram) and incubated the samples on ice for 20 min. Following centrifugation at 14,000*g* for 20 min, we added Coomassie dye for protein separation by BN-PAGE. The BN-PAGE gels were prepared using polyacrylamide (acrylamide:bis-acrylamide, 37.5:1) at either a 5–15% or a 3–12% gradient. The cathode buffers contained 50 mM Tricine, 15 mM Bis-Tris, pH 7.0, and 0.02% Coomassie Blue G250. The anode buffer consisted of 50 mM Bis-Tris pH 7.0. Electrophoresis was performed at 4°C using high blue buffer at 100 V for 1 h, followed by low blue buffer at 250 V for 1.5 h.

Size exclusion chromatography (SEC)

SEC was carried out at room temperature on a Sepharose 6 Increase 10/300 column (GE Healthcare) equilibrated with PBS at a flow rate of 0.5 ml/min. The column was calibrated using both low and high molecular weight calibration kits (GE Healthcare) by plotting molecular weights of standards versus their relative elution volume. Elution position was determined by measuring the absorbance of the eluted fractions at 280 nm. Eluted peaks were concentrated approximately 10-fold on Amicon Ultra-4 spin columns. Following SEC separation, the fractions were analyzed either by Blue Native PAGE or by immunblotting following SDS-PAGE. For the latter, the fractions were loaded onto a denaturing SDS-PAGE gel and the proteins were transferred to PVDF membranes. An anti-PAICS polyclonal antibody (Millipore Sigma, AV46049) was used as the 1° antibody (1:1000 dilution). A streptavidin-linked anti-rabbit 2°antibodies (1:1000 dilution) was used to detect PAICS immunoreactivity.

Mass spectrometric protein identification

Proteins excised from BN-PAGE gels were identified by liquid chromatograph (LC) ESI MS/MS, after the in-gel trypsin digestion as previously described with modifications [393]. For in-gel digestion, Coomassie-stained BN-PAGE gel bands were cut into 1-mm^3 plugs and incubated in 100 mM triethylammonium bicarbonate (TEA-BC; Sigma) at room temperature for 15 min. Acetonitrile was then added to the TEA-BC solution, and the gel plugs were incubated at room temperature for 15 min with gentle vortexing. The solvent was removed, and the washing process was repeated until the Coomassie Blue stain was no longer visible. Solvent was removed, and the gel plugs were dried in a SpeedVac for 5 min. The dried plugs were incubated in DTT (20 mM DTT, 100 mM TEA-BC) at 56°C for 45 min, followed by iodoacetamide (55 mM iodoacetamide, 100 mM TEA-BC) at room temperature for 30 min. Iodoacetamide was removed, and gels were washed in 50 mM TEA-BC at room temperature for 15 min, followed by gentle vortexing in acetonitrile for 15 min at room temperature. The gel plugs were again dried for 5 min in a SpeedVac and incubated in digestion buffer [20 ng/µl modified Trypsin (Promega) in 50 mM TEA-BC] for 10 min. Then, 50 mM TEA-BC was added to the plugs, followed by 37°C overnight incubation in a shaker. Peptides were extracted from the gel pieces [394] and the digest was diluted with one volume of LC-MS grade water, four volumes of 1:2 5% v/v formic acid in water:acetonitrile, and incubated for 15 min in a shaker at 100 RPM. The extract was dried in a SpeedVac, dissolved in 20 µl 2% v/v acetonitrile/0.1% v/v

formic acid, and filtered through a 0.45 µm regenerated cellulose syringe filter (ThermoFisher); 4 µl were used for analysis.

For protein identification, peptides were analyzed using an Easy-nLC 1000 system and an Orbitrap Elite - ETD mass spectrometer (ThermoFisher Scientific, Waltham, MA) with 1.6 kV of spray voltage and 225°C at the heated desolvation capillary. A 100 µm ID fused silica needle tip packed in-house with 12 cm of Aeris Peptide 3.6 µm XB-C18 material (Phenomenex, Torrance, CA) was used with the EASY-nLC 1000. Samples were separated with a 45 min linear gradient from 0% B (100% A) to 50% B at 250 nl/min, followed by a 5 min linear gradient from 50% B to 95% B and linear flow ramp from 250 to 300 nl/min, and a 10 min wash with 95% B at 300 nl/min. Solvent A = A 2% v/v acetonitrile / 0.1% v/v formic and B = 80% v/v acetonitrile / 0.1% v/v formic acid. A Nanospray Flex source (ThermoFisher) was used to introduce sample to the Orbitrap Elite. An Nth Order Double Play method was created in Xcalibur v2.2 (ThermoFisher) to acquire data with the Elite. Scan event one obtained an FTMS MS1 scan (normal mass range; 240,000 resolution, full scan type, positive polarity, profile data type) for the range 300–2000 m/z. Scan event two obtained ITMS MS2 scans (normal mass range, rapid scan rate, centroid data type) on up to twenty peaks with a minimum signal threshold of 5,000 counts from scan event one. The lock mass option was enabled (0% lock mass abundance) using the 371.101236m/z polysiloxane peak. The data was analyzed in Proteome Discoverer v1.4.1.114 (ThermoFisher) using Mascot v2.5.1 and SequestHT and the 2/6/2017 version of the UniprotKB *Mus musculus* reference proteome canonical and isoform sequences (Proteome ID

UP000000589). The enzyme specified was trypsin (maximum two missed cleavages; inhibition by P) with Carbamidomethyl(C) as static and Oxidation(M) as dynamic modifications. Tolerances were 1.0 Da (monoisotopic) for fragments and 50ppm (monoisotopic) for parent masses. The result files from Proteome Discoverer were loaded into Scaffold Q+S v4.4.5. The false discovery rate was calculated using the Scaffold Local FDR and Protein Prophet algorithms. Peptides were accepted if the identification had probability greater than 99.9% and parent mass error within 2 ppm. Proteins were accepted if they had a probability greater than 99.9% and at least one peptide. Proteins were grouped into clusters to satisfy the parsimony principle. Following iBAQ normalization, proteins in selected bands were subjected to univariate and multivariate tests.

Statistical Analyses

No data were excluded for statistical purposes. Differences in pool total abundances and isotopologue total abundances were calculated using one-way or two-way ANOVA, respectively, with Tukey's multiple comparison. For examining protein complexes, ANOVA was used with an FDR cutoff of 0.05. Correlation coefficients, PLS-DA analysis, and VIP score analyses were assessed using Metaboanalyst software (https://www.metaboanalyst.ca). Analyses were performed using GraphPad Prism 9 (La Jolla, CA, USA) and significance was assigned when $p<0.05$.

RESULTS

Standardization of liquid diet feeding: To examine the utility of *in vivo* deep network tracing for cardiac metabolic phenotyping, we first standardized dietary delivery of $^{13}C_6$-glucose in adult C57BL/6J and FVB/NJ mouse strains, which are commonly used in biomedical research.[395,396] Although both strains of mice could consume up to ~20 g of this diet in a 24 h period, consumption of ~14 g of liquid diet was sufficient to maintain body weight in both strains (**Fig. 22A-B**). We then placed individual mice and liquid diet in metabolic chambers to assess ambulatory activity and diet-induced changes in the respiratory exchange ratio (RER) (**Fig. 22C**). Although strain-specific differences in ambulatory activity were identified, we found that the liquid diet did not significantly influence ambulatory activity (**Fig. 22D-F**). As expected from the higher carbohydrate levels in liquid diet (**Table 2**), the respiratory exchange ratio was higher in both mouse strains on liquid diet when compared with normal chow (**Fig. 22G**). At the end of the feeding period, we found that the liquid diet did not significantly affect circulating free fatty acid or insulin levels compared with normal chow (**Fig. 22H-I**).

PFK1 activity influences ancillary biosynthetic pathways that branch from G6P and F6P: After standardization of liquid diet delivery, we examined the metabolic fate of dietary $^{13}C_6$-glucose in FVB mice that constitutively express kinase- or phosphatase-deficient 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in the heart; these mice are termed Glyco^{Lo} or Glyco^{Hi} mice, respectively. By controlling levels of fructose-2,6-bisphosphate, expression of the Glyco^{Lo} or

Glyco^{Hi} transgenes regulate the activity of cardiac phosphofructokinase.[145,214,388,389] Glyco^{Lo} and Glyco^{Hi} mice were fed the $^{13}C_6$-containing liquid diet for 18 h, after which the hearts were freeze-clamped *in situ*. Metabolite extracts from excised heart tissue were then analyzed by NMR and ion exchange chromatography mass spectrometry (IC-MS) to quantify metabolite abundance

Figure 22

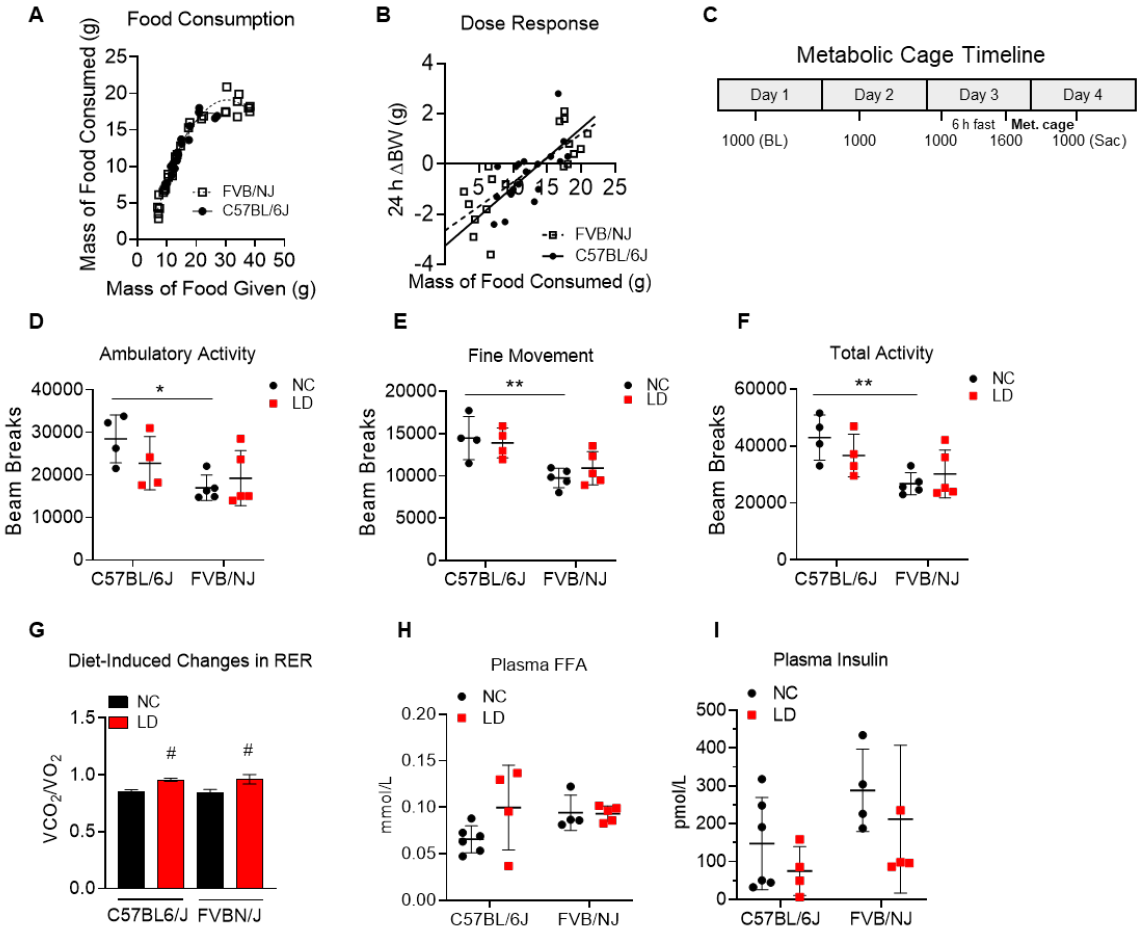


Figure 22: Standardization of liquid diet feeding. (**A**) Twenty-four hour food consumption and (**B**) dose response of liquid diet feeding in male C57BL/6J and FVB/NJ mice, and (**C**) timeline to test metabolic considerations of diet. Panels **D-F** show activity data while panel (**G**) shows RER measurements in chow and liquid diet-fed mice. (**H**) Circulating fatty acid levels and (**I**) plasma insulin in chow and liquid diet-fed mice. Figures **D-I**, *n*=4–6/gp. *p<0.05, **p<0.01, 2-way ANOVA with Bonferroni's correction. #p<0.0001, 2-way ANOVA with Tukey's post-hoc test normal vs. liquid diet. RER = respiratory exchange ratio, FFA = free fatty acid.

Table 2

Ingredient	g/kg Mix for Glucose Base Liquid Diet	%kcal
Casein	53.39	17.7
L-Cystine	0.8	0.3
Soybean oil	18.68	16
Vitamins/Minerals	29.23	<0.1
$^{12}C_6$/$^{13}C_6$-glucose	164.87/170.32	66
Water	733	0

Table 2: Composition of Liquid Diet. Basal murine diet supplied in powdered form and mixed with water and carbohydrate source. Liquid diet was supplied to mice for *ad libitum* consumption in glass feeders.

and ^{13}C metabolite enrichment (**Fig. 23A**). The genotypes consumed equivalent amounts of the $^{13}C_6$-glucose labeled diet during the feeding protocol (**Fig. 23B**). Moreover, we found no differences in circulating glucose or lactate levels between the groups (**Fig. 23C-D**).

Because PFK is expected to exert strong control over glycolytic metabolite levels,[240] we first constructed modified cross-over plots of relative metabolite abundance. These plots have been used to assess the influence of effectors on metabolite levels in enzymatic reaction chains[397]; here, they are used to present metabolite data in graphical form to assess which metabolic pathways are most responsive to differences in PFK1 activity. High PFK activity in Glyco^{Hi} hearts reduced levels of G6P and F6P relative to WT littermates, whereas low PFK activity in Glyco^{Lo} hearts increased G6P and F6P (**Fig. 23E**); however, the levels of other branchpoint metabolites, such as 3PG and pyruvate were not different between Glyco^{Lo} and Glyco^{Hi} hearts.

Because G6P and F6P contribute to glycogen synthesis, the hexosamine biosynthetic pathway (HBP), the pentose phosphate pathway (PPP), and nucleotide biosynthesis pathways, we also constructed cross-over plots for these pathways. As predicted from our previous results,[214] low PFK activity is associated with a higher abundance of glycogen synthetic metabolites such as glucose-1-phosphate (G1P) and glycogen (**Fig. 23F**); however, UDP-glucose levels were not different between the genotypes. Isotopologue analysis revealed higher levels of both unlabeled G1P (m+0) and fully labeled $^{13}C_6$-G1P (m+6) in Glyco^{Lo} hearts (**Fig. 24A-B**). We next performed NMR spectral analysis as represented in **Fig. 24C**,

Figure 23

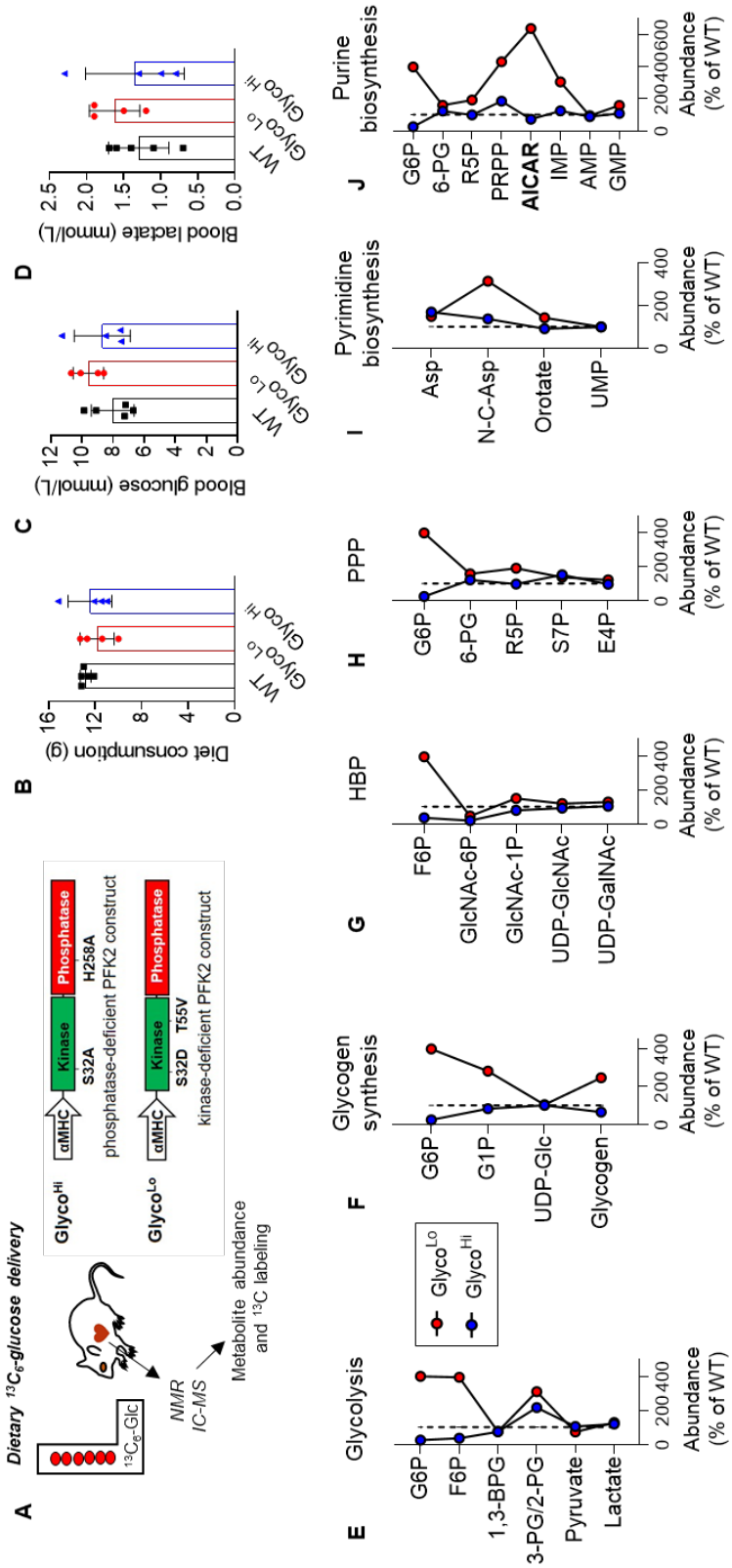

A
Dietary $^{13}C_6$-glucose delivery
$^{13}C_6$-Glc
NMR
IC-MS
Metabolite abundance and ^{13}C labeling
Glyco^{Hi}
αMHC
Kinase
Phosphatase
S32A H258A
phosphatase-deficient PFK2 construct
Glyco^{Lo}
αMHC
Kinase
Phosphatase
S32D T55V
kinase-deficient PFK2 construct
B
Diet consumption (g)
C
Blood glucose (mmol/L)
D
Blood lactate (mmol/L)
WT
Glyco^{Lo}
Glyco^{Hi}
E
Glycolysis
G6P
F6P
1,3-BPG
3-PG/2-PG
Pyruvate
Lactate
F
Glycogen synthesis
G6P
G1P
UDP-Glc
Glycogen
G
HBP
F6P
GlcNAc-6P
GlcNAc-1P
UDP-GlcNAc
UDP-GalNAc
H
PPP
G6P
6-PG
R5P
S7P
E4P
I
Pyrimidine biosynthesis
Asp
N-C-Asp
Orotate
UMP
J
Purine biosynthesis
G6P
6-PG
R5P
PRPP
AICAR
IMP
AMP
GMP
Glyco^{Lo}
Glyco^{Hi}
Abundance (% of WT)

Figure 23: Effect of phosphofructokinase activity on the relative abundances of ancillary biosynthetic pathway metabolites. (**A**) Schematic of study design and transgenic mouse model. (**B**) Consumption of $^{13}C_6$-glucose-containing liquid diet over the course of 18 h. (**C**) Circulating glucose levels after 18 h of liquid diet feeding. (**D**) Circulating lactate levels after 18 h of liquid diet feeding. (**E-J**) Crossover plots: Influence of cardiac phosphofructokinase activity on average relative abundances of metabolites in glycolysis, glycogen synthesis, the hexosamine biosynthetic pathway (HBP), the pentose phosphate pathway (PPP), and the pyrimidine and purine biosynthetic pathways. *n*=4–5 hearts/group.

Figure 24

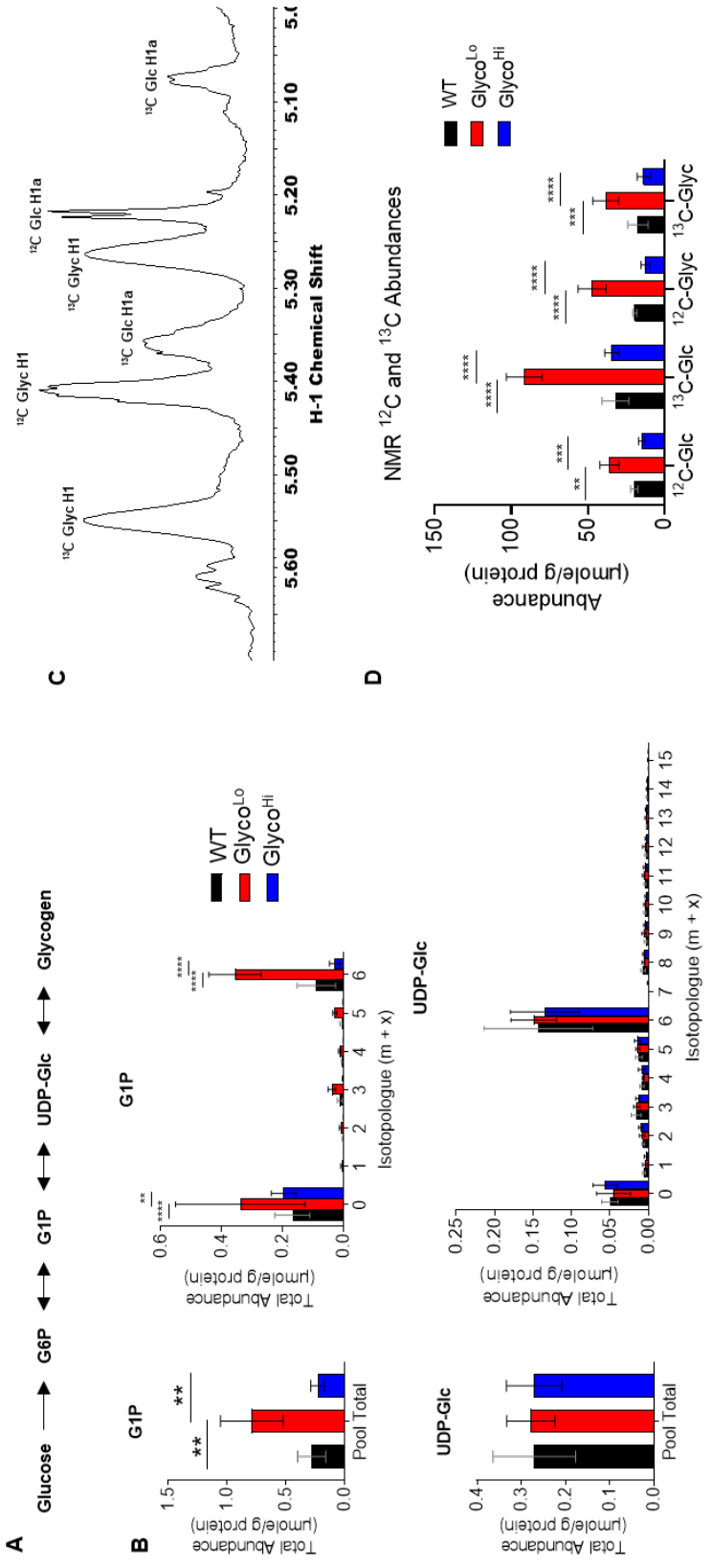

A
Glucose
G6P
G1P
UDP-Glc
Glycogen
B
G1P
Total Abundance (umole/g protein)
Pool Total
UDP-Glc
C
13C Glyc H1
13C Glc H1a
12C Glyc H1
12C Glc H1a
H-1 Chemical Shift
D
NMR 12C and 13C Abundances
WT
GlycoLo
GlycoHi
Abundance (umole/g protein)
12C-Glc
13C-Glc
12C-Glyc
13C-Glyc
G1P
UDP-Glc
Total Abundance (umole/g protein)
Isotopologue (m + x)

Figure 24: Low phosphofructokinase activity increases glycogen biosynthesis in the heart. (**A**) Schematic of glycogen biosynthesis pathway. (**B**) Pool totals and isotopologue abundances for glycogen intermediates. (**C**) Representative NMR trace for glucose (Glc) and glycogen (Glyc) in WT mouse heart. (**D**) Abundance of ^{12}C- and ^{13}C-labeled glucose (Glc) and glycogen (Glyc) in the heart. *n*=4–5 hearts per group, **p<0.01, ***p<0.001, ****p<0.0001, one-way (panel **B**) or two-way (panel **D**) ANOVA with Tukey's multiple comparison test.

which indicated higher levels of both unlabeled and ^{13}C-labeled glycogen in Glyco^{Lo} hearts (**Fig. 24D**).

Although PFK activity influenced glycogen biosynthesis, PFK had little effect on HBP metabolite abundance (**Fig. 23G**). Despite modest increases in N-acetylglucosamine-1-phosphate (GlcNAc-1P) levels and ^{13}C enrichment, overall abundance and ^{13}C enrichment of UDP-GlcNAc and UDP-GalNAc were largely unaffected by differences in PFK activity (**Fig. 25**), which is consistent with recent studies suggesting that HBP flux is rather unresponsive to changes in glucose availability in the heart.[398] Similarly, cardiac PFK activity had relatively modest effects on PPP intermediates (**Fig. 23H**). Nevertheless, we found higher levels of metabolic intermediates in the pyrimidine and purine biosynthetic pathways in Glyco^{Lo} hearts (**Fig. 23I-J**). While the total abundances and ^{13}C labeling of most pyrimidine intermediates and end products were only modestly enhanced by lower PFK activity, total abundance and isotopologue abundance plots showed marked changes in the total levels and ^{13}C labeling of the pyrimidine precursor, carbamoyl aspartate (**Fig. 26**).

As shown in **Fig. 27**, analysis of purine biosynthetic pathway metabolites revealed marked changes in Glyco^{Lo} hearts. We found higher levels of unlabeled ribose 5-phosphate (m+0), as well as higher levels of unlabeled and $^{13}C_5$-phosphoribosyl pyrophosphate (PRPP) in Glyco^{Lo} hearts (**Fig. 27A**). Furthermore, isotopologue analysis suggests routing of glucose-derived carbon to the purine synthesis pathway, with marked increases in unlabeled and ^{13}C-labeled 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR). However, the end

Figure 25

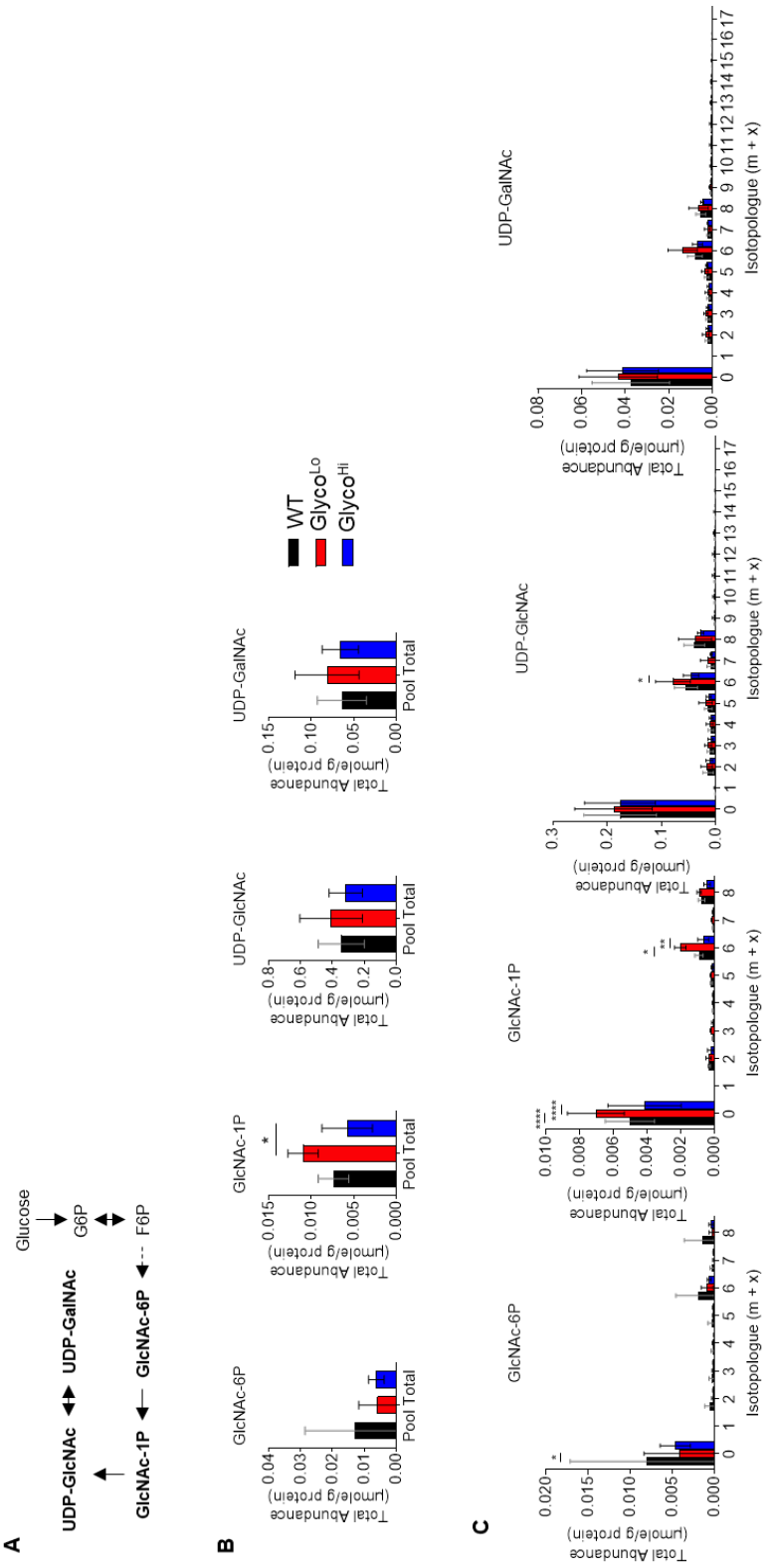

A
Glucose
G6P
F6P
UDP-GlcNAc
UDP-GalNAc
GlcNAc-1P
GlcNAc-6P
B
GlcNAc-6P
GlcNAc-1P
UDP-GlcNAc
UDP-GalNAc
Total Abundance (μmole/g protein)
Pool Total
WT
Glyco^Lo
Glyco^Hi
C
GlcNAc-6P
GlcNAc-1P
UDP-GlcNAc
UDP-GalNAc
Total Abundance (μmole/g protein)
Isotopologue (m + x)

Figure 25: Phosphofructokinase has little effect on hexosamine biosynthetic pathway activity in the heart. (**A**) Schematic of hexosamine biosynthetic pathway. (**B**) Pool totals and (**C**) isotopologue abundances of hexosamine biosynthetic pathway intermediates in hearts of $Glyco^{Lo}$, $Glyco^{Hi}$, and WT hearts. *p<0.05, **p<0.01, ****p<0.0001, (panel B) one-way ANOVA or (panel **C**) two-way ANOVA with Tukey's multiple comparison test, *n*=4–5 hearts per group.

Figure 26

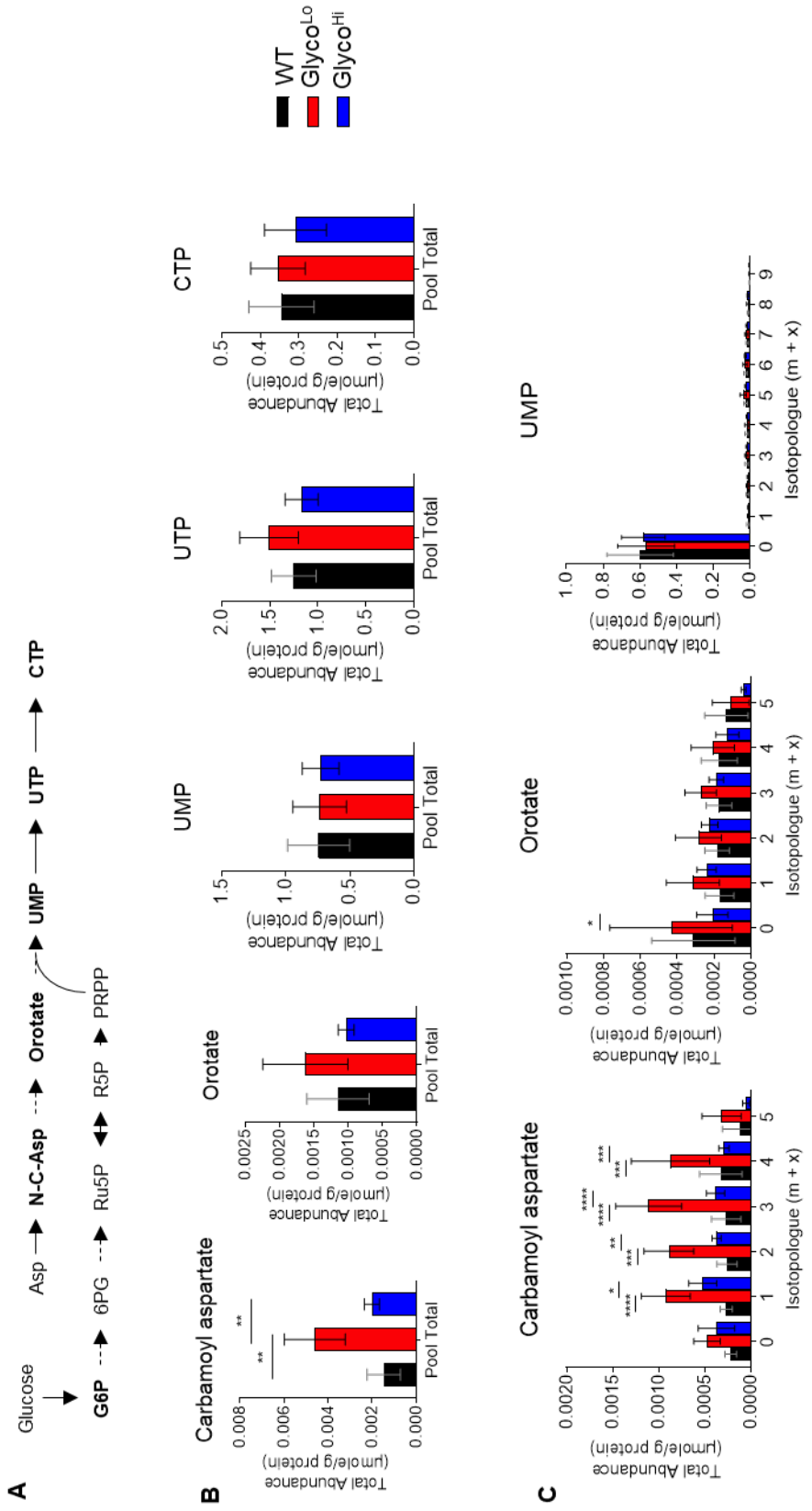

A
Glucose
Asp
N-C-Asp
Orotate
UMP
UTP
CTP
G6P
6PG
Ru5P
R5P
PRPP
B
Carbamoyl aspartate
Orotate
UMP
UTP
CTP
Total Abundance (µmole/g protein)
Pool Total
WT
Glyco^Lo
Glyco^Hi
C
Carbamoyl aspartate
Orotate
UMP
Total Abundance (µmole/g protein)
Isotopologue (m + x)

Figure 26: Influence of phosphofructokinase on pyrimidine biosynthesis in the heart. (**A**) Schematic of pyrimidine biosynthetic pathway. (**B**) Pool total and (**C**) isotopologue abundances of pyrimidine biosynthetic pathway metabolites in hearts of $Glyco^{Lo}$, WT, and $Glyco^{Hi}$ mice. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, (panel **B**) one-way ANOVA or (panel **C**) two-way ANOVA with Tukey's multiple comparison test, *n*=4–5 hearts per group.

Figure 27

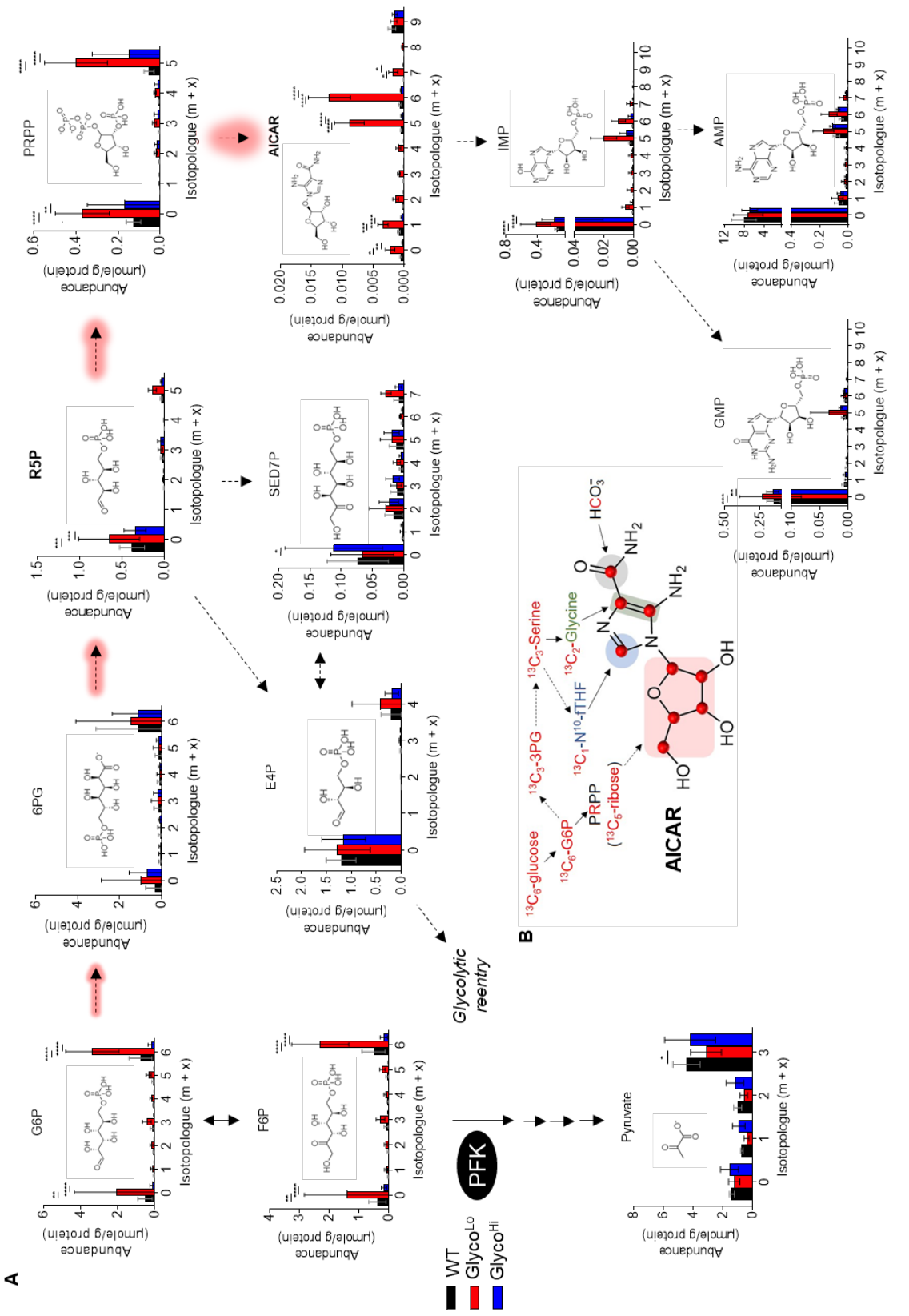

A
WT
Glyco^Lo
Glyco^Hi
PFK
Glycolytic reentry
G6P
F6P
Pyruvate
6PG
E4P
R5P
SED7P
PRPP
AICAR
IMP
AMP
GMP
Abundance (μmole/g protein)
Isotopologue (m + x)
B
AICAR
$^{13}C_6$-glucose
$^{13}C_6$-G6P
PRPP
($^{13}C_5$-ribose)
$^{13}C_3$-3PG
$^{13}C_3$-Serine
$^{13}C_2$-Glycine
$^{13}C_1$-N^{10}-fTHF
HCO_3^-

Figure 27: Low cardiac phosphofructokinase activity promotes channeling of glucose-derived carbon to form AICAR. (**A**) Metabolite isotopologue abundances for intermediates in the pentose phosphate and purine biosynthetic pathways in hearts of Glyco^{Lo}, WT, and Glyco^{Hi} mice. (**B**) Schematic of $^{13}C_6$-glucose-derived carbon incorporation into AICAR biosynthesis. *n*=4–5 hearts per group,*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, two-way ANOVA with Tukey's multiple comparison test.

products of the purine biosynthesis pathway (IMP, AMP, GMP) were much less affected by differences in PFK activity, and there were no differences in overall cardiac energy charge (WT, 0.73±0.03; Glyco^{Hi}; 0.75±0.04, Glyco^{Lo}, 0.77±0.03; p=0.22). In Glyco^{Hi} hearts, the abundances of unlabeled and ^{13}C labeled AICAR were lower than that in WT and Glyco^{Lo} hearts.

PFK activity influences cytosolic enzyme complexes in the heart: The accumulation of unlabeled and labeled R5P, PRPP, and AICAR caused by low PFK activity could be due to a decrease in AICAR utilization or an increase in its formation. Although many possibilities exist, findings from studies in cultured cells suggest the presence of cytosolic, multi-enzyme complexes (e.g., purinosomes) that could facilitate carbon channeling in the purine biosynthetic pathway.[399,400] To determine whether such complexes might exist *in vivo* and whether they could explain the preferential routing of glucose carbon to AICAR, we separated cytosolic cardiac proteins in their native state via BN-PAGE and identified protein complexes migrating to molecular masses between ~480–1000 kDa. As shown in **Fig. 28A**, we identified at least seven bands, designated by Greek letters. Proteomics analysis of each band revealed that each complex contains structural, metabolic, and chaperone proteins, with metabolic proteins comprising a large proportion of the highly abundant proteins. To determine whether PFK activity influences these complexes, we isolated cytosolic fractions from WT, Glyco^{Lo}, and Glyco^{Hi} hearts and examined gross changes in protein complex migration. Interestingly, samples from Glyco^{Hi} hearts showed molecular mass shifts in the β1 and β2 complexes

Figure 28

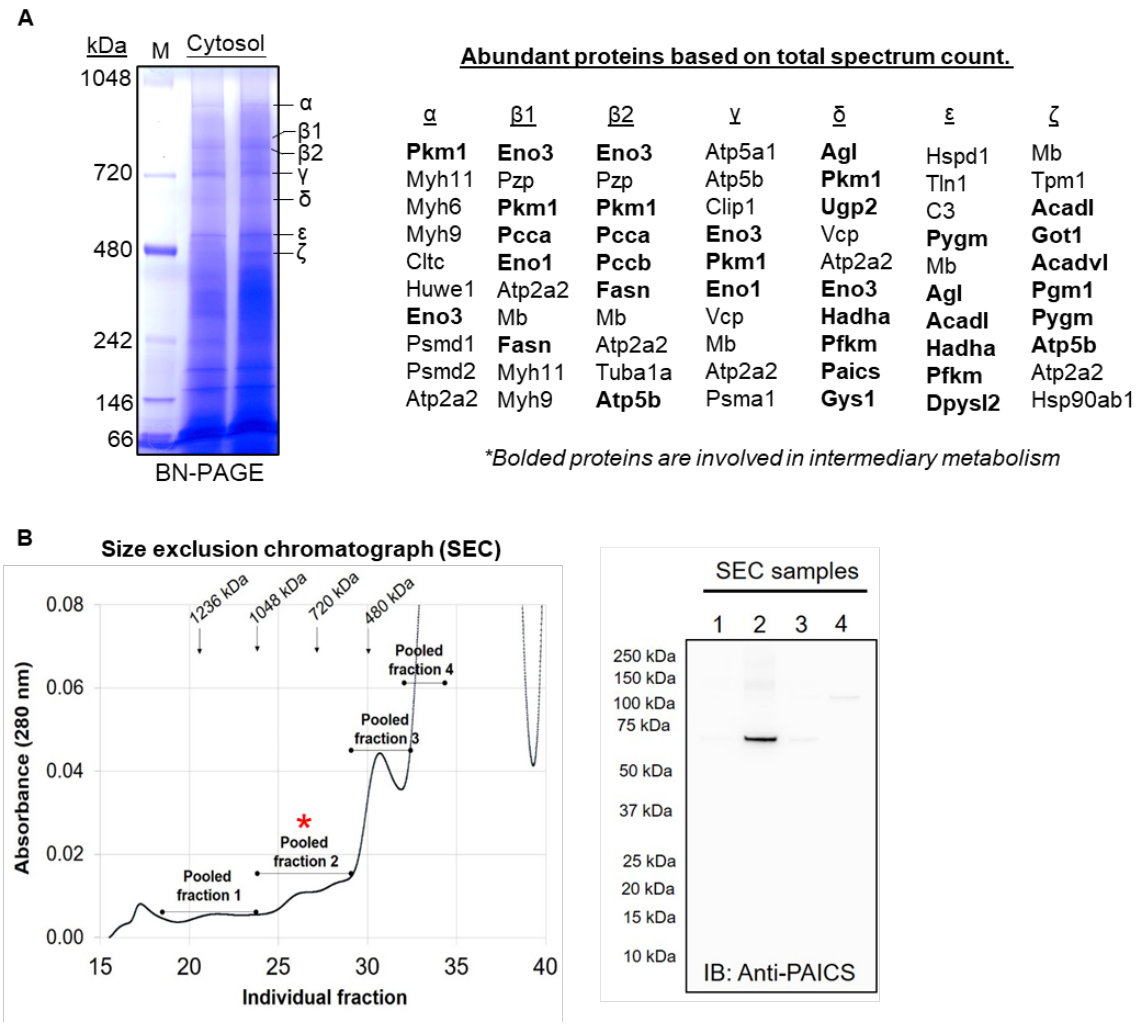


Figure 28: High molecular mass multi-enzyme complexes in cardiac cytosolic fractions. (**A**) Representative Blue-Native PAGE and abundant protein in the identified bands, measured via mass spectrometry. (**B**) Large protein complexes were separated by size exclusion chromatography, and PAICS abundance in each fraction was assessed by Western blotting using anti-PAICS antibodies.

(**Fig. 29A**). To see how the composition of these bands differed between the genotypes, we excised each band and identified proteins by mass spectrometry followed by iBAQ normalization and univariate or multivariate analyses. ANOVA results suggested the purine biosynthetic enzyme phosphoribosylaminoimidazole carboxylase (PAICS) to be the most significantly different constituent between the groups (**Fig. 29B**). To further ensure that PAICS is present in complexes at or near this size, we separated cardiac cytosolic complexes by size exclusion chromatography (SEC) and assessed PAICS in the fractions by immunoblotting. As shown in **Fig. 28B,** PAICS was present in SEC fraction 2, which contained complexes between ~500–1000 kDa. Also markedly different by ANOVA were transgene-derived PFKFB1, as well as several metabolic enzymes involved in fatty acid metabolism (fatty acid synthase, FASN; ATP citrate lyase, ACLY; carnitine palmitoyl transferase 1b, CPT1B), in caveolae formation (caveolae associated protein 5, MURC; caveolae associated protein 1, PTRF), and in components of the chaperonin-containing T-complex (TCP1, CCT3, CCT6A, CCT7, CCT5) (**Fig. 29B**).

Because chaperones such as HSP90 are required for purinosome formation,[401,402] we next determined which proteins in the complexes were most strongly associated with HSP90. As shown in **Fig. 29C**, correlation analysis demonstrated high correlation coefficients for PAICS as well as several other cytosolic metabolic enzymes (FASN, ENO1, ACLY, ENO3), some mitochondrial and endoplasmic reticulum enzymes (MDH2, DLAT, VCP), chaperones (CRYAB), and components of the proteasome (PSMA1, PSMB6, PSMB5, PSMA7), among

Figure 29

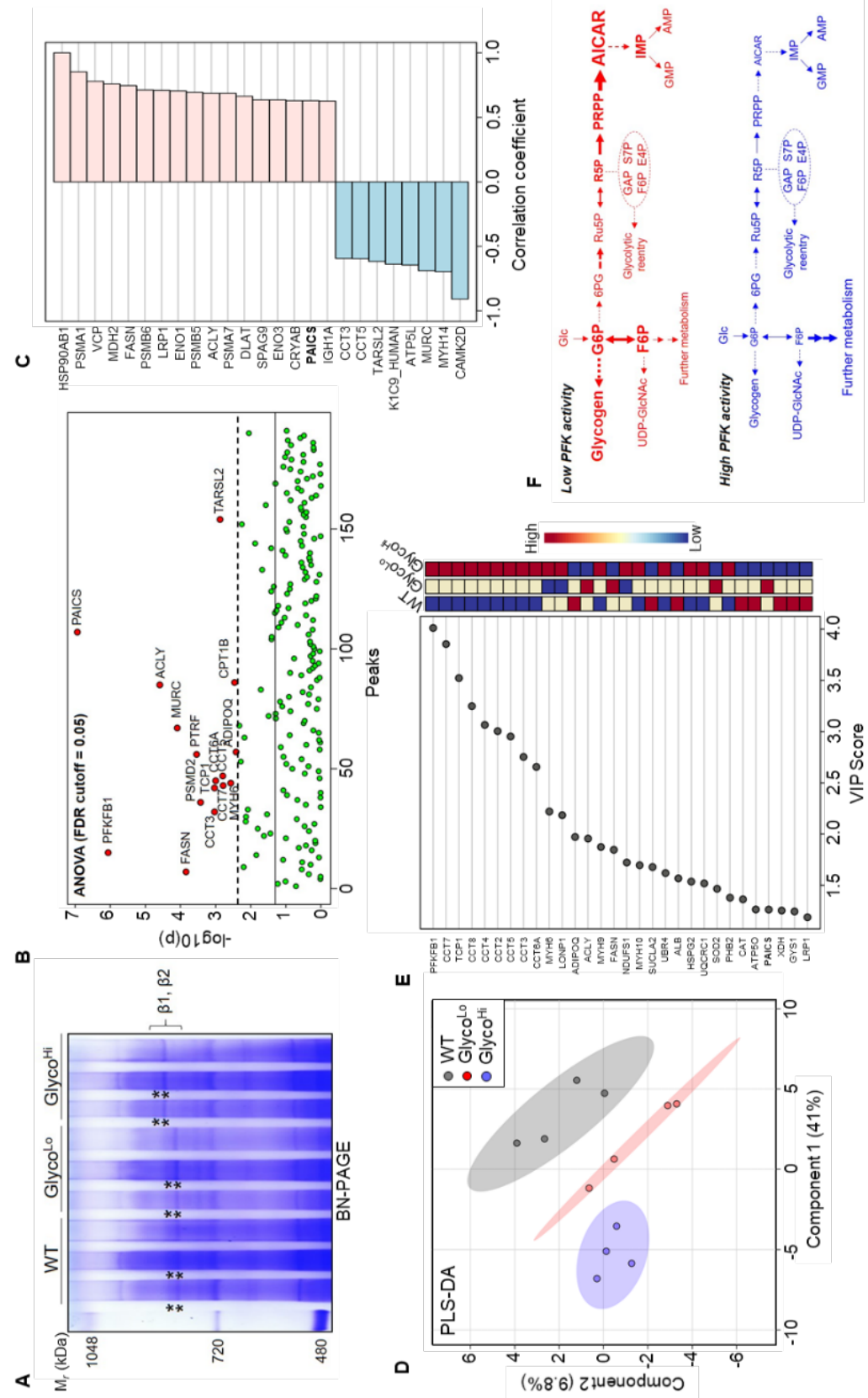

A
WT
Glyco^Lo
Glyco^Hi
M_r (kDa)
1048
720
480
β1, β2
BN-PAGE
B
ANOVA (FDR cutoff = 0.05)
-log10(p)
Peaks
PAICS
PFKFB1
ACLY
MURC
PTRF
PSMD2
TCP1
CCT3
CCT7
CCT6A
CCT4
ADIPOQ
MYH9
CPT1B
FASN
TARSL2
C
Correlation coefficient
HSP90AB1
PSMA1
VCP
MDH2
FASN
PSMB6
LRP1
ENO1
PSMB5
ACLY
PSMA7
DLAT
SPAG9
ENO3
CRYAB
PAICS
IGH1A
CCT3
CCT5
TARSL2
K1C9_HUMAN
ATP5L
MURC
MYH14
CAMK2D
D
PLS-DA
WT
Glyco^Lo
Glyco^Hi
Component 1 (41%)
Component 2 (9.8%)
E
WT
Glyco^Lo
Glyco^Hi
High
Low
VIP Score
PFKFB1
CCT7
TCP1
CCT8
CCT4
CCT2
CCT5
CCT3
CCT6A
MYH9
LONP1
ADIPOQ
ACLY
MYH9
FASN
NDUFS1
MYH10
SUCLA2
UBR4
ALB
HSP92
UQCRC1
SOD2
PHB2
CAT
ATP5O
PAICS
XDH
GYS1
LRP1
F
Low PFK activity
Glc
Glycogen
G6P
6PG
Ru5P
R5P
PRPP
AICAR
IMP
GMP
AMP
UDP-GlcNAc
F6P
Glycolytic reentry
GAP
S7P
F6P
E4P
Further metabolism
High PFK activity
Glc
Glycogen
G6P
6PG
Ru5P
R5P
PRPP
AICAR
IMP
GMP
AMP
UDP-GlcNAc
F6P
Glycolytic reentry
GAP
S7P
F6P
E4P
Further metabolism

Figure 29: Phosphofructokinase coordinates metabolic complex assembly in the heart. (**A**) Blue Native (BN)-PAGE of cytosolic fractions from WT, Glyco^{Lo}, and Glyco^{Hi} hearts. Asterisks indicate bands of the β1 and β2 complexes that were excised for proteomic analyses. *N*=3 hearts per group. (**B**) ANOVA analyses using iBAQ normalized protein abundances for β complex bands. *n*=4 bands per genotype (2 hearts per group). (**C**) Correlation of protein abundances in the β complex with HSP90. (**D**) Partial least squares discriminant analysis (PLS-DA) of protein abundances in the β complex. I Variable importance in projection (VIP) scores showing the contribution of proteins in the β complexes to group separation in panel **D**. (**F**) Working model suggesting the role of phosphofructokinase in ancillary biosynthetic pathway activity in the heart.

others (LRP1, SPAG9). Negatively correlating with HSP90 were components of the chaperonin-containing T-complex (CCT3, CCT5), TARSL2, MURC, MYH14, CAMK2D, and ATP5L, which suggests that these proteins were not associated with HSP90 in the protein complexes.

Given that PAICS in the β protein complexes was most significantly different between the genotypes and was associated strongly with HSP90, we next performed multivariate analyses to identify which protein constituents contributed most to phosphofructokinase-mediated differences in β complex composition. As shown in **Fig. 29D** and **29E**, partial least square discriminant analysis followed by variable importance in projection (VIP) score analysis suggested PFKFB1 contributed most to group separation, which is not surprising given that PFKFB1 mutants comprise the overexpressed, mutant transgenes. Mirroring contribution by PFKFB1, several members of the T-complex were elevated in $Glyco^{Hi}$ and $Glyco^{Lo}$ hearts. Interestingly, PAICS also contributed largely to group separation, with higher levels of PAICS in $Glyco^{Lo}$ hearts and lower levels in $Glyco^{Hi}$ hearts; other metabolic enzymes such as FASN and ACLY paralleled the PAICS pattern. Collectively, these findings insinuate phosphofructokinase-driven compositional changes in multimeric protein complexes, which support the idea that metabolic channeling identified by deep network tracing *in vivo* could be due to the formation of protein assemblies that influence glucose-derived carbon fate.

DISCUSSION

In this study, we delivered ^{13}C-labeled glucose via diet to mice with low or high cardiac glycolytic activity and then performed *in vivo* deep network tracing to examine ancillary biosynthetic pathway activity. We found that the amphibolic metabolites G6P and F6P accumulated in hearts of mice with constitutively low PFK activity and were associated with elevated glycogen levels; however, HBP activity was relatively insensitive to differences in PFK activity. Interestingly, we found that PFK activity influences routing of glucose-derived carbon to the purine biosynthetic pathway intermediate, AICAR (**Fig. 29F**). These changes were associated with the presence of large multimeric complexes containing chaperones and metabolic enzymes, including PAICS, which is required for AICAR biosynthesis. Collectively, these studies illustrate the utility of deep network tracing for cardiac metabolic phenotyping and for developing new insights into the differential regulation of biosynthetic pathways *in vivo*.

Of practical importance, we found that both the C57 and FVB genetic mouse backgrounds consumed similar amounts of diet and that the diet did not significantly affect circulating levels of free fatty acids or insulin. These standardization studies were important because a preponderance of genetic mouse models are in these backgrounds.[395,396] Moreover, our studies suggest that deep network tracing using dietary ^{13}C-glucose introduction can be used to measure biosynthetic pathway activity in the heart. Typically, fast turnover pathways such as glycolysis and the TCA cycle require relatively short durations of labeling to achieve isotopic steady state, whereas some biosynthetic pathways

may take numerous hours or even days to see significant labeling and reach isotopic steady state.[387,403] For the purposes of assessing biosynthetic pathway activity in the heart *in vivo*, we strove to incorporate enough glucose-derived ^{13}C into the biosynthetic pathway metabolite pools for confident isotopologue detection, while also staying within the dynamic phase of labeling, which allows assessment of relative flux in biosynthetic pathways.[387,403] Indeed, in pilot studies comparing 18 h versus 36 h ^{13}C-glucose feeding, we found that 36 h of feeding led to generally higher ^{13}C labeling enrichment in intermediates and end products of ancillary biosynthetic pathways compared with the 18 h feeding group (**Fig. 30**). Collectively, these findings indicate that 18 h of dietary ^{13}C-glucose provision is sufficient to label biosynthetic pathway pools for adequate detection and to stay within the dynamic phase of labeling. Furthermore, the findings suggest that this duration of feeding should be adequate for analysis of biosynthetic pathway activity in other genetic models or in mouse models of heart failure or physiologic cardiac growth.

We chose to examine how PFK regulates biosynthetic pathways because of its relevance to glucose metabolism and because changes in its activity influence cardiac remodeling. The reaction catalyzed by PFK1 is a strongly exergonic, committed step in glycolysis[404,405] and appears to exert strong control on glycolytic flux[240] Our findings suggest that PFK activity strongly influences the abundance of G6P and F6P, thereby controlling levels of precursors for several biosynthetic pathways, including the PPP, nucleotide biosynthetic pathways, glycogen synthesis, and the HBP. Consistent with the idea that low PFK activity

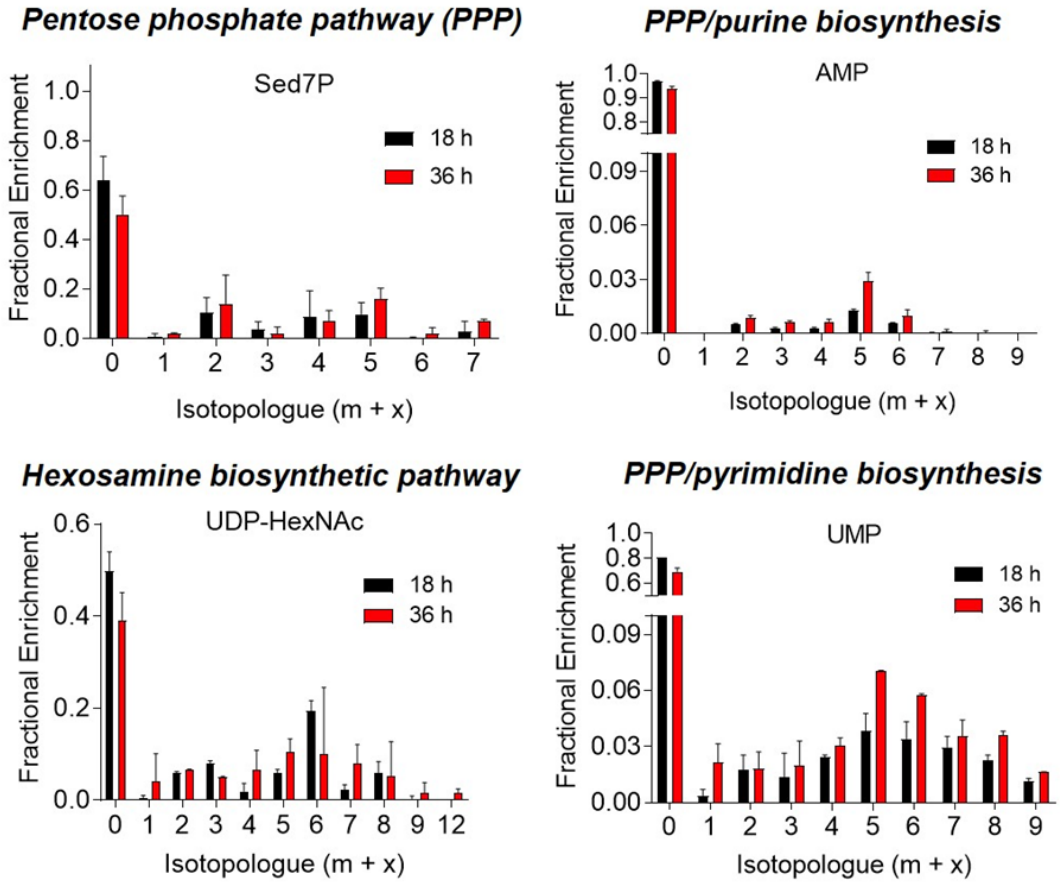


Figure 30: Time course of cardiac metabolite ^{13}C incorporation from mice provided with a $^{13}C_6$-glucose-labeled liquid diet for 18 h or 36 h. Pilot data showing fractional enrichment values for a sampling of biosynthetic pathway intermediates and end products. Sed7P = seduheptolose-7-phosphate, UMP = uridine monophosphate, AMP = adenosine monophosphate, *n*=2–3/gp.

reroutes glucose to glycogen synthesis,[197,406-408] high levels of sugar phosphates in Glyco^{Lo} hearts were associated with accumulation of unlabeled and ^{13}C-glycogen. While glycogen levels were influenced by levels of sugar phosphates, HBP activity was mostly unaffected by increased levels of G6P and F6P, which is consistent with recent studies showing that HBP flux is independent of changes in glucose availability.[398] This may be due to the multiple metabolic inputs required for UDP-GlcNAc synthesis, which effectively diminish PFK control over the pathway.

By tracking the fate of ^{13}C through the PPP and nucleotide biosynthetic pathways, we found that low PFK activity was associated with increased glucose carbon partitioning into PRPP and AICAR. We interpret this metabolic phenotype as a form of metabolic channeling for several reasons. A general flooding paradigm of pathways by elevated G6P levels would predict more uniform ^{13}C deposition in all possible pathways, including pentose phosphate products and the final nucleotide products; however, enhanced ^{13}C labeling only became apparent at the amphibolic metabolite R5P and increased successively through PRPP and AICAR, while ^{13}C labeling of the final nucleotide products was not altered. Thus, the occurrence of such selective partitioning of glucose-derived carbon downstream of metabolic branchpoints provides evidence of metabolic channeling.

It remains unclear if the form of channeling observed in this study fits the simple substrate channeling paradigm. The general paradigm of substrate channeling implicates stoichiometric assemblies of enzymes that catalyze sequential transfer of metabolic intermediates from one enzyme to the next without

dilution of products in the bulk medium. Such substrate channeling has been proposed to promote pathway efficiency, regulate entry of intermediates into competing metabolic pathways, and provide a means to segregate and microcompartmentalize metabolites in the cell.[409-412] Consistent with the idea of spatial organization of metabolism, we found evidence of metabolic enzymes in high molecular weight complexes in cytosolic heart extracts. In particular, higher molecular weight α, β1, β2, and γ complexes contained numerous glycolytic enzymes, as well as fatty acid metabolism enzymes, and the lower molecular weight δ, ε, and ζ complexes showed generally higher levels of glycogen metabolism enzymes (e.g., GYS1, AGL, UGP2, PYGM) as well as enzymes important for nucleotide biosynthesis (e.g., PAICS, GOT1). We also found by proteomics that PAICS in β complexes was influenced by PFK activity and correlated strongly with the presence of the purinosome chaperone HSP90 [401,402]. In addition, some of the complexes also contained relatively high levels of myofibrillar components, the sarcoplasmic reticulum calcium ATPase, and mitochondrial proteins, indicating spatial coordination of energy consuming and providing processes in the heart. These data are consistent with the presence of higher order, quinary protein structures consisting of functionally related proteins, some of which respond to changes in PFK activity.

Although the channeling to AICAR, a known signaling molecule,[413] observed by deep network tracing could occur via purinosome-like metabolons, several issues and limitations must be addressed for further elucidation. Metabolons found in mammalian systems, such as glucosomes[414-416] and

purinosomes,[399,400] are thought to be loosely associated and thus could dissociate during extraction or native separation. Although loose associations may help provide sensitivity for altering carbon fate in response to stimuli *in vivo*, it limits our ability to isolate an intact, active metabolon, which could otherwise be tested *in vitro* for confirmation of direct substrate channeling. Also, it is possible that direct channeling represents only one form of spatial regulation used to direct carbon flow in tissues. It is possible that metabolic enzymes could become sequestered or made unavailable to some metabolic conduits, resulting in accumulation of upstream metabolic intermediates. Nevertheless, our *in vivo* tracer and native separation experiments insinuate control over cardiac glucose-derived carbon allocation, dependent on phase-separated condensates of metabolic complexes. We propose that this is the first evidence of cytosolic metabolons in the heart, which have been previously inferred to exist from experimental results of *in vitro* and cultured cell studies.[411,417-422]

Findings of this study are also important in light of the evidence that PFK activity influences cardiac remodeling. For example, low PFK activity during exercise appears to activate the cardiac growth transcriptional program,[214] and high levels of PFK activity, such as that occurs in the pressure-overloaded heart [385], are sufficient to promote a deleterious form of remodeling.[214] Such metabolic regulation of cardiac remodeling may be due in part to the ability of PFK to influence biosynthetic pathways, which not only provide building blocks for myocyte structure, but also regulate redox state and cell signaling.[294,423] Understanding how PFK-mediated changes in biosynthetic pathways activity

coordinate anabolic pathways and transcriptional programs to elicit cardiac remodeling remains an exciting challenge for future studies.

Study Limitations

Several limitations of this study deserve mention. First, these studies used mice with constitutive overexpression of kinase- or phosphatase-deficient 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase transgenes in the heart to regulate PFK1 activity. This creates a metabolic phenotype from birth and promotes metabolic inflexibility at the level of PFK1, which may not mimic the dynamic changes in PFK activity that occur with physiological or pathological stress.[214] Nevertheless, these studies reveal how different levels of PFK activity influence cardiac biosynthetic pathway activity, which may be important for understanding how even transient changes in PFK activity may influence biosynthetic pathways in other contexts. Also, because the heart is not composed exclusively of cardiomyocytes, it is possible that some of the results could be influenced by non-cardiomyocyte contributions to metabolism; however, the mutant transgenes used to modulate PFK1 activity are expressed only in cardiac myocytes (under the αMHC promoter), which suggests that the findings represent changes predominantly in cardiomyocytes. Second, although label scrambling can be a significant impediment in *in vivo* ^{13}C labeling, our principal focus on anabolic pathways of glucose metabolism largely negates this issue because a primary endpoint was the net incorporation of glucose-derived ^{13}C into biosynthetic pathway metabolites. Furthermore, because adult cardiac myocytes lack significant phosphoenolpyruvate carboxykinase activity,[424] there is little concern of

incorporation of ^{13}C from circulating lactate or pyruvate, especially in those isotopologues in biosynthetic pathway metabolites that branch from G6P or F6P. Finally, although our deep network tracing studies demonstrate apparent metabolic channeling, identifying the exact composition of the complexes that contributed to the elevations in AICAR remains a difficult, yet exciting challenge.

This chapter previously appeared as an article in the journal entitled Journal of Molecular and Cellular Cardiology. The original citation is as follows: Fulghum KL et al. In vivo deep network tracing reveals phosphofructokinase-mediated coordination of biosynthetic pathway activity in the myocardium. *JMCC* 162:32-42. 2021.

CHAPTER V

DEEP NETWORK TRACING IDENTIFIES EXERCISE-INDUCED CHANGES IN MYOCARDIAL GLUCOSE UTILIZATION DURING PERIODS OF ACITVE CARDIAC GROWTH

INTRODUCTION

The cardiovascular benefits of aerobic exercise training occur as an adaptive response to transient, yet repetitive increases in cardiac workload[21]. Aerobic exercise increases the demand for oxygen and delivery of energy-providing substrates throughout the body, and over time, the compounding effects of these heightened demands play a key role in promoting adaptive physiological hypertrophy of the heart while preserving, or even enhancing, overall cardiovascular function and chamber compliance.[425] Previous studies imply the necessity of certain signaling pathways and gene expression in the progression of exercise-induced cardiac growth[224,255,426] while other studies suggest that changes in cardiac metabolism are necessary for physiological cardiac hypertrophy.[214,276,379] Because cellular growth requires synthesis of macromolecules, the metabolic adaptations associated with exercise might occur to promote utilization of substrates that favor anabolic activity in the heart. In support of this concept, over 40% of glucose consumed by rat neonatal

cardiomyocytes is not catabolized to form pyruvate, but instead utilized in pathways ancillary to glucose catabolism.[281]

Glucose metabolism is an important consideration in cardiac growth because most glucose that enters the heart is either stored or utilized in biosynthetic pathways. With respect to exercise, a recent study showed that exercise transiently reduces myocardial activity of phosphofructokinase,[214] which is a key, regulatory step in glycolysis that may control enrichment of glucose-derived carbons into biosynthetic pathways.[281] Therefore, we posit that exercise training alters myocardial glucose utilization by augmenting biosynthetic pathway activity in the heart to promote physiological cardiac growth.

In this study, we created a metabolic atlas outlining exercise-induced changes in cardiac glucose utilization and how they could associate with various stages of physiological growth in the heart. We first created a timeline of exercise-induced cardiac growth, and then we investigated the metabolic responses to acute (1 day), early (1 week), and established (4 week) stages of exercise training and subsequent cardiac growth. Because we know that changes in cardiac glucose metabolism are associated with this physiological growth process, we delivered ^{13}C-labeled glucose via liquid diet to mice to visualize glucose utilization and relationships between biosynthetic pathways *in vivo*. We used only male, FVB/NJ mice in this study because our preliminary data suggest that exercise-induced cardiac growth occurs in male, but not female, FVB/NJ mice subjected to forced treadmill running (shown in Chapter II, **Fig. 5**). Finally, we report that exercised male hearts utilize glucose-derived carbons for biosynthesis in the acute

and early phases of physiological growth, but that glucose utilization returns to sedentary levels once the heart is fully adapted.

EXPERIMENTAL METHODS

Experimental Animals: All procedures were approved by the Institutional Animal Care and Use Committee at the University of Louisville. Adult, male FVB/NJ mice were kept on 12 h: 12 h light:dark cycle with both chow and water provided *ad libitum* unless fasted (6 h) for untargeted metabolomics or provided liquid diet for deep network tracing experiments. Upon completion of each experiment, mice were anesthetized with sodium pentobarbital (150 mg/kg, i.p.) and mice were euthanized via excision of the heart. Additional tissues were harvested following euthanasia. These procedures are consistent with the AVMA *Guidelines on Euthanasia*.

Exercise Training Protocol: Mice were acclimated to treadmill running and trained as described previously.[293] Briefly, mice were first exercised to exhaustion to determine initial exercise capacity and then exercise training was set at 75% maximal capacity for increasing amounts of time each week (40 min/d week 1, 50 min/d week 2, 60 min/d weeks 3–4). To determine a timeline of exercise-induced cardiac growth, mice were exercise for one day, one week, or two weeks. The initial exercise capacity test (ECT) served as the acute bout of exercise in each study and the ECT was repeated on final days of exercise as a gauge of exercise adaptation.

Circulating Substrate Measurements: Blood samples for circulating glucose, lactate, and ketones were obtained via tail clip and measured in about 0.7 µL blood using the Accu-Check Aviva meter (Roche), the Lactate Plus meter (Nova Biomedical), and the Keto-Mojo ketone meter, respectively.

Liquid Diet Feeding Protocol: We previously standardized liquid diet delivery of $^{13}C_6$-glucose in both FVB/NJ and C57BL/6J mice,[427] and we used this method to deliver stable isotope-labeled glucose to male, FVB/NJ mice in the final 3 d of an exercise training protocol. The liquid diet was switched from unlabeled glucose diet to labeled ($^{13}C_6$-glucose) diet for 18 h following the final bout of exercise.

Stable Isotope-Resolved Metabolomics: Following exercise and feeding protocols, mice were anesthetized with sodium pentobarbital (150 mg/kg, i.p.) and euthanized. Hearts were freeze-clamped *in situ* using liquid N_2-cooled Wollenberger tongs and then pulverized for extraction of polar, lipid, and protein fractions in acetonitrile/water/chloroform (V/V 2:1.5:1). Lyophilized polar fractions were analyzed via Dionex ICS-5000+ion chromatograph interfaced to Orbitrap Fusion Tribrid mass spectrometer and data were analyzed as previously described.[427] Nuclear magnetic resonance of polar metabolite extracts was analyzed as previously described.[427]

Enzyme Activity Assays: Activity assays for cardiac pyruvate dehydrogenase, malic enzyme, and pyruvate carboxylase (BioRad) were performed per manufacturer instructions.

Immunoblots: Hearts were pulverized under liquid nitrogen and tissue powder (~20 mg) was added to lysis buffer (20 mM HEPES, 100 mM KCl, 1 mM EDTA, 1% IGEPAL, 0.1% SDS, pH=7.4) containing phosphatase and protease inhibitors. Tissue was vortexed, sonicated, and protein supernatant was quantification using Bio-Rad DC assay. Ten or 20 µg cardiac protein sample were resolved via SDS-PAGE using AnyKD gels (BioRad) and proteins were transferred to 0.2 µm Immun-Blot PVDF membrane (BioRad). Membranes were blocked in 5% milk before the following antibodies were used: 1:5000 anti-pAMPK (Thr172, CST), 1:5000 anti-pAKT (Ser473, CST), 1:5000 anti-pmTOR (Ser2448, CST), 1:2000 anti-pPFK2 (Ser483, CST), 1:2500 anti-AMPK (CST), 1:3000 anti-AKT (CST), 1:1000 anti-mTOR (Invitrogen), 1:3000 anti-GAPDH with HRP (CST), and 1:2500 anti-Rabbit (CST) or anti-Mouse (CST) secondary antibodies. Membranes were imaged using BioRad Imager and densitometry was calculated using ImageLab.

Statistical Analyses: Data are represented as mean +/- S.D. and significance was assumed when $p<0.05$. For analyses using MetaboAnalyst 5.0 software (https://www.metaboanalyst.ca), data were filtered first by omitting analytes with missing values, then by interquartile range, and data were finally log-transformed before presenting in figures and performing statistical analyses. An FDR cutoff with

$p<0.10$ was additionally implemented to assume significance for the large dataset. We used one-way and two-way ANOVA where appropriate and confirmed significance with Tukey post-hoc test or as otherwise indicated.

RESULTS

Aerobic exercise leads to cardiovascular adaptations.

To resolve the timeline of exercise-induced cardiac growth, we subjected an additional cohort of male FVB/NJ mice to an acute bout of exercise, one week of exercise, or two weeks of exercise. We first measured initial exercise capacity (**Fig. 31A**) to determine a training speed that ensured equal, relative workload by all mice, then we measured heart weight in response to training duration (**Fig. 31B**). We found that one week of exercise training appears to be a dynamic timepoint in physiological cardiac growth while two weeks of training leads to a more established hypertrophic response in the heart. Together, these data imply that changes in metabolism likely occur early in a training program and that training duration influences the cardiovascular response exercise.

We did not observe any effects of exercise on circulating blood lactate, glucose, or 3-hydroxybutyrate levels (**Fig. 31C–E**). Furthermore, exercise training did not influence body weight (**Fig. 31F**). We next looked at the phosphorylation of major kinases in the heart and found that acute exercise resulted in a trend toward increased phosphorylation of AMPK, but there were no effects of exercise on mTOR or AKT following any duration of exercise training (**Fig. 31G–L**).

Figure 31

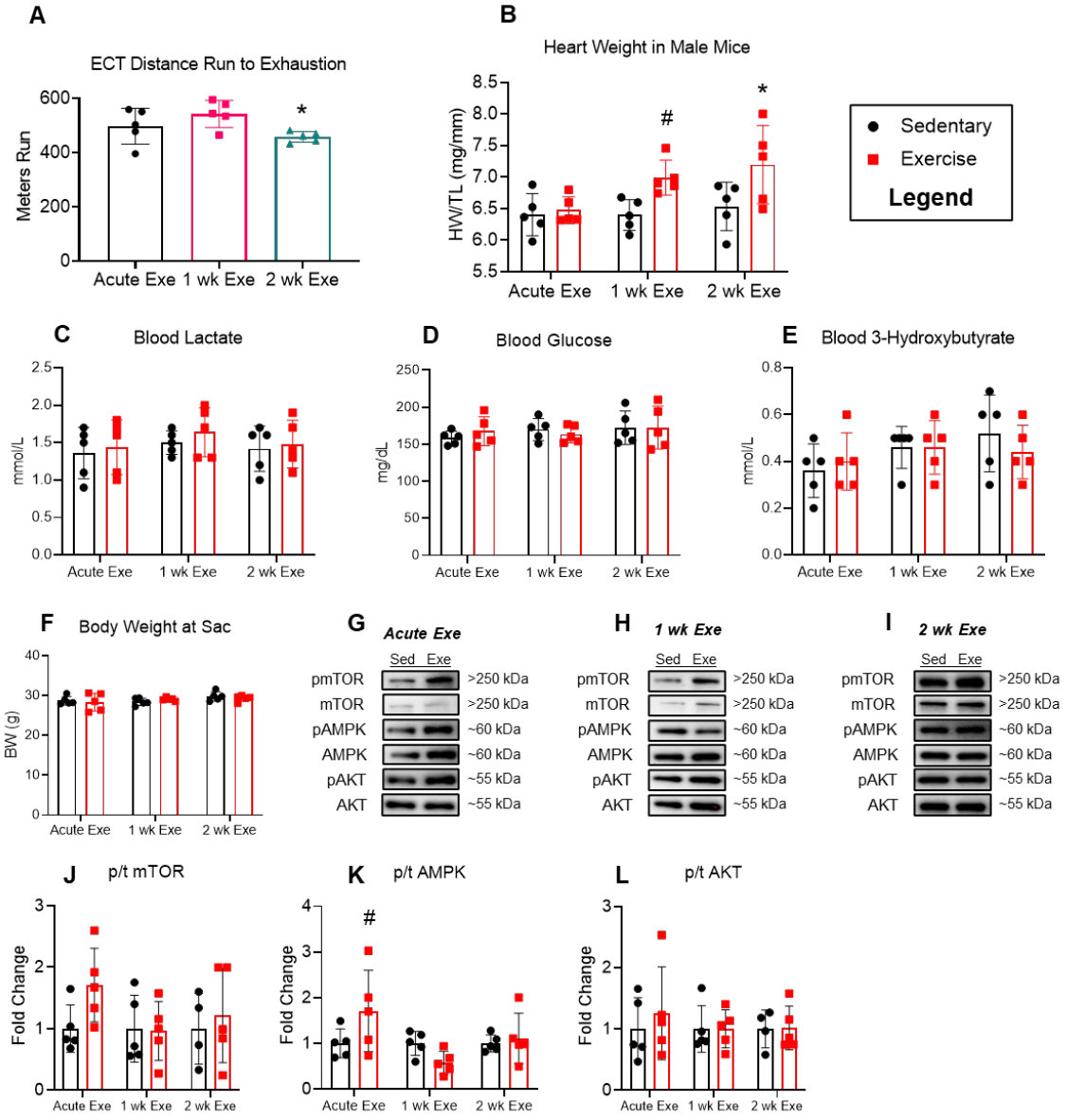


Figure 31: Timeline of exercise-induced cardiac growth. Initial exercise capacity in male, FVB/NJ mice (**A**) and normalized heart weights following 1 and 2 week forced treadmill running (**B**). Effect of acute, 1 week, and 2-week exercise training on resting levels of circulating lactate (**C**), glucose (**D**), and 3-hydroxybutyrate (**E**), as well as body weight (**F**), in male mice. Phosphorylation of mTOR, AMPK, and AKT 24 h following acute exercise (**G**), 1 week exercise training (**H**), and 2-week exercise training (**I**) in hearts of male mice (fold change plotted in **J-L**). *n*=5/gp **A-F**, *n*=4–5/gp **G-L**. Panel **A** *p<0.05 one-way ANOVA with Bonferroni post-hoc, panel **B** #p<0.10, *p<0.05, **p<0.01 two-way ANOVA with Sidak post-hoc test. Sed = sedentary, Exe = exercise.

Stable isotope-resolved metabolomics traces the fate of glucose-sourced carbon.

Previous studies[214] indicate that exercise-induced changes in cardiac glucose metabolism facilitate physiological growth in the heart. To visualize glucose utilization in response to exercise training, we subjected male FVB/NJ mice to 1 day, 1 week, or 4 weeks of exercise training and then provided mice a liquid diet containing ^{13}C-enriched glucose for 18 hours following the final session of exercise (**Fig. 32A**). These timepoints model acute, dynamic, and established phases of cardiac remodeling during exercise. Because we only saw exercise-induced growth in male hearts (Chapter II), we used only male mice for deep network tracing studies. Mice that trained for one or four weeks displayed higher work performance and distance run to exhaustion than acutely exercised mice or initial, pre-trained values (**Fig. 32B–C**). Blood glucose levels decreased following an initial exercise capacity test, but increased following final exercise capacity tests for all groups (**Fig. 32D**). Blood lactate was significantly increased after each exercise capacity test, indicating that mice ran to exhaustion (**Fig. 32E**), and there were no differences in pre-training body weight, liquid diet consumption, or body weight at time of euthanasia (**Fig. 32F–H**).

In response to exercise training, we saw marginal changes in the total abundances of glycolytic intermediates. While not significant, the abundances of 6-carbon intermediates were generally higher in hearts of acutely exercised mice compared to all other groups while abundances of 3-carbon intermediates such as pyruvate were lower in hearts of acutely exercised mice compared to hearts of sedentary and 4 wk exercised mice; however, there were no significant changes

Figure 32

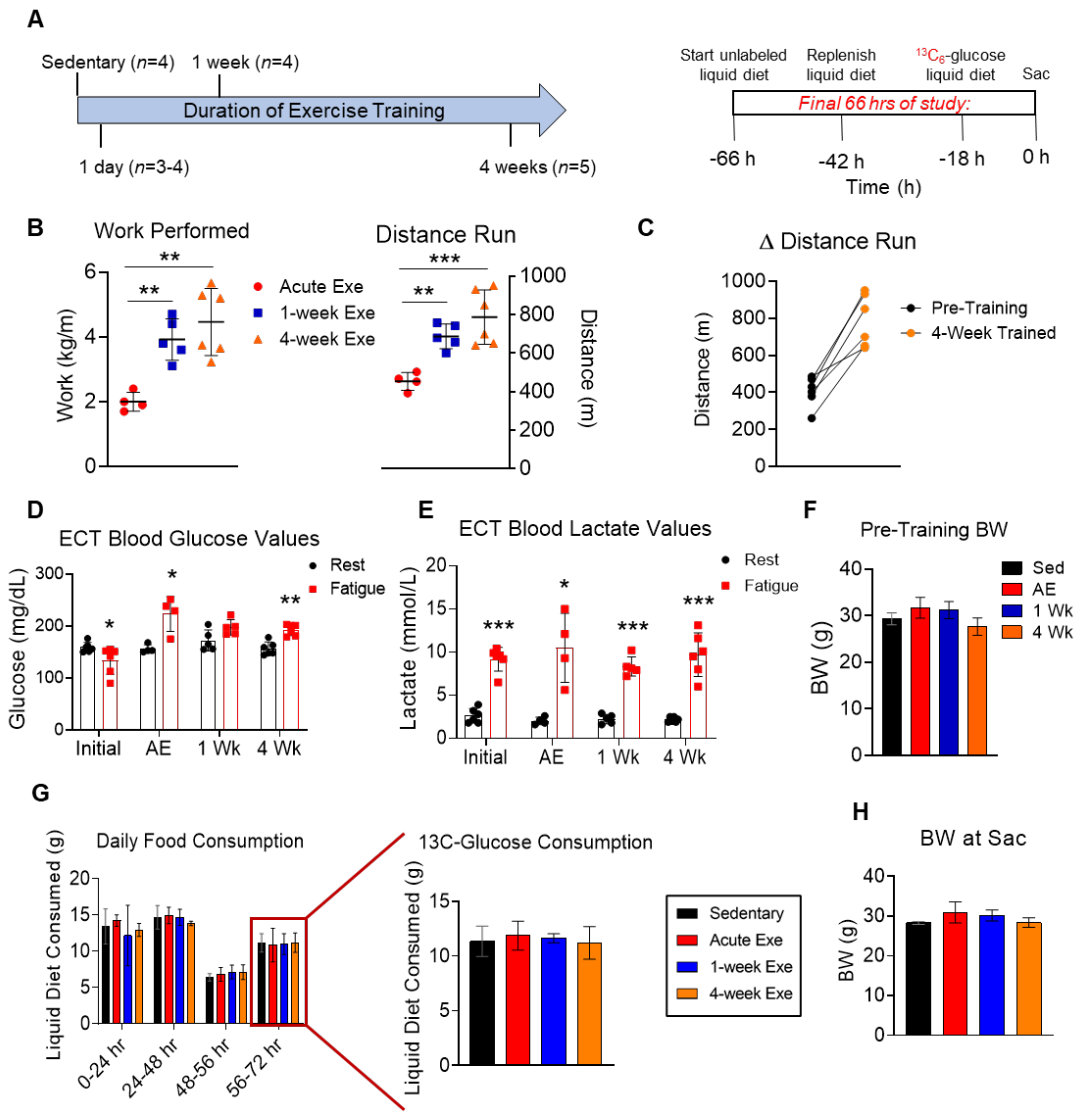


Figure 32: SIRM Exercise Training Data. Timeline **(A)** of exercise training in study. Differences in work and distance run to exhaustion following training protocol **(B)** and paired change in ECT distance run in 4-week trained mice **(C)**, **p<0.01, ***p<0.001, One-Way ANOVA with Holm-Sidak post hoc. Circulating values of blood glucose **(D)** and blood lactate **(E)** at rest and exhaustion in final ECT, *p<0.05, **p<0.01, ***p<0.001 paired test rest vs. fatigue. Group assignments **(F)** were similar in BW with acute exercise and 4 week assigned groups showed difference (p<0.05) after post-hoc test. Daily liquid diet food consumption and labeled glucose consumption **(G)** during feeding protocol, and body weight at euthanasia **(H)** showed no differences after post-hoc test. Sed = sedentary, Exe = exercise, AE = acute exercise, ECT = exercise capacity test, BW = body weight.

in ^{13}C enrichment of glycolytic intermediates (**Fig. 33A–B**). Exercise had more prominent effects on the total abundances of TCA cycle intermediates, with reductions in abundances of citrate, aconitate, and α-ketoglutarate after 1 d of training and reductions in α-ketoglutarate remaining after 1 wk of training. There were no changes in total abundances of succinate, fumarate, or malate. We observed greater ^{13}C-enrichment in aconitate, isocitrate, fumarate, and malate (logit transformed, $p<0.10$) in hearts of mice exercised for 1 d than in sedentary hearts. Higher enrichment paired with lower abundance indicates greater turnover of TCA cycle metabolites in the early cardiac responses to exercise. Following 4 weeks of training, cardiac metabolite abundances and ^{13}C-enrichment patterns appeared to mirror that of sedentary mice. After this duration of training, exercise-induced increases in cardiac mass have plateaued, as indicated by data showing no further increases in cardiac mass after 2 weeks of training (Chapter II). This could imply that the catabolic and anabolic need for glucose-derived carbons is increased only during active phases of cardiac growth.

Our feeding protocol was delivered to mice *ad libitum* and did not supply glucose exclusively to the heart, which could allow other tissues such as skeletal muscle and liver to utilize glucose and extrude into circulation myocardial substrates such as lactate or pyruvate. Because this potential scrambling of ^{13}C-label could influence the results of this study, we also measured plasma enrichment and abundance of ^{13}C-labeled glucose, lactate, and pyruvate (**Fig. 33F–G**). We found slight differences in total abundance of plasma glucose, lactate, and pyruvate, but no differences in fractional enrichment.

Figure 33

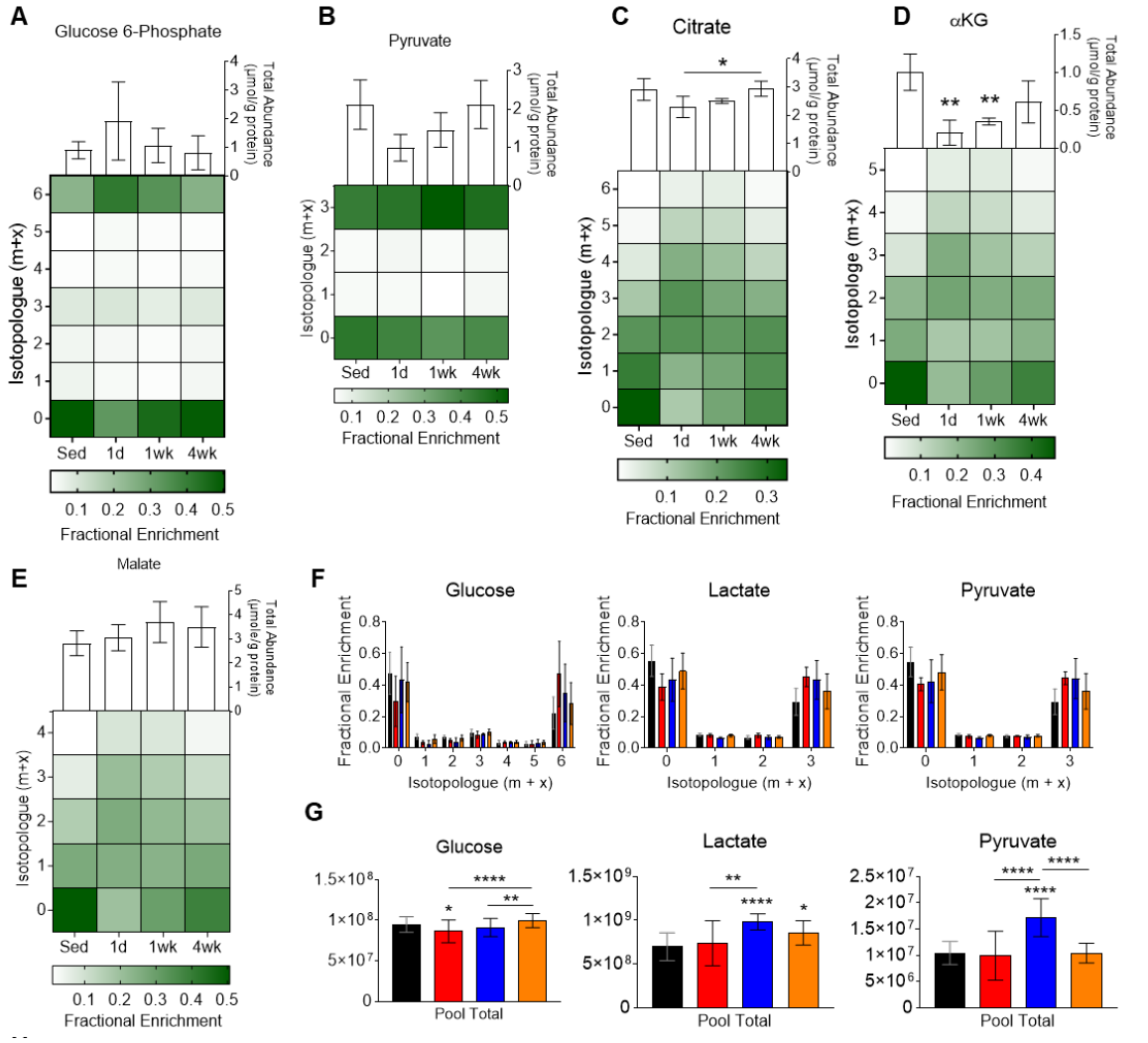


Figure 33: Exercise training influences cardiac glycolytic and Krebs Cycle intermediates. Comparison of exercise training effects on total and isotopologue abundances in representative cardiac glycolytic products (**A-B**) and Krebs cycle intermediates (**C-E**). Fractional enrichment (**F**) and pool totals (**G**) of plasma circulating metabolites, and enzyme activity within cardiac tissue. All figures *n*=3–6/gp, *p<0.05, **p<0.01, ****p<0.0001 one-way ANOVA (pool total) or two-way ANOVA (isotopologue abundance) with Tukey's multiple comparison test. Significance denoted vs. sedentary unless noted. Sed = sedentary, AE = acute exercise.

One benefit of deep network tracing is the ability to visualize the overall incorporation of substrate-derived atoms into specific metabolites. To explain the discrepancy in abundances of TCA cycle metabolites, we first considered cataplerosis that could occur through selective routing of carbon to glutamate synthesis. Similar to α-ketoglutarate, we saw appreciable reductions in the abundances of glutamate and aspartate following various durations of exercise training (**Fig. 34A–B**). Importantly, ^{13}C-enrichment of glutamate and aspartate in the heart was greatest following 1 day of exercise (logit transformed, $p<0.10$); however, ^{13}C-enrichment returned to sedentary levels by 4 weeks of training. Because glutamate and aspartate metabolism have been implicated in cardiac hypertrophy,[428] this finding could imply an increased demand for these amino acids to support the material demands for cell growth. Additionally, following 1 d and 1 wk of exercise, we saw about 25% and 16% increases in ^{13}C-enrichment of orotate, respectively, which is a metabolite precursor for pyrimidine biosynthesis and could imply that an expansion of nucleotides might be important for growing or dividing tissues (**Fig. 34C**).

We observed reductions in levels of AMP and ADP early in an exercise training program (**Fig. 34D**) which mostly recovered following 4 wk of exercise; however, there were no changes in ATP levels following any duration of training. Compared to sedentary hearts, there was a higher energy charge in hearts of mice exercised for 1 d or for 1 wk (**Fig. 34E**), as calculated with the abundance of adenine nucleotides as follows: energy charge ratio = ([ATP]+0.5[ADP]) / ([ATP]+[ADP]+[AMP]). Given that biosynthetic reactions require energy, the higher

Figure 34

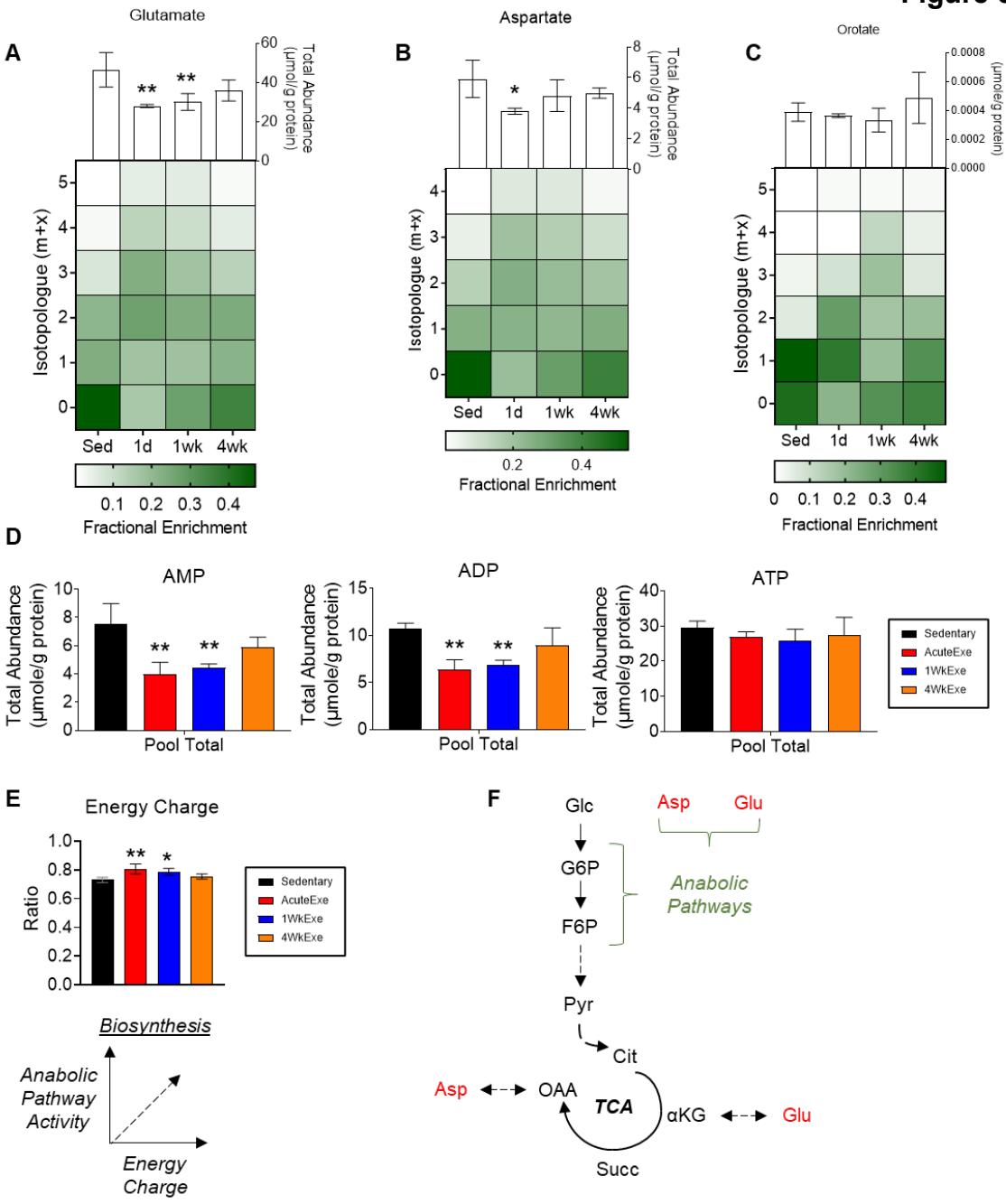


Figure 34: Acute exercise promotes a biosynthetic environment in the heart. Cardiac pool totals and isotopologue abundances of the amino acids glutamate (A) and aspartate (B), with corresponding fractional enrichment plots from $^{13}C_6$-glucose-derived carbon delivered to mice in liquid diet. Abundance of adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP) in the heart (C) with corresponding energy charge calculation for the cardiac environment (D). Schematic of biosynthetic environment (E). *n*=3–5/gp, *p<0.05, **p<0.01, one-way ANOVA (pool totals and energy charge) with Tukey's multiple comparison test. Enrichment data were logit-transformed, and p-values reflect one-way ANOVA with Tukey's multiple comparison test.

energy charge observed 1 d and 1 wk after exercise training indicates a favorable redox environment for biosynthetic activity. We summarize these findings of exercise-induced effects on cardiac glucose utilization and anabolic metabolism in **Fig. 34F**.

We also find that cardiac glycogenesis appears greatest following 1 d of exercise training, which would be consistent with an anabolic state in the heart.[214,429] Hearts of mice exercised for 1 d show the greatest ^{1}H-NMR spectral intensity of glycogen, which indicates its abundance is greatest in hearts of these mice (**Fig. 35A**). Supporting the notion of glucose-sourced glycogenesis during this timeframe, we found over 20% increase in the abundance of glucose-1-phosphate (G1P) and nearly 50% increase in total ^{13}C-enrichment of G1P in hearts of mice exercised for 1 d compared to hearts of sedentary mice. Furthermore, we also observed close to 30% increase in ^{13}C-enrichment of UDP-glucose in hearts of mice exercised for 1 d compared to hearts of sedentary mice (**Fig. 35B–C**), suggesting glucose-sourced glycogenesis in the heart following exercise.

DISCUSSION

In this study, we established a time-course of exercise-induced cardiac growth showing active growth following 1 wk of exercise training and confirming established growth in as little as 2 wk of exercise training. We then exercised mice for 1 d, 1 wk, or 4 wk and delivered $^{13}C_6$-glucose to mice via liquid diet to visualize cardiac glucose utilization during acute, dynamic, and established phases of cardiac remodeling. We found acute reductions in the abundance of pyruvate and

Figure 35

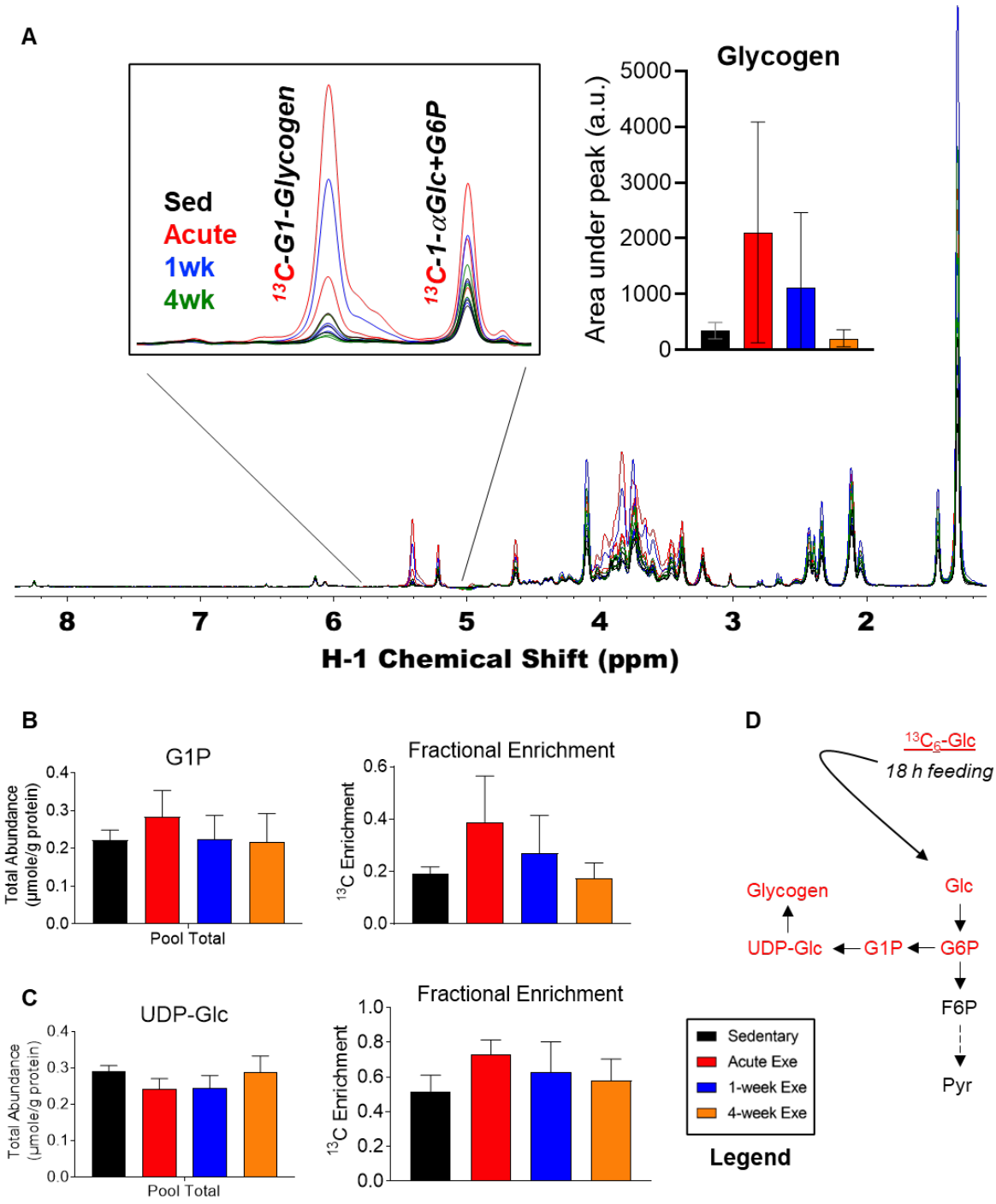


Figure 35: Exercise training alters glycogen utilization in the heart. Representative NMR trace (**A**) of cardiac metabolites with glycogen highlighted in subset. Total abundance and fractional enrichment of G1P (**B**) and UDP-Glc (**C**), with schematic of the incorporation of glucose-derived carbon into glycogen biosynthesis (**D**). G1P=glucose-1-phosphate. *n*=3–5/gp.

in proximal TCA cycle intermediates. The reductions in abundance of TCA cycle metabolites were paired with increased ^{13}C-enrichment, indicating greater turnover during active cardiac growth; however, all changes observed during growth periods returned to levels seen in sedentary hearts after 4 wk of exercise. Finally, we observed greater turnover of hypertrophy-promoting amino acids glutamate and aspartate during active phases of cardiac growth, which was accompanied by higher energy charge and increased glycogenesis when compared to hearts of sedentary and 4 wk exercised mice.

Because we previously showed that cardiovascular adaptation to exercise occurs in male FVB/NJ mice and plateaus following 2 wk of training (Chapter III), it was important to expand the timeline of exercise-induced cardiac remodeling to determine a timepoint of dynamic growth. While we verified cardiac growth through gravimetry, we did not see significant changes in mTOR, AMPK, or AKT phosphorylation 24 h following the final bout of exercise. Since the mice were not fasted prior to euthanasia, the immunoblot results likely reflect feeding status more than hypertrophic changes. Other groups have found evidence of dynamic cardiac adaptations to exercise early in a training program,[430,431] similar to what we show in this study. The importance of establishing a dynamic timepoint of exercise-induced cardiac growth is to investigate processes and visualize metabolic relationships which lead to physiological growth.

We chose to investigate metabolic changes in the heart during acute, dynamic, and established phases of exercise-induced cardiac growth. Because changes in cardiac glucose metabolism influence growth and remodeling,[214] we

examined how cardiac glucose utilization is influenced by various durations of exercise training. While recent studies show comprehensive coverage of human plasma metabolite abundances[432] and murine cardiac metabolite abundances[362] following acute exercise, we are the first to use isotope tracing *in vivo* to visualize glucose utilization in the heart in response to exercise. Using deep network tracing, we observed distinct patterns of glucose-sourced metabolite abundance and ^{13}C-enrichment during acute, dynamic, and established phases of exercise-induced cardiac growth. While there were no significant differences in abundance of most glycolytic intermediates, hearts of mice exercised for 1 d showed acute reduction in hexose abundance ($p<0.05$, data not shown) compared to hearts of sedentary mice.

The effects of exercise were more pronounced when observing abundance and ^{13}C-enrichment of TCA cycle metabolites. We saw a 20% reduction in the abundance of citrate ($p<0.10$) in hearts of acutely exercised mice, but deep network tracing highlighted a general increase in the fractional enrichment of *m*+4 and *m*+6 isotopologues compared to hearts of sedentary mice, indicating a greater incorporation of glucose-derived ^{13}C, which is likely sourced from acetyl CoA in successive turns of the TCA cycle. This phenomenon was maintained, and even magnified to significance, in the metabolites aconitate and α-ketoglutarate, which indicates a great turnover of these metabolites in the heart following exercise. Interestingly, there was no effect of exercise on cardiac abundances of succinate, fumarate, or malate; however, there was appreciable increase in ^{13}C-enrichment of *m*+2 and *m*+4 isotopologues in each metabolite. These results could imply

greater synthesis and utilization of succinate, fumarate, and malate because ^{13}C-enrichment is increased while total abundance remains unaffected. Taken together, glucose-sourced enrichment and utilization of TCA cycle metabolites in the heart is an important early response to exercise and appears to prioritize utilization through α-ketoglutarate.

The promotion of physiological cardiac growth in response to exercise requires coordination of catabolism with biosynthetic metabolic events. We found reductions in the abundances of glutamate and aspartate in hearts of acutely exercised mice compared to sedentary hearts, but ^{13}C-enrichment in these amino acids were higher (logit-transformed, $p<0.10$) than in hearts of sedentary mice, which again indicates augmented synthesis and utilization in hearts of acutely exercised mice. A recent study suggested these amino acids were important in hypertrophic responses in the heart,[428] which could be further supported by our data showing acute increases in glucose-sourced glutamate and aspartate turnover. Furthermore, another study implicated glucose-sourced changes that promote hypertrophic signaling in the heart following a single bout of exercise.[245] Indeed, we provide evidence suggesting the metabolic changes we observe in the heart following an acute bout of exercise likely supports hypertrophic events because energy charge is highest at this timepoint, which could align with energy requirements for biosynthetic reactions.

The finding that cardiac ^{13}C-enrichment of orotate could be influenced by acute exercise further supports that biosynthesis occurs in the heart following exercise. Orotate is a precursor to pyrimidine synthesis and is formed in a series

of reactions, which include carbamoyl phosphate and aspartate. One study suggested its importance in maintaining cardiac function during early stages of hypertrophy.[433] Fractional enrichment data could identify glucose-sourced contributions to its synthesis, since orotate is essentially a 5-carbon metabolite comprised of carbamoyl phosphate, aspartate, and a few enzymatic steps. The enrichment pattern of orotate indicates the *m*+2 isotopologue has greatest enrichment, which would likely be sourced from aspartate with *m*+2 enrichment as well. Interestingly, hearts of acutely exercised mice showed significant reductions in the abundance of UMP, but unfortunately there were no significant effects on fractional enrichment. Taken together, these data could imply that there is greater synthesis of orotate and potentially greater utilization of UMP in the heart following an acute bout of exercise; however, this interpretation could be strengthened with additional data on metabolite abundance and ^{13}C enrichment of other intermediates in pyrimidine biosynthesis.

Finally, we observed increased glycogen accumulation in hearts of acutely exercised mice. Previous studies report increased myocardial glycogen content resulting from exercise,[214,434] which could potentially result from an accumulation of G6P in the heart and subsequent activation of glycogen synthase.[435] While we did not see significant differences in many cardiac glycolytic metabolites due to exercise, there was >2-fold increase in the abundance of G6P in hearts of acutely exercised mice compared to hearts of sedentary mice; however, we did not measure activity of glycogen synthase or other enzymes involved in glycogenesis. By tracing glucose-derived carbon into metabolites in the heart, we found that

glycogen accumulation occurred within 18 h following exercise. Furthermore, we saw 21% increase in abundance of G1P and >2-fold increase in ^{13}C-enrichment of its *m*+6 isotopologue in hearts of acutely exercised mice compared to hearts of sedentary mice, indicating glucose-sourced enrichment of glycogen synthesis following exercise.

The findings in this study are important because we provide a temporal view of cardiac glucose metabolism in acute, dynamic, and established phases of exercise-induced cardiac growth. Using deep network tracing, we provide insight into metabolic flux and biosynthetic pathway activities of glucose metabolism that promote physiological growth in the heart. The observation that many metabolic changes that occur during acute and dynamic phases of remodeling revert after established remodeling in the heart suggests important coordination and regulation of substrate utilization. Understanding the signals for promoting and subsequently inhibiting anabolic pathway activity in the heart remains an exciting field for future study. Additionally, there are important future studies that could discern how cardiac utilization of non-glucose substrates influences glucose metabolism in the heart. Nevertheless, these data could be useful in identifying strategies or interventions to optimize cardiovascular benefits of exercise and promote beneficial remodeling in the heart.

CHAPTER VI

SUMMARY AND CONDLUDING REMARKS

The collection of studies presented in this dissertation addresses how exercise influences cardiac metabolism and how these changes could promote growth in the heart. Because changes in metabolism alone have been associated with physiological cardiac growth,[214,436] we assert that exercise-induced changes in cardiac metabolism provide building block materials for biosynthetic processes by altering cardiac utilization of glucose-derived carbons. Our general hypothesis was that exercise leads to cardiac growth by regulating cardiac PFK activity and coordinating anabolic pathway activity in the heart. To test this, we first characterized how cardiac metabolism responds to exercise and then tested the control exerted by cardiac PFK activity on anabolic pathway activity in the heart. We found biological sex-dependent effects on murine cardiac metabolism and the cardiac response to exercise. Furthermore, we found that mobilization of BCAAs during exercise is associated with time-dependent changes in cardiac PFK activity and anabolic pathway activity that promotes exercise-induced cardiac growth.

The first step in testing our hypothesis involved characterizing exercise-induced changes in cardiac metabolism. In Chapter II, we measured cardiac mitochondrial respiration on several oxidizable substrates, as well as mitochondrial ADP sensitivity, following acute and chronic exercise. We emphasized the

potential contribution of lactate to cardiac mitochondrial respiration because cardiac reliance on lactate is greatly elevated during exercise (**Fig. 3**). We found that exercise training did not influence cardiac mitochondrial respiration in the isolated organelle and that ADP sensitivity was mostly unaffected by exercise training. Interestingly, we did observe a sex-dependent effect on ADP sensitivity of cardiac mitochondria following an acute, exhaustive bout of exercise with mitochondria isolated from female hearts showing greater sensitivity than males (**Fig. 8B**). Nevertheless, while exercise training alters substrate concentrations in circulation, it does not appear to have significant effects on mitochondrial electron chain capacity or ADP sensitivity in the isolated organelle. Because we did not test other factors which could influence mitochondrial respiration in the exercising heart, this leaves possibilities that cardiac mitochondrial respiration during exercise may increase due to other factors such as substrate availability, intracellular calcium concentration,[437] or even sensitivity of substrate transporters. An important observation resulting from this study was the determination that LDHB is primarily a cytosolic enzyme and is not located in the mitochondrial matrix (**Fig. 11**). The cellular localization of this enzyme has been heavily debated and we provided evidence through a protease protection assay that LDHB is not present in the matrix of mitochondria isolated from the heart. This finding could help explain why isolated mitochondria were able to respire on glutamate, malate, and ADP but cannot respire when provided lactate for respiration.

In Chapter III, we further characterized exercise-induced changes in cardiac metabolism by subjecting male and female FVB/NJ mice to an exhaustive bout of

exercise and then measuring metabolite abundances in the heart immediately following exercise, 1 hour following exercise, and 24 hours following exercise. We found that, at baseline, male and female hearts showed major differences in the composition of the cardiac metabolome (**Fig. 15**), marked by higher levels of glycerophospholipid species and sphingomyelin in female hearts compared to male hearts. Additionally, there were higher levels of tryptophan, pyridoxamine, and homoarginine in female hearts that could contribute to the cardioprotective[346,349] and resilient[348] phenotypes observed in female hearts when compared with male hearts. In both male and female hearts, corticosterone was significantly increased during exercise. This glucocorticoid response could be important in the regulation of energy metabolism, providing a means to maintain adequate substrate availability for tissues in the exercising mouse. While glucocorticoids have been implicated to promote cardiomyocyte hypertrophy,[438] it is unclear if the transient increase during exercise has growth-promoting effects. It could be possible, however, that corticosterone binds nuclear glucocorticoid response elements and influences transcription that favors hypertrophic effects in the heart.[439]

Exercise influenced metabolite abundances more in female hearts than in male hearts. Notably, female hearts appeared to have greater increases in tyrosine, tryptophan, BCAAs, and 3-hydroxybutyrate than male hearts following exercise; however, all exercise-induced changes returned to levels seen in sedentary hearts 24 hours following exercise. Coupled with our data from Chapter II, we notice that while hearts of female mice show a greater number of metabolite

changes following exercise, these metabolite changes did not promote lasting growth in hearts from exercise-trained females. Collectively, the findings suggest there are intrinsic differences in male and female hearts and that, following exercise, the cardiac metabolome in female hearts changes to a greater extent than the male cardiac metabolome.

To understand how some of the exercise-induced changes in cardiac metabolite levels contribute to cardiac growth, we investigated the role of BCAAs in exercise-induced cardiac growth because BCAAs are known to promote cardiomyocyte hypertrophy and protein synthesis.[326,327] We used only male mice for this study because we consistently see no physiological cardiac growth in female hearts (on the FVB/NJ background) following a forced exercise training program (**Fig. 5B**). Mice were provided low- or high-BCAA diets and exercised at 75% intensity for two weeks to elicit physiological cardiac growth. We observed cardiac growth in high-BCAA fed mice, but not low-BCAA fed mice, following two weeks of exercise training. Interestingly, we did not see significant growth in sedentary hearts of high-BCAA fed mice compared with low-BCAA fed mice, implying that the stimulus of exercise in addition to BCAAs was important in promoting physiological growth of the heart. While BCAAs could promote growth in the heart by stimulating mTOR signaling,[326] less is known regarding the effects of BCAAs on other growth-promoting properties. A recent study investigating the relationship between BCAAs and glucose metabolism in the heart suggested a coordinated response is required for cardiomyocyte hypertrophy.[180] A promising future direction to come from these data is the hypothesis that elevations in

myocardial BCAAs following exercise reduce cardiac glycolytic rate and spare glucose-derived carbons for biosynthetic purposes.

In Chapter IV, we examined how constitutive changes in cardiac phosphofructokinase activity regulate cardiac glucose utilization *in vivo*. Because cardiac PFK activity decreases during exercise,[214] we investigated how this phenomenon controls biosynthetic pathway activity and levels of amphibolic metabolites in the heart. Using male mice with cardiac specific kinase- or phosphatase-deficient constructs of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (termed Glyco^{Lo} and Glyco^{Hi}, respectively), we delivered $^{13}C_6$-glucose via liquid diet for 18 hours and then freeze-clamped hearts to measure abundance of, and ^{13}C enrichment in, cardiac metabolites. We found greater abundance of amphibolic metabolites glucose-6-phosphate and fructose-6-phosphate in hearts of Glyco^{Lo} mice than wild-type or Glyco^{Hi} mice. This finding could be important for understanding exercise-induced cardiac growth because glucose-6-phosphate increases mTOR activity.[436] Transient reductions in cardiac PFK activity during exercise may increase the abundance of glucose-6-phosphate and work concomitantly with elevations in BCAAs to promote mTOR signaling and subsequent hypertrophy in the heart. Additionally, we observed that low cardiac PFK activity was associated with greater abundance and enrichment of glucose-derived carbon (measured via ^{13}C-enrichment) in intermediates for the pentose phosphate pathway and the purine biosynthetic intermediate, AICAR. Associated with the increased abundance of AICAR was the existence of multimeric complexes that contained the metabolic enzyme, PAICS, which is required for

biosynthesis of AICAR and suggests a potential channeling mechanism selectively routing carbon toward AICAR biosynthesis. The phenomenon of metabolic channeling is relevant to understanding hypertrophic responses in the heart because it demonstrates efficiency of glucose-derived metabolite synthesis which could support demands of growth, such as lipids for membrane expansion and nucleic acids for genetic material.

To gain insight into how exercise influences cardiac glucose utilization and anabolic pathway activity, we standardized a noninvasive method of dietary delivery of $^{13}C_6$-labeled glucose to mice and measured fractional enrichment of ^{13}C in cardiac metabolites following various durations of exercise training. We chose to use this method to trace glucose utilization in male, FVB/NJ murine hearts following one day, one week, and four weeks of exercise training which represent acute, dynamic, and established phenotypes of exercise-induced cardiac growth. Summarized in Chapter V, we found remarkable changes in cardiac glucose utilization following one day and one week of exercise; however, cardiac glucose utilization following four weeks of exercise training appeared to match that in sedentary hearts. Indeed, we observed a general reduction in the abundances of amino acids and TCA cycle intermediates, but higher fractional enrichment, in hearts of acutely exercised mice compared to hearts of sedentary and four-week exercised mice. This could imply greater synthesis and utilization of these metabolites in the acute response to exercise, which would facilitate macromolecule biosynthesis required for structural hypertrophy. Supporting this premise is the observation that energy charge in hearts of mice exercised one day

and one week is significantly greater than energy charge in hearts of sedentary and four-week exercised mice. Energy charge in the heart could be influenced by mitochondrial oxidation of non-glucose substrates. Indeed, we found that exercise increases circulating lactate, ketone bodies, and BCAAs, all of which are dependent upon the intensity of training. Furthermore, myocardial abundance of non-glucose substrates increases one hour following exercise, which could increase ATP:ADP ratio while sparing glucose-sourced carbons for biosynthetic purposes. Finally, we find that acute exercise increases glycogen storage in the heart. This could be due to the effects of exercise on cardiac PFK activity,[214] or even a result of myocardial elevations in corticosterone following exercise.[440] Nevertheless, these data collectively suggest that during periods of active physiological cardiac growth, glucose-derived carbons are likely preserved for biosynthetic purposes and to support anabolic pathway activity before cardiac glucose utilization returns to baseline levels after hypertrophy is established.

Taken together, the data obtained from these studies provide insight into metabolic changes occurring in the heart during and after exercise, highlighting how exercise training modifies cardiac glucose utilization to promote physiological growth. The coordination of catabolic and anabolic activities in the heart maintains energy supply while providing substrates to promote growth. Importantly, exercise increased circulating and myocardial abundance of BCAAs, which appeared necessary to promote the hypertrophic response to exercise and likely have an important role in regulation of glucose metabolism in the heart. These findings are summarized in **Fig. 36**.

Figure 36

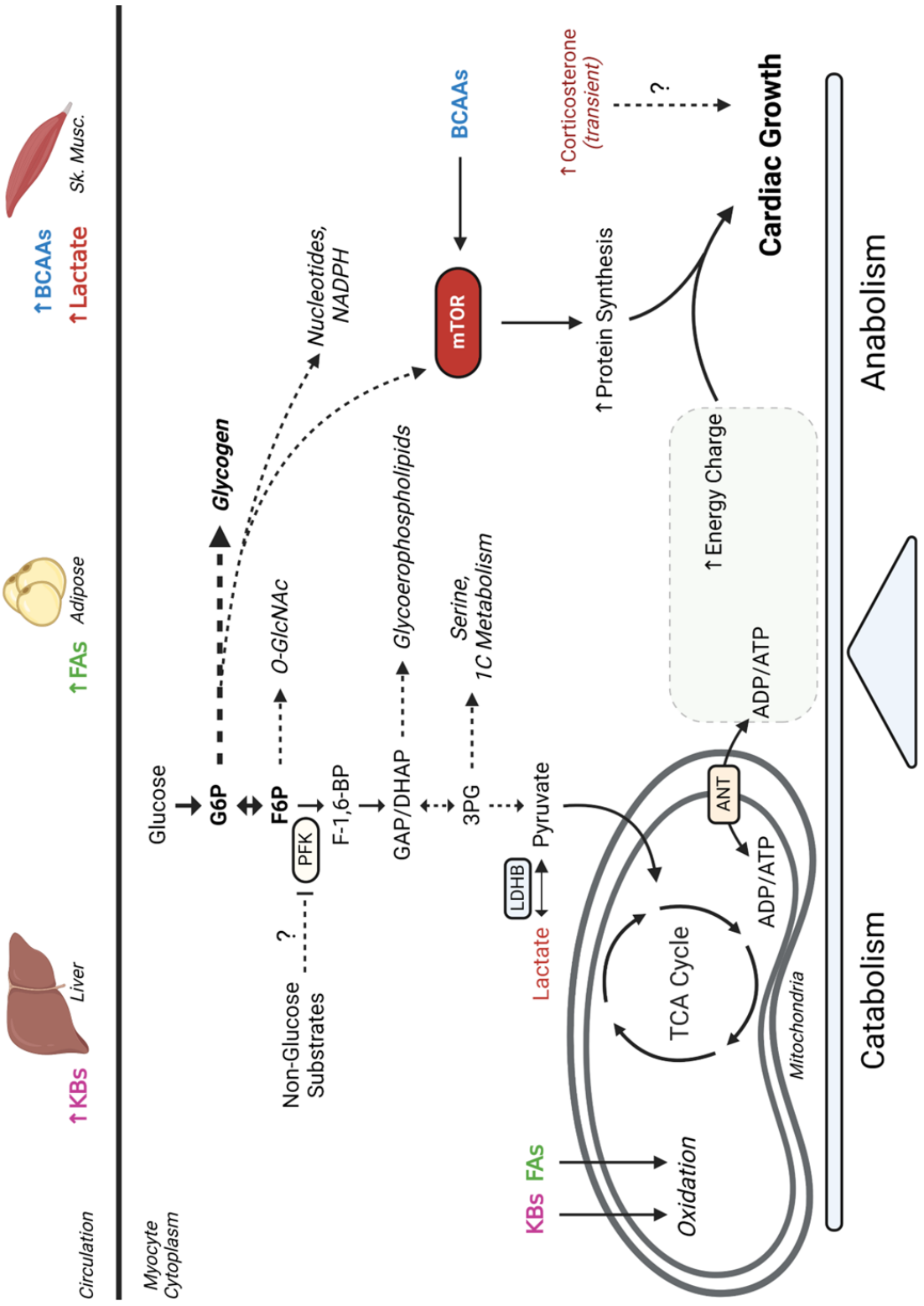

Circulation
Myocyte Cytoplasm
Liver
↑ KBs
Adipose
↑ FAs
Sk. Musc.
↑ BCAAs
↑ Lactate
Glucose
G6P
Glycogen
F6P
O-GlcNAc
Nucleotides, NADPH
Non-Glucose Substrates
?
PFK
F-1,6-BP
GAP/DHAP
Glycoerophospholipids
3PG
Serine, 1C Metabolism
BCAAs
mTOR
↑ Protein Synthesis
↑ Corticosterone (transient)
?
Cardiac Growth
LDHB
Pyruvate
Lactate
KBs
FAs
Oxidation
TCA Cycle
ANT
ADP/ATP
ADP/ATP
↑ Energy Charge
Mitochondria
Catabolism
Anabolism

Figure 36: Metabolic foundations of exercise-induced cardiac growth. A revised working model of exercise-induced changes in cardiac metabolism that support physiological growth. Elevations in circulating substrates are taken into the heart to maintain cardiac ATP production while potentially increasing energy charge following exercise. Coordination between BCAA and glucose metabolism spare glucose-derived carbons for biosynthetic purposes and promote growth in the heart. Non-glucose substrates: KB = ketone bodies, FA = fatty acids, BCAA = branched-chain amino acids.

Metabolic Foundations of Exercise-Induced Cardiac Growth

Exercise is one of the best interventions for cardiovascular wellness and longevity, but the mechanisms by which exercise supports beneficial adaptations in the heart remain unclear. In these studies, we show the following:

1) Exercise training has minimal effects on cardiac mitochondrial respiration in the isolated organelle, and LDHB is primarily a cytosolic enzyme.[441]
2) Biological sex influences the cardiac metabolome at rest and impacts the cardiac metabolic response to exercise[442]; and, branched-chain amino acids appear necessary to facilitate exercise-induced cardiac growth. (*unpublished data*)
3) Deep network tracing is a useful tool for examining glucose utilization and pathway activities *in vivo*. Reductions in cardiac PFK activity are associated with channeling of glucose-derived carbon to AICAR biosynthesis, mediated by multimeric complexes containing the purine biosynthetic enzyme, PAICS.[375]
4) During periods of active cardiac growth following exercise, there is increased turnover of glycolytic and TCA cycle metabolites in the heart that, coupled with an increased energy charge, promote glucose-derived biosynthesis of glycogen, nucleotide intermediates, and amino acids. (*unpublished data*)

These findings are relevant to cardiovascular health and could help identify ways to optimize cardiovascular responses to exercise. In particular, understanding the role of biological sex in cardiovascular responses to exercise could identify

mechanisms to promote cardiac growth or resilience. Additionally, understanding the role of BCAAs and other substrates in cardiac remodeling could reveal actionable targets which promote beneficial, or prevent deleterious, remodeling in the heart. Finally, the utilization of deep network tracing could highlight strategies for effective metabolic interventions in treating heart failure.

Future Directions

We found that a low-BCAA diet prevented exercise-induced cardiac growth following two weeks of exercise (Chapter III), but the mechanisms by which BCAAs promote exercise-induced cardiac growth remain unknown. Our study design was limited to observing the general phenomenon that elevations in BCAAs are required for cardiac growth. These observations could result from enhanced oxidation of BCAAs in the heart, which could potentially augment the pool of TCA metabolites to produce reducing equivalents for the electron transport chain, or it could maintain the pool of TCA metabolites if cataplerotic reactions drive synthesis of glutamate from alpha-ketoglutarate. This could be tested by increasing BCAA oxidation (via i.p. administration of BT2, up to 60 mg/kg/d) and observing the effects on exercise-induced cardiac growth. We would expect to see reduced activation of mTOR, and we may see a reduction in exercise-induced cardiac growth if transient elevations in BCAA abundance are necessary for mTOR signaling and protein synthesis associated with physiological growth. Or, coupling our observations with the results of Chapters IV and V, we could propose a new hypothesis that myocardial BCAAs influence cardiac PFK activity and augment the

activity of biosynthetic pathways of glucose metabolism during periods of active cardiac growth. Nevertheless, the simplest explanation for our observations would be that transient elevations in myocardial BCAAs result from exercise and activate mTOR signaling to promote protein synthesis. Future studies that target the role of BCAAs in cardiac remodeling remain an exciting field with potential to clarify the mechanisms of exercise-induced cardiac growth. A recent study found that dietary consumption of just one high-BCAA diet leads to significant increases in cardiac mass and cardiomyocyte cross-sectional area, mediated at least in part by cardiac mTOR signaling.[327] Interestingly, the cardiac effects of this high-BCAA diet required its consumption at the end, but not the beginning, of the active phase in mouse light/dark cycle, which indicates that BCAAs may influence cardiac remodeling in a circadian-dependent manner.

One strength associated with these studies is that the time of day at which exercise was performed was consistent throughout studies. This is important because time of day has been implicated to influence cardiovascular function[443] and metabolism[444] even in the absence of exercise. The influence of the circadian cycle on metabolism is particularly conspicuous in the context of exercise. Compared with exercise in the early rest phase, exercise during the early active phase has more robust effects on the number of metabolites changed in several tissues, including the heart.[362] Skeletal muscle BCAA and ketone body metabolism was shown to be higher in the early active phase than in the rest phase, suggesting that the metabolic response in muscle is dependent upon the time of day at which exercise is performed.[362] Because BCAAs are known to stimulate cell growth, it is

not surprising that many amino acid degradation enzymes peak in the active hours, which would help provide rhythmicity to catabolic-anabolic phases of metabolism and could be important for tissue remodeling in response to exercise. It is likely that circadian BCAA oscillations may influence exercise-induced cardiac growth, given the tight coordination between BCAA and glucose utilization, which work through Kruppel-like factor 15 (Klf15) to integrate metabolic pathway activity with hypertrophic signaling.[180] Collectively, these findings imply that circadian variations in metabolism could influence the degree to which cardiac growth and remodeling occur after exercise. Future studies targeting the relationships between exercise, cardiac metabolism, and circadian biology could reveal insights useful for optimizing cardiometabolic health and cardiovascular responses to exercise.

In addition to examining the effects of circadian influences and BCAAs on cardiac remodeling, future studies should be designed to better understand biological sex-dependent differences in cardiac metabolism and adaptation to exercise. While we did not observe physiological growth in hearts of female mice subjected to exercise training, there were robust changes in cardiac metabolite abundances and enhanced mitochondrial ADP sensitivity following an acute bout of exercise when compared with hearts of male mice subjected to the same training. Nevertheless, it is surprising that exercise training did not have lasting effects on mitochondrial electron transport chain capacity when we tested respiration in the isolated organelle. Considering that we isolated mitochondria from the cellular environment, it is possible that mitochondrial networks could

remodel in response to exercise training or that metabolic enzyme activity could be influenced by exercise; however, that remains a separate question for future studies. We could design experiments to test if sex-dependent differences result from sex hormones as follows: first, we could perform ovariectomy in female mice or orchiectomy in male mice and subject to exercise training, then we could compare indices of exercise and cardiac adaptations to sham-operated male and female mice subjected to the same training.

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APPENDIX

The contents of Chapters 1, 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. A portion of the future directions has been submitted to *Current Opinions in Physiology* as a review article describing the relationship between exercise, cardiac metabolism, and circadian rhythm. This is currently under review.

CURRICULUM VITAE

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EDUCATION

07/2013	B.S. in Biological Chemistry, Evangel University, Springfield, MO
12/2015	M.S. in Cell and Molecular Biology, Missouri State University, Springfield, MO
08/2020	M.S. in Physiology, University of Louisville, Louisville, KY
12/2022	*Ph.D. in Physiology, University of Louisville, Louisville, KY*

ACADEMIC APPOINTMENTS

08/2018 – present	Doctoral Candidate Department of Physiology University of Louisville Louisville, KY
08/2013 – 05/2015	Graduate Assistant Department of Computer Information Systems Missouri State University Springfield, MO

OTHER POSITIONS AND EMPLOYMENT

04/2016-07/2018	Research Technologist II, University of Louisville, Louisville, KY
08/2015-04/2016	Clinical Research Technician, QPS Biokinetic, Springfield, MO
08/2015-04/2016	Medical Assistant, CoxHealth Neurological Surgery, Springfield, MO
09/2013-01/2014	Inpatient Pharmacy Technician, Mercy Hospital, Springfield, MO
08/2011-07/2013	Science Tutor, Evangel University, Springfield, MO

HONORS AND AWARDS

SHVM William C. Stanley Early Investigator Award	October 2022
UofL Graduate Student Council Travel Award	November 2018
IPIBS Graduate Fellowship	August 2018 – June 2020
Alpha Chi National Honor Society	May 2011 - Present
Dean's List	Fall 2010 – July 2013
Leadership Fellow	May 2013
Outstanding Graduate in Biological Chemistry	May 2013
Sigma Zeta National Honor Society	September 2011 – July 2013

Evangel University Founders Scholar Fall 2010 – Summer 2013
Sigma Alpha Sigma Honor Society Inducted August 2012

EDUCATIONAL ACTIVITIES

Course Instruction

Department of Computer Information Systems August 2013 – May 2015
Missouri State University, Springfield, MO
Assisted with instruction of CIS 101: Computers for Learning. Prepared lecture material pertaining to weekly assignments in Microsoft Office, Windows 8, Webpress (2013), and Adobe

General Chemistry I and II Summer 2013
Evangel University, Springfield, MO
Assisted in preparation and delivery of course lecture material and supervised problem-solving sessions for summer classes

Lab Assistant, General and Organic Chemistry August 2011 – July 2013
Evangel University, Springfield, MO
Monitored and instructed student laboratory activities, evaluated student progress through lab practicums and written exams, delivered lab-specific lectures to explain concepts and techniques

GRANTS AND CONTRACTS

Extramural

NIH F31 Predoctoral Fellowship (1F31HL154663)
Metabolic regulation of exercise-induced adaptation in striated muscle
08/21/20 – 08/21/23
Direct costs: $110,320
Role: PI

Completed grant support

Sigma Zeta National Science and Mathematics Honor Society
Optical Activity in Isomers of Alcohols
11/20/2012 – 5/4/2013
Direct Costs: $421.20
Role: Student, Co-PI (**Fulghum**, Engebretson, Thomas)

ABSTRACTS AND PRESENTATIONS

Invited Talks

Scientific Sessions, American Heart Association November 6, 2022
Coordinated Metabolic Responses Facilitate Exercise-Induced Cardiac Growth

Co-Authored Presentations

Scientific Sessions, American Heart Association November 5, 2022
Altered Nucleotide Biosynthesis is a Prominent Metabolic Feature of Cardiac and Liver Aging.
Authors: Zimple Kurlawala, **Kyle L. Fulghum**, Teresa Cassel, Kenneth R. Brittain, Jing-Juan Zheng, Ernesto Pena Calderin, Teresa WM Fan, Pawel K. Lorkiewicz, Jason L. Hellmann, Matthew N. Nystoriak, Bradford G. Hill.
Hyatt Regency, Chicago, IL

MD/PhD National Student Conference July 8-10, 2022
The Role of TAK1 in Cardiac Fibroblast Activity
Authors: Daniel C. Nguyen, **Kyle Fulghum**, Helen Collins, Ken Brittian, Steven P. Jones, Bradford G. Hill.
Cooper Mountain Resort, Frisco, CO

Society for Heart and Vascular Metabolism September 23-24, 2021
Changes in the Metabolome and Transcriptome of the Maternal Heart During Pregnancy
Authors: Helen E. Collins[1*], **Kyle L. Fulghum[1]**, Lindsey A. McNally[1], Kenneth R. Brittian[1], Julia Cariker[2], Shizuka Uchida[1#], Pawel Lorkiewicz[1], Steven P. Jones[1], Bradford G. Hill[1]. [1]Division of Environmental Medicine, Diabetes and Obesity Center, [2]KY-INBRE Bioinformatics Core, University of Louisville, Louisville, KY. *Corresponding and presenting author. [#]Current Affiliation: Department of Clinical Medicine, Aalborg University, Denmark.

Basic Cardiovascular Sciences July 26-30, 2020
Examination of the Transcriptomic and Metabolic Signatures of Pregnancy Induced Cardiac Growth
Authors: Helen E. Collins, **Kyle Fulghum**, Lindsey A McNally, Mallory L Foster, Kenneth Brittian, Shizuka Uchida, Matthew A Nystoriak, Steven P Jones, Bradford G Hill, University of Louisville, Louisville, KY
Hilton Chicago, Chicago, IL (Online format due to COVID-19)

American Society for Mass Spectrometry June 1-12, 2020
Integrating MetaboScape and Polly™ for the analysis of LC-TIMS-MS and LC-MS based fluxomics
Authors: Heino M. Heyman[1]; Heiko Neuweger[2]; Pawel Konrad Lorkiewicz[3]; Bradford G. Hill[3]; **Kyle Fulghum[3]**; Shefali Lathwal[4]; Avijit Zutshi[5]; Brian Dranka[6]; Swetabh Pathak[5]; Abhishek Jha[6]
[1]Bruker Scientific LLC, Billerica, MA; [2]Bruker Daltonik GmbH, Bremen, Germany; [3]University of Louisville, Louisville, KY; [4]Elucidata, New Delhi, India; [5]Elucidata, Delhi, India; [6]Elucidata, Cambridge, MA
Houston, TX (Online format due to COVID-19)

American Heart Association, Scientific Sessions November 10-14, 2018
Electronic Cigarette Aerosols Alter Cardiac Expression of Genes Key to Electrical and Structural Remodeling in Mice
Authors: Alex P Carll, **Kyle Fulghum**, Daniel J Conklin, Aruni Bhatnagar, Sanjay Srivastava, University of Louisville, Louisville, KY
Chicago, IL

Poster Presentations

Society for Heart and Vascular Metabolism September 23-24, 2021
Phosphofructokinase-mediated metabolic channeling in the heart
Jena, Germany (Online format due to COVID-19)

International Society for Heart Research September 13-16, 2021
In vivo deep network tracing reveals metabolic changes in murine pressure overload hearts
Curtis Hotel, Denver, CO

Basic Cardiovascular Sciences July 26-30, 2020
Phosphofructokinase coordinates anabolic pathways in the heart
Hilton Chicago, Chicago, IL (Online format due to COVID-19)
Published: *Circ Res* 2020;127:A524

Society for Redox Biology in Medicine November 20-23, 2019
Stable isotope resolved metabolomics to assess cardiac metabolism *in vivo*
Planet Hollywood, Las Vegas, NV

Society for Redox Biology in Medicine November 20-23, 2019
Aerobic exercise does not promote significant adaptations in cardiac mitochondrial respiration
Planet Hollywood, Las Vegas, NV

Southeast IDeA November 6-8, 2019
In vivo stable isotope metabolomics reveals increases in cardiac glucose utilization following acute exercise
Galt House, Louisville, KY

Mitochondrial Biology Symposium September 26-27, 2019
Aerobic exercise alters cardiac mitochondrial respiration independent of ADP sensitivity
NHLBI, Bethesda, MD

Society for Redox Biology in Medicine November 22-24, 2018
Mitochondria-associated lactate dehydrogenase is not a significant contributor to bioenergetic function in striated muscle
The Palmer House, Chicago, IL

Cell Symposia: Exercise Metabolism July 12 – 14, 2015
Systemic insulin sensitivity and skeletal muscle Akt signaling in rats artificially selected for low intrinsic aerobic capacity
Amsterdam, The Netherlands

Interdisciplinary Graduate Forum* April 25, 2015
Systemic insulin sensitivity and skeletal muscle Akt signaling in rats artificially selected for low intrinsic aerobic capacity
Missouri State University, Springfield, MO
*Honorable mention presentation

College of Health and Human Services Symposium April 23, 2015
Systemic insulin sensitivity and skeletal muscle Akt signaling in rats artificially selected for low intrinsic aerobic capacity
Missouri State University, Springfield, MO

Oral Presentations

Society for Heart and Vascular Metabolism October 8, 2022
Coordinated metabolic responses facilitate exercise-induced cardiac growth
Walkerhill Hotel, Seoul, South Korea

American Heart Association, Scientific Sessions November 13-15, 2021
Temporal changes in metabolism and gene expression in murine heart following exercise
Boston, MA (Online format due to COVID-19)
International Society for Heart Research September 13-16, 2021
In vivo deep network tracing reveals metabolic changes in murine pressure overload hearts
Curtis Hotel, Denver, CO

International Society for Heart Research October 9-10, 2020
Phosphofructokinase regulates substrate channeling and metabolic signaling in the heart
Curtis Hotel, Denver, CO (Online format due to COVID-19)

Missouri Academy of Science April 18, 2015
Systemic insulin sensitivity and *in vivo* skeletal muscle Akt signaling in rats artificially selected for high and low intrinsic aerobic capacities
Missouri Western University, St. Joseph, MO

Missouri Academy of Science April 20, 2013
Optical resolution and enantiomeric separation in isomers of hexan-2-ol
College of the Ozarks, Point Lookout, MO

Alpha Chi National Convention April 4 – 6, 2013
The effect of molecular branching on specific rotation in isomers of alcohols: separation of racemic alcohols into pure enantiomers
Vanderbilt University, Nashville, TN

Sigma Zeta Nation Convention March 21 – 23, 2013
Folic acid deficiency in women of childbearing age
Decatur University, Decatur, IL

Missouri Academy of Science April 14, 2012
Optical resolution and enantiomeric separation in isomers of hexan-2-ol
University of Missouri, Columbia, MO

SERVICE

Graduate Student Council Fall 2020 – present
Physiology Department Representative

Selection Committee Member Fall 2019
Trainee-choice, environmental medicine grand rounds

BOOK CHAPTERS

Gibb AA, **Fulghum K**, Hill BG, Quindry J, and Lopaschuk GD. Influence of exercise on cardiac metabolism and resilience. *APS eBook chapter in press.*

PUBLICATIONS

In preparation:

Fulghum K, Dassanayaka S, Collins HEC, Cassel T, Lorkiewicz PK, Brainard E, Fan TWM, Hill BG, Jones SP. *In vivo* deep network tracing reveals metabolic changes in murine pressure overload hearts.

Fulghum K, Collins HE, Cassel T, Lorkiewicz PK, Fan TWM, Hill BG. Deep network tracing identifies exercise-induced changes in myocardial glucose utilization during periods of active cardiac growth.

Under review:

Fulghum K and Hill BG. Interplay between Exercise, Circadian Rhythm, and Cardiac Metabolism and Remodeling. *Curr Opinion Phsyiol. Under review.*

Peer-reviewed:

1. Carll AP, Arab C, Salatini R, Miles, MD, Nystoriak MA, **Fulghum KL**, Riggs DW, Shirk GA, Theis WS, Talebi N, Bhatnagar A, and Conklin DJ. E-cigarettes and their lone constituents induce cardiac arrhythmia and conduction defects in mice. *Nat Comm* 13:6088 (2022). https://doi.org/10.1038/s41467-022-33203-1
2. **Fulghum KL,** Smith JB, Chariker J, Brittian KR, Lorkiewicz P, McNAlly LA, Uchida S, Jones SP, Hill BG, and Collins HE. Metabolic signatures of pregnancy-induced cardiac growth. *Am J Physiol Heart Circ Physiol* 323(1):H146-H164 (2022). 10.1152/ajpheart.00105.2022
3. **Fulghum K**, Collins HE, Jones SP, and Hill BG. Influence of biological sex and exercise on murine cardiac metabolism. *J Sport Health Sci* (2022). 10.1016/j.jshs.2022.06.001
4. Audam TN, Howard CM, Garrett LF, Zheng YW, Bradley JA, Brittian KR, Frank MW, **Fulghum KL**, Polos M, Herczeg S, Merkely B, Radovits T, Uchida S, Hill BG, Dassanayaka S, Jackowski S, Jones SP. Cardiac PANK1 deletion exacerbates ventricular dysfunction during pressure overload. *Am J Physiol Heart Circ Physiol* 321(4):H784-H797 (2021). 10.1152/ajpheart.00411.2021
5. **Fulghum KL**, Audam TN, Lorkiewicz PK, Zheng Y, Merchant M, Cummins TD, Dean WL, Cassel TA, Fan TWM, Hill BG. *In vivo* deep network tracing reveals phosphofructokinase-mediated coordination of biosynthetic pathway activity in the myocardium. *J Mol Cell Caridol* 162:32-42 (2021). 10.1016/j.yjmcc.2021.08.013
6. McNally LA, Altimimi T, **Fulghum K**, Hill BG. Considerations for using isolated cell systems to understand cardiac metabolism and biology. *J Mol Cell Cardiol* 153:26-41 (2020). 10.1016/j.yjmcc.2020.12.007

7. **Fulghum KL**, Rood BR, Shang VO, McNally LA, Riggs DW, Zheng YT, Hill GB. Mitochondria-associated lactate dehydrogenase is not a biologically significant contributor to bioenergetic function in murine striated muscle. *Redox Biology* (2019). 10.1016/j.redox.2019.101177
8. **Fulghum K**, and Hill BG. Metabolic mechanisms of exercise-induced cardiac remodeling. *Frontiers in Cardiovascular Medicine* 5:127 (2018). 10.3389/fcvm.2018.00127
9. Arab C, Vanderlei LCM, Paiva LS, **Fulghum K**, Fristachi CE, Nazario ACP, Elias S, Gebrim LH, Filho CF, Gidron Y, and Ferreira C. Cardiac autonomic modulation impairments in advanced breast cancer patients. *Clinical Research in Cardiology* 107(10):924-936 (2018). 10.1007/s00392-018-1264-9