DIABETIC CARDIOMYOPATHY: PHENOTYPES, MECHANISMS, AND THERAPEUTIC TARGETS

By

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To the Faculty of Washington State University:

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Diabetic cardiomyopathy (DCM) is a syndrome of cardiac dysfunction, fibrosis, and hypertrophy that is often associated with type 2 diabetes mellitus (T2DM) independent of classical risk factors for cardiovascular disease. Identifying specific therapies for DCM has been elusive because the etiology and natural history of this condition are poorly understood. Therefore, the purpose of this dissertation was to investigate the hypothesis that DCM has a unique etiology from other types of hypertrophic disorders, to study the phenotypes and mechanisms of this disease in preclinical animal models of DCM, and investigate new therapeutic targets for the treatment of DCM. Studies in type 2 diabetic (db/db) mice reveal that post-translational protein glycosylation and the activity of histone deacetylase (HDAC) enzymes may be contributing factors to the development of DCM. Several methodological advances are also identified that can be used to improve preclinical DCM research, including a standard protocol for exercise training diabetic mice with a “human” exercise prescription. Finally, systematic analysis of the phenotype of DCM in preclinical animal models highlights confounding effects of species, diabetogenic agents, and laboratory methods that need to be standardized to improve the accuracy and precision of research in this field. Cumulatively, this dissertation provides insight into the preclinical presentation of DCM and potential underlying
mechanisms that can be used to inform future research into the etiology and management of this condition.
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Dedicated to my wife Rebecca
CHAPTER 1

Introduction

Diabetic cardiomyopathy (DCM): presentation and management

Type 2 diabetes mellitus (T2DM) currently affects an estimated 9.3% of the US population (diagnosed and undiagnosed cases, CDC National Diabetes Statistic Report, 2014). If current trends continue, the prevalence of diagnosed and undiagnosed T2DM will likely increase to 25-28% of the US population by 2050 [1]. T2DM is a disease of insulin resistance that is strongly associated with preventable factors, such as a sedentary lifestyle, obesity, and consumption of a Western diet. It is diagnosed in adults as a glycosylated hemoglobin (HbA1c) concentration ≥6.5%, fasting blood glucose ≥126 mg/dL (7.0 mM), or random glucose test ≥200 mg/dL [2]. The progression of T2DM consists of an initial state of insulin resistance characterized by hyperglycemia, hyperlipidemia, and hyperinsulinemia; in later stages, T2DM progresses to a state of β-cell exhaustion characterized by hyperglycemia and hypoinsulinemia that is similar to the phenotype observed in type 1 diabetes. T2DM is managed in its early stages by lifestyle modifications and/or oral hypoglycemic agents and in later stages by the addition of exogenous insulin.

Cardiovascular disorders are the leading complications of T2DM [3, 4] and incur the majority of diabetes-related mortality and healthcare costs in the US [5]. Coronary artery disease and autonomic neuropathy are the most studied cardiovascular complications of T2DM [6]; however, a third, less understood syndrome is largely responsible for the two- to three-fold increase in heart failure risk among patients with T2DM. Diabetic
cardiomyopathy (DCM), sometimes termed insulin-resistant cardiomyopathy [7], is a unique cardiovascular syndrome that occurs secondary to T2DM. DCM approximately doubles the risk of cardiovascular events, particularly heart failure and stroke, and cardiovascular-related mortality. Although prevalence is difficult to estimate due to differences in population study designs and rapidly evolving diagnostic criteria, between 16 and 60% of patients with T2DM exhibit DCM [8-13]. More critically, about 19-26% of patients with T2DM develop heart failure secondary to DCM [14-16], with a threefold higher risk in women compared to men [17].

DCM is characterized by left ventricular hypertrophy (LVH) and impaired lusitropy (diastolic relaxation) that degenerates to diastolic dysfunction, a reduced ejection fraction, and heart failure [17]. In normoglycemic humans, the development of LVH is typically load-dependent and arises secondary to high central or peripheral blood pressure or another preexisting cardiovascular risk factor. However, in DCM, both LVH and diastolic dysfunction appear to arise directly from T2DM independent of blood pressure, coronary artery disease, and other classical risk factors [18-20]. Diastolic dysfunction is the main functional impairment in DCM, although systolic dysfunction may be observed during exercise [21] and at later stages of the disease [22, 23].

Pharmaceutical management of DCM in humans focuses on reducing cardiac workload. First-line therapy consists of antihypertensive therapy with angiotensin converting enzyme inhibitors or angiotensin receptor blockers; however, patients with T2DM often require more aggressive treatment to achieve blood pressure goals, typically combination therapy with thiazide diuretics or third-generation β-blockers [4, 24]. While reducing central and peripheral blood pressure with these agents staves off the development of heart failure, it does not address the underlying defect in DCM, which is
not hemodynamic overload but rather the metabolic insult of diabetes. For this reason it is highly desirable to identify the underlying mechanisms in DCM and investigate new therapeutic targets specific to the diabetic heart. Therefore, the purpose of this dissertation was to study the phenotypes and mechanisms of DCM and investigate new therapeutic targets for the treatment of DCM. The subsequent chapters investigate the following Specific Aims to support the hypothesis that DCM has a unique, non-load-dependent etiology that requires novel research approaches and specific therapeutic targets:

1. **Specific Aim 1**: Investigate and characterize the presentation of DCM in currently used preclinical models of diabetes and identify confounding effects of methodology on the development of DCM.

2. **Specific Aim 2**: Investigate the trainability and characteristics of exercising VO\(_2\) in type 2 diabetic mice.

3. **Specific Aim 3**: Characterize the effects of diabetes and exercise training on the expression and activity of the class 1 histone deacetylases (HDACs) and translational control of hypertrophy in type 2 diabetic mice.

Cumulatively, the following chapters in this dissertation provide new data in support of the hypothesis that cardiac hypertrophy in DCM has a unique etiology from load-dependent cardiomyopathy and hypertrophy and identify mechanisms underlying its development.

*Diabetic cardiomyopathy results from an energy insult*

Impaired contractility in the diabetic heart is associated with both structural and molecular changes. Structurally, fibrosis and myocardial hypertrophy are the main
changes that impair diastolic filling. Many molecular mechanisms have been suggested as the underlying defect in DCM, and it is difficult to disentangle the effects of diabetes on the heart from the effects of common cardiovascular comorbidities, such as hypertension. However, the preponderance of evidence supports our hypothesis that DCM itself (i.e. subclinical diastolic dysfunction, fibrosis, and hypertrophy occurring independent of other cardiac risk factors) arises from the metabolic insult of diabetes.

The etiology of peripheral insulin resistance is not established. However, the initial insult is likely to be defective skeletal muscle glucose uptake precipitated by elevated circulating free fatty acids (FFAs) and intracellular lipid accumulation, which inhibit ligand stimulation of the insulin receptor 1 (IRS-1)-phosphotidylinositol 3 kinase (PIP3) pathway in skeletal muscle cells [25]. This is a similar etiology to the development of hepatic insulin resistance, which is thought to involve elevated intracellular diacylglycerol-induced inhibition of the PIP3 pathway [26].
At rest, the heart uses FFAs as the predominant metabolic substrate. However, under stressful conditions such as chronic hypertension, substrate switching allows the heart to use glucose as the primary energy source. Hearts exhibiting load-dependent hypertrophy are almost always characterized by this elevated glucose metabolism [27, 28]. Insulin resistance compromises this conversion because it limits the supply of intracellular glucose; instead, the diabetic heart is almost exclusively reliant on FFAs as the major metabolic substrates [29, 30]. This has three major consequences. Stoichiometrically, FFA oxidation produces less ATP than glycolysis for a given level of oxygen consumption; therefore, FFAs are less efficient cardiac energy sources than glucose. Fat oxidation also down-regulates glucose metabolism through an extensive
series of mechanisms summarized in the Randle hypothesis [31], which further depresses the heart’s ability to metabolize glucose. Finally, elevated FFA metabolism promotes mitochondrial uncoupling, which reduces the efficiency of ATP production. For these three reasons, elevated reliance on FFA in the diabetic heart is associated with increased cardiac oxygen consumption and reduced myocardial efficiency. This metabolic insult has been proposed as the underlying cause of DCM [7] (Figure 1.1).

Because insulin signaling is normally required for hypertrophy of skeletal muscle [32, 33] and cardiomyocytes [34, 35], it follows that hypertrophy in DCM incurs different mechanisms from other types of load-dependent cardiac hypertrophy. This is corroborated by the opposite effects that diabetes and pressure overload have on a group of genes that is frequently used as a biomarker of hypertrophy, called the fetal gene program. The fetal gene program earns its name because it is highly expressed during rapid in utero and immediately postnatal cardiac growth. In adult hearts, recapitulation of fetal gene expression occurs during pressure-overload hypertrophy [36-39] and is associated with increased glucose metabolism [40, 41]. However, the fetal gene program is not associated with hypertrophy in rodent models of diabetes and is even downregulated in some experimental studies of diabetic rodents [42]. This supports our hypothesis that ventricular hypertrophy in DCM has a different etiology than load-dependent hypertrophy and underscores the importance of investigating specific mechanisms for DCM.

**Epigenetic and cell signaling factors in DCM**

An emerging body of evidence implicates epigenetic mechanisms in the development of DCM. Histone deacetylases (HDACs) are enzymes involved in chromatin compaction
and relaxation; they are named for their paradigmatic function as deacetylases of lysine residues on histone tails. HDAC activity returns chromatin to its compacted state and is generally repressive of transcription [43]. However, the indirect (i.e. downstream) effects of HDAC activity are more complex. HDAC inhibitors decrease and increase the expression of approximately equal numbers of genes, and HDAC inhibition causes about 10% of mammalian genes to increase or decrease in expression by at least two-fold [44]. Moreover, HDACs have signaling roles: these enzymes evolved before histones [45] and deacetylate lysine residues on many non-histone nuclear and cytosolic proteins including STAT3, oncoproteins, and the androgen receptor [46]. These diverse functions have sparked a surge of research into the many potential applications of HDAC inhibitors.

There are four FDA-approved HDAC inhibitors used clinically as chemotherapeutics: vorinostat, romidepsin, chidamide, and panobinostat. However, emerging evidence implicates HDACs in diseases as diverse as multiple sclerosis, lupus, and type 2 diabetes [47, 48]. Recent studies suggest that HDAC inhibitors could be therapeutic targets for diabetes and its organ-specific complications [49]. HDAC inhibition prevents cardiac hypertrophy in vitro and in vivo [36, 50-58], protects pancreatic β-cell function in response to inflammatory insults [47-49, 59-65], and enhances skeletal muscle insulin sensitivity [66]. The attractiveness of applying HDAC inhibitor (HDACi) drugs specifically to T2DM lies in their insulin sensitizing effects, summarized in a recent review [49], as well as their specific effects on the heart. Class 1 HDACs are an intrinsic component of the cardiac hypertrophic response: phosphorylation of HDAC2 is required for cardiomyocyte hypertrophy [52], and acetylation of HDAC2 appears to have the same effect [67]. Similarly, non-class specific pan-HDAC inhibition completely prevents cardiac hypertrophy in response to a variety of stressors [36, 55, 56]. In one study, the effect of
HDAC2 on cardiac hypertrophy appeared to be mediated by inhibition of glycogen synthase kinase (GSK) 3β downstream of protein kinase B (PKB, aka AKT) [58].

Although by different mechanisms, HDAC inhibition also appears to have therapeutic effects on the pancreas and peripheral insulin sensitivity. HDACi treatment prevents β-cell death induced by inflammatory cytokines in vitro [61, 68], and pan-HDACi with trichostatin A increases insulin release from β-cells [65]. In mice, pan-HDACi treatment appears to improve skeletal muscle insulin sensitivity [66], and a recent study shows that pan-HDACi treatment prevents diabetes in non-obese diabetic mice by suppressing inflammation [69]. These data suggest that HDAC inhibitors, particularly class 1 inhibitors, may be therapeutic targets in T2DM and/or DCM.

In the heart, HDAC1/2 form a complex with mammalian switch-independent 3A (mSin3A) and repressor element-1 silencing transcription factor (REST). The HDAC/mSin3A/REST complex regulates fetal gene expression [70], which is associated with cardiomyocyte hypertrophy. These transcription factors are modified post-translationally by O-linked β-N-acetylglucosamine (O-GlcNAc) [71], a glucose derivative produced via the hexosamine biosynthetic pathway that is ubiquitous in the diabetic and failing heart [72-75]. O-GlcNAcylation of proteins is catalyzed by O-GlcNAc transferase and hydrolyzed by O-GlcNAcase and reciprocates with phosphorylation on serine and threonine residues on many nuclear and cytosolic proteins [73, 76-78]. Therefore, O-GlcNAc transferase and O-GlcNAcase are thought to have signaling functions akin to kinases and phosphatases. Because O-GlcNAcylation is paradoxically associated with both cardiac dysfunction [72, 75, 79-81] and cardioprotection [82, 83] and changes in response to exercise [80, 84], it is currently under study as a component of the cardiac stress response.
Data presented in this dissertation demonstrate that diabetes alters the O-GlcNAc modification and association of components of the HDAC/mSin3A/REST complex as well as the activity of the class 1 HDACs in the heart, suggesting that the actions of pro-hypertrophic transcription factors may be linked to changes in glucose metabolism in the diabetic heart. Additionally, we have recently shown that class 1 HDAC inhibition is anti-diabetic in the type 2 diabetic db/db mouse and that class 1 HDAC knockdown in vitro potentiates insulin-like growth factor 1 (IGF-1) signaling by promoting AKT and GSK3β phosphorylation (data not shown).

**Translational challenges to the study of DCM**

**Model phenotypes**

Part of the difficulty elucidating mechanisms and therapeutic targets for DCM arises from inconsistent research methodologies in preclinical literature. Previously we have shown that these inconsistencies are most likely attributable to the choice of experimental model and the definition and measurement of DCM [42]. There are three major ways of inducing experimental diabetes for the study of DCM in preclinical animal models: dietary methods, transgenic methods, and pharmaceutical methods. Dietary methods typically involve chronic high-fat, high-sugar feeding, but are not used routinely in cardiac studies because it takes over a year to observe impairments in contractility and lusitropy [85]. Among transgenic and spontaneously diabetic animal models of T2DM, the db/db mouse is the most frequently used [42]. The db/db exhibits a mutation in the long-form of the leptin receptor that causes leptin resistance, and obesity and type 2 diabetes are probably secondary effects of this phenotype [86]. These mice develop
overt diabetes and obesity by 8 weeks of age [87]. Other frequently used models include the Zucker diabetic rat [88-92], which develops diabetes secondary to a mutation in the leptin receptor; and the Otsuka Long-Evans Tokushima Fatty (OLETF) rat [93-96], which has a mutation in the cholecystokinin receptor that may underlie its development of obesity and insulin resistance [97]. The non-obese Goto-Kakizaki rat is considered a model of T2DM [98-101] but the underlying etiology of its diabetes is not fully characterized, and may comprise inflammatory insults to pancreatic islets [102] or hypercorticosteronemia [103].

Finally, experimental diabetes is frequently induced with toxic glucose analogs that cause either gradual or rapid death of pancreatic β-cells, depending on the dosing regimen. It is important to clarify that animal models of diabetes induced with injectable toxic glucose analogues are fundamentally models of T1DM, which exhibit cachexia, cardiac atrophy, and ketoacidosis. Therefore, their use in studying DCM is limited. Streptozotocin is by far the most common injectable diabetogenic drug [42].

Whether animal models display DCM is debated, perhaps because of the wide variety of diabetic models as well as differences in age and diagnostic methods. For example, cardiomyocyte hypertrophy occurs in the Goto-Kakizaki rat [99] and the OLETF rat [93], and studies have reported both the presence [104] and absence [105, 106] of cardiac hypertrophy in the db/db mouse. In the Zucker rat, cardiac function is probably not impaired [88-92], although studies have reported both the presence [89, 90] and absence [91] of cardiac hypertrophy. Further, as we have previously discussed [42], the presence of DCM depends on strain as well as diagnostic methods. For example, preclinical studies of diabetic cardiomyopathy may diagnose hypertrophy based on gross heart weight, left ventricular weight, the heart weight:body weight ratio or the heart
weight:tibia length ratio, each of which produces a different estimate of hypertrophy. Therefore, additional careful and systematic research is needed to characterize the presentation of DCM in rodent models of diabetes.

**Preclinical exercise research**

Once considered highly unsafe for cardiac patients, aerobic exercise training has now become a standard of care for preventing or recovering from cardiac events [107]. Large-scale meta-analysis has shown that exercise regimens are comparable to drug interventions for preventing cardiovascular morbidity and mortality as well as diabetes [108]. In humans with T2DM, regular physical activity consisting of low- to moderate-intensity aerobic exercise training modestly improves glucose control, blood lipids, and cardiac and microvascular hemodynamics [109, 110]. A comprehensive meta-analysis of exercise regimens showed that chronic exercise training achieves a net 0.8% reduction in HbA1c [111]. Similarly, other studies have shown that chronic aerobic exercise increases peak heart rate and peak gradient during walking [112] and improves diastolic function in patients with T2DM [113]. Even a moderate level of aerobic exercise is consistently associated with a reduced risk of heart failure in both men and women [114]. In human patients, the obvious barrier to exercise therapy is adherence [115]. However, the advantage of exercise is that it is a single, simple therapy, and patients with T2DM tend to adhere much more strictly to monotherapies than complicated combinations [115, 116]. Cumulatively, these data indicate that exercise is at least a moderately effective therapy for reducing cardiovascular complications in patients with T2DM and highly effective for lowering the risk of cardiovascular morbidity and mortality in these patients.
Previously, we have proposed that clinical exercise programs could be strategically “titrated” for functional improvements in different types of pathological cardiac remodeling [117]. For example, a short-duration, high-frequency exercise protocol could be used to produce a consistent unloading effect in the hypertensive patient, and combination aerobic and resistance exercise training could be used to promote glucose control in the patient with T2DM. However, it is difficult to verify these hypotheses based on preclinical research, largely because translational research investigating the effects of exercise in diabetic rodents shows considerable variability in exercise protocols; for example, mouse exercise protocols include treadmill running at moderate speeds (8-10 m/min) [118, 119] and fast speeds (15-20 m/min) [120], voluntary wheel running [121], and intensive swimming exercise for upwards of an hour each day [80, 82]. Exercise protocols for rodents are highly disparate, are usually more intensive than those prescribed for humans with T2DM, and are not usually calibrated to the animals’ exercise capacity. Consequently, their findings may not reflect the effects of moderate exercise on the cardiac physiology of a human with T2DM, and there is a clear need for standardized exercise regimens that can be used for preclinical exercise research in diabetic mice.

Standard practice for prescribing exercise to humans with T2DM is to set the exercise level at a low to moderate percentage of a measured or predicted maximal oxygen consumption (\(\text{VO}_{2\text{max}}\)) [122, 123]. This is difficult to accomplish in mice for multiple reasons: \(\text{VO}_{2\text{max}}\) is unknown for many rodent models and probably varies by strain, and the equipment required to perform these analyses is prohibitively expensive. To solve this problem, we identified a systematic exercise protocol using the \(\text{db/db}\) mouse compared to the non-diabetic C57BL/6 control strain that mirrors the exercise level prescribed for human diabetic patients. The results of this study show that a “human”
exercise protocol set to as little as 45-70% of these mouse strains’ peak VO₂ significantly improves absolute peak VO₂ in both strains.

**Conclusion**

Management of DCM in human patients currently focuses on the use of anti-hypertensives to reduce cardiac workload. However, DCM does not have a load-dependent etiology and does not invoke the same biomarkers as load-dependent hypertrophy. Novel therapies for DCM may include specific exercise programs and HDACi agents. However, to study the efficacy of these therapies and elucidate the underlying mechanisms of DCM, more systematic preclinical research methods are needed. This dissertation provides novel insight into the unique etiology of DCM, challenges in preclinical research, and potential new therapies for this condition (Figure 1.2). The following chapters investigate the hypothesis that DCM does not have the same etiology as load-dependent hypertrophy, systematically characterize the presentation of DCM in preclinical models, and provide new data implicating protein glycosylation and HDAC enzymes in the development of DCM.
Map of the topic areas of this dissertation. This dissertation provides new insight into molecular mechanisms of the etiology of DCM as well as translational issues that affect DCM research. The chapters in this dissertation also explore the clinical implications of these findings.
CHAPTER 2
Evidence for distinct effects of exercise in different cardiac hypertrophic disorders

Context
This chapter is a published mini-review that covers the etiology and phenotype of three different types of pathological cardiac remodeling, as well as the potential beneficial effects of a targeted exercise prescription for each condition. We also address the development of physiological hypertrophy and its relevance to heart patients. Additionally, this Chapter describes the structural and functional phenotype of diabetic cardiomyopathy in human patients and current evidence to support exercise as a therapy for diabetic cardiomyopathy.

Citation for published paper

Author contributions:
Wrote the manuscript: EJJ, BPD, SAM.
Evidence for distinct effects of exercise in different cardiac hypertrophic disorders

Abstract
Aerobic exercise training (AET) attenuates or reverses pathological cardiac remodeling after insults such as chronic hypertension and myocardial infarction. The phenotype of the pathologically hypertrophied heart depends on the insult; therefore, it is likely that distinct types of pathological hypertrophy require different exercise regimens. However, the mechanisms by which AET improves the structure and function of the pathologically hypertrophied heart are not well understood, and exercise research uses highly inconsistent exercise regimens in diverse patient populations. There is a clear need for systematic research to identify precise exercise prescriptions for different conditions of pathological hypertrophy. Therefore, this review synthesizes existing evidence for the distinct mechanisms by which AET benefits the heart in different pathological hypertrophy conditions, suggests strategic exercise prescriptions for these conditions, and highlights areas for future research.

Introduction
Cardiac hypertrophy is enlargement of the heart that occurs in response to metabolic stress, hemodynamic insults, or inherent genetic defects. It is characterized by increases in ventricular wall thickness and/or internal chamber dimensions. With the exception of the physiological hypertrophy that occurs in response to pregnancy or exercise, hypertrophic cardiac remodeling is a response to a pathological condition, and precedes or causes impaired cardiac function [124]. However, the prognoses as well as the structural, metabolic, and functional phenotypes of different hypertrophic disorders are distinct, and depend on the initial insult as well as the presence of cardiovascular comorbidities (Figure 2.1).
Aerobic exercise training (AET) reduces the risk of cardiac events with an efficacy comparable to pharmacological therapy [108]. Increasingly, AET is prescribed for the prevention, management, or rehabilitation of those cardiovascular diseases that are characterized by cardiac hypertrophy, including hypertension, myocardial infarction (MI), and diabetic cardiomyopathy [123, 125]. The rationale for prescribing AET is based on evidence that it reduces cardiovascular mortality and cardiac event recidivism rates, and improves cardiovascular risk factors such as high blood pressure and overweight [126-129]. Translational studies have shown that improvements in cardiovascular risk factors can be improved by both interval-based and continuous AET [130-132]. Therefore, a key question is what modes and intensities of exercise elicit the greatest benefit in individuals with various hypertrophic conditions.

Evidence for the effects of exercise training in humans with cardiovascular disease is mixed, and the exercise programs that have been used to investigate these effects use highly varied methods and outcome measures [133, 134]. The Heart Failure: A Controlled Trial Investigating Outcomes of Exercise Training (HF-ACTION) Trial is the
largest clinical trial to date examining the effects of AET in patients with reduced ejection fraction or New York Heart Association class II-IV heart failure who were normalized to exercise training or usual care [135, 136]. Thirty-six weeks of supervised cardiac rehabilitation followed by home-based AET until the median follow-up point of 30 months was associated with modest but significant reductions in rehospitalization and all-cause mortality, after these outcomes were adjusted for key prognostic indicators such as atrial arrhythmias [135]. Similarly, exercise training improved self-reported wellbeing assessed by the Kansas City Cardiomyopathy Questionnaire [136].

These data indicate that AET is an effective therapy for improving outcomes in patients with pathological cardiac remodeling and cardiac dysfunction. However, evidence for the structural and functional effects of aerobic exercise on different types of pathological cardiac hypertrophy is lacking, and the effects may differ depending on the mode or duration of training. Therefore, the purpose of this review is to summarize current evidence for the therapeutic mechanisms and efficacy of AET in different types of cardiac hypertrophy.

**Physiological hypertrophy**

Chronic AET, such as running, rowing or cycling, is associated with 12-lead electrocardiogram (ECG) changes indicative of increases in ventricular mass [137, 138]. Echocardiographic studies unequivocally support the existence of an “athlete’s heart” [139], characterized by eccentric ventricular remodeling, an increase in septal thickness and ventricular wall thickness (Left ventricular hypertrophy in athletes), and normal or improved ejection fraction (EF) [140]. In male athletes, left ventricular wall thickness may be between 12 and 16 mm in male athletes [141]; in females, this increase is about 23%
less [142]. This remodeling is beneficial to cardiac function and is associated with improved oxygen delivery, angiogenesis, and nitric oxide sensitivity [143].

Classic “physiological” hypertrophy results from AET and not from resistance exercise training. Indeed, it is important to clarify that resistance strength training actually results in concentric cardiac hypertrophy, and a reduction in internal ventricular chamber dimensions [144, 145]. The resistance-trained heart is therefore morphologically similar to a heart with pressure overload-induced pathological hypertrophy, although the important distinction is that resistance training-induced hypertrophy does not result in cardiac dysfunction in healthy human subjects [146, 147]. This review will focus on the effects of endurance AET on physiological hypertrophic remodeling in the heart.

It is probable that a relatively high exercise intensity, frequency, and duration are required to induce the actual “athlete’s heart.” Therefore, it is unlikely that patients with pathological hypertrophy or post-infarct remodeling will achieve the phenotype of the “athlete’s heart” or a clinically relevant level of physiological hypertrophy. However, an eight-year longitudinal study in mildly hypertensive individuals showed that chronic moderate physical activity did not induce physiological hypertrophic remodeling, but merely prevented pathological remodeling [148]. Therefore, AET may improve or prevent cardiac remodeling following cardiac insults.

Physical activity or exercise training is the result of repeated exposures to individual exercise sessions, and translational studies suggest that the effects of exercise at the molecular level occur immediately and have acute effects on hypertrophic signaling. We recently reported that 15 minutes of moderate-to-high intensity treadmill running in mice reduces the association of the histone deacetylases (HDACs) 4 and 5 with the
mSin3A/REST corepressor complex in the mouse heart, an event that may permit transcription of pro-hypertrophic genes [149]. This is consistent with the findings of McGee and Hargreaves, who showed that 60 minutes of cycling at 70% peak oxygen consumption (VO₂) decreases the association of HDAC5 with myocyte enhancer factor 2 in skeletal muscle, permitting hypertrophic signaling and sarcomeric protein expression [150]. Though preliminary, these data suggest that physiological cardiac hypertrophic signaling occurs with moderate- to high-intensity exercise, and begins either during or immediately after an exercise session.

The chronic effects of exercise on physiological cardiac hypertrophy are mediated by several required growth factors, although insulin-like growth factor 1 (IGF-1) and its receptor, IGF-1R, appear to be a primary stimulus in vivo. Ligand-bound IGF-1R induces growth signaling in cardiomyocytes by inducing the protein kinase B (PKB, aka AKT) signaling pathway; in addition, and less potently, IGF-1 activates the insulin receptor [151]. Because of this dual effect IGF-1 is a potent mitogenic agent that activates protein synthesis through the AKT and mTOR pathways. (For an excellent review, see [152].) Circulating IGF-1 is elevated in exercise-trained mice [153], rats [154], and humans [155-157], and IGF-1 signaling is essential for physiological cardiac hypertrophy in mice [35]. However, the action of IGF-1 and IGF-1R appears to be mediated by IRS-1 and IRS-2, since IRS-1/2 knockout mice do not develop cardiac hypertrophy in response to exercise training [158]. Similarly, expression of a dominant negative PI3K in mice prevents physiological hypertrophy, but has no effect on the development of pathological transaortic constriction-induced hypertrophy [159], indicating that PI3K specifically mediates physiological hypertrophic signaling. Translational studies also indicate that IGF-1 signaling is cardioprotective, and improves insulin sensitivity, cardiomyocyte
depolarization, and endothelial dysfunction, and low circulating IGF-1 may be an independent risk factor for heart disease [160].

It is important to note that chronic AET induces metabolic changes in the myocardium that may underlie structural and functional changes to the whole organ. In mice, short-term high-intensity interval training reduces fatty acid oxidation and increases glucose utilization [161]. However, ten weeks of treadmill training is associated with reduced glycolytic flux and higher rates of palmitate oxidation in isolated rat hearts [162], and seven weeks of treadmill training increases cardiac expression of genes that regulate lipid metabolism such as peroxisome proliferator-activated receptor alpha (PPARα) [163], fatty acid translocase (CD36) and uncoupling protein 2 (UCP2) [164]. This metabolic phenotype of the endurance trained heart is clearly distinct from the phenotype of the pathologically hypertrophied heart, which is a preferential glucose consumer [28, 40, 165]. Whether lipid and glucose metabolism influence hypertrophic remodeling is still unknown, but upregulation of fatty acid oxidation (FAO) protects the heart against pathological hypertrophy [166], and increased glucose utilization is strongly associated with pathological hypertrophy and cardiac dysfunction [167]. Therefore, it is possible that the therapeutic effects of AET on the pathologically hypertrophied heart are partly due to increased flexibility in substrate utilization and increased myocardial metabolic efficiency.

**Pathological hypertrophy**

**Pressure overload**

Conditions that increase LV afterload, such as hypertension or aortic stenosis, induce concentric hypertrophy that is characterized by fibrosis [124], increased ventricular wall
thickness, and reduced ventricular cavity dimensions [168]. Concentric LV hypertrophy in response to hypertension initially normalizes wall strain by increasing wall thickness [169]. However, this increase in myocardial mass is not energetically sustainable, and progresses to decompensation and heart failure [168]. Indeed, concentric hypertrophy is a strong positive risk factor for heart failure and cardiac-related mortality [170].

Treatment for concentric cardiac hypertrophy typically focuses on reducing the primary insult, i.e. the use of antihypertensives to reduce blood pressure resulting from high afterload [171]. Blood pressure reduction is clearly the most efficacious therapy for regression of hypertension-induced LV hypertrophy [172, 173] and reduces the risk of cardiovascular events by over 50% [174]. There are several classes of antihypertensives that can reduce pathological hypertrophic remodeling via different mechanistic targets; a meta-analysis concluded that efficacy of these compounds for the treatment of hypertension-associated hypertrophy, from most to least effective are: angiotensin-converting enzyme inhibitors, angiotensin receptor antagonists, calcium channel antagonists, diuretics, and beta-blockers [175]. However, epidemiological studies suggest that antihypertensives are associated with long-term complications such as idiopathic new-onset diabetes [176, 177]. While AET lowers blood pressure with virtually no detrimental side effects, the efficacy of AET for LV mass reduction and attenuation or reversal of pathological hypertrophic remodeling in hypertensive individuals has not been systematically researched.

**Effects of exercise on hypertrophy and function**

AET has both acute and chronic benefits in hypertensive individuals. Acutely, exercise elicits a transient unloading effect called post-exercise hypotension (PEH) [178]; this phenomenon may permit myocardial repair processes to occur while the heart is
unloaded. Chronic AET lowers basal catecholamine concentrations and resting heart rate [178, 179] and generally reduces systolic blood pressure by 5-10 mmHg [180]. The net effect of these acute and chronic unloading affects is a reduction in cardiac afterload, which reduces the stimulus for concentric remodeling.

Unlike pharmaceutical therapies, there is almost no uniformity in exercise regimens utilized in hypertension research, in which exercise is prescribed in highly varied modes, intensities, frequencies and durations. Therefore, it is perhaps not surprising that there is mixed evidence regarding the effects of AET on the morphology of the hypertensive heart. For example, 15 months of AET reduced blood pressure but did not change echocardiographically-determined ventricular mass in hypertensive individuals [181], while others reported that 26 weeks of AET had no effect on estimates of heart size produced by echocardiography or magnetic resonance imaging [182]. However, six months of AET decreased echocardiographically-determined LV wall thickness in hypertensive patients [183], 16 weeks of AET in hypertensive African American males caused significant reductions in ventricular mass and wall thickness compared to sedentary controls [184], and 12 weeks of supervised exercise decreased LV mass in mildly hypertensive sedentary humans [185]. Nevertheless, one study showed that 10 weeks of military training increased LV mass as determined by MRI [186], while another reported that AET increased LV mass in hypertensive participants [187]. These conflicting results highlight the need for additional research to identify more specific exercise regimens to improve afterload-induced cardiac hypertrophy.

Effect of exercise on molecular characteristics

The therapeutic effects of AET on concentrically hypertrophied hearts may also be mediated by changes in cardiac metabolism. As mentioned above, a distinguishing
characteristic of concentrically hypertrophied hearts is preferential glucose utilization rather than fatty acid oxidation (FAO) [28]. The preferential use of glucose is characteristic of fetal hearts and is thought to be a stress response in adult hearts [40, 41]. Translational studies have shown that treadmill exercise improves both glucose and fatty acid utilization in tandem in hypertensive and hypertrophied rat hearts [188]. However, there is very limited evidence for this effect, and additional studies are needed to confirm this hypothesis.

**Myocardial Infarction (MI)**

The process of ventricular remodeling after MI has been extensively reviewed elsewhere [189]. Briefly, the early phase of MI is characterized by inflammation of the infarcted area, expression of the stretch-responsive hormones atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), and increased expression of fetal isoforms of myosin heavy chain [190, 191]. “Late” or long-term remodeling after infarction includes fibrosis and a loss of contractile activity in the infarcted area, increased wall stress in the ventricular region surrounding the infarct, and progressive decompensatory dilatation of the LV that predisposes the infarcted heart to failure [192, 193].

**Effects of exercise on hypertrophy and function**

AET training reduces MI-related mortality, regardless of whether training occurs before or after the infarct occurs [194]. Outpatient cardiac rehabilitation programs that utilize AET after infarction improve LV performance and reduce mortality by 20-26% [126, 195-197]. Six months of AET successfully attenuates ventricular remodeling after MI in humans [198]. In rats, eight weeks of treadmill exercise improved ventricular fibrosis and systolic function, although it had no effect on ventricular dilatation [199]. Similarly,
voluntary wheel running in mice improved fractional shortening but not cardiac hypertrophy post-infarction [200].

Exercise consistently and significantly improves cardiac function after MI. Indeed, AET induces the same magnitude of improvement in ejection fraction (EF) as angiotensin-converting enzyme inhibitors or pacing with cardiac resynchronization therapy in humans with heart failure [201]. AET attenuates a decline in EF after MI [202, 203], and six months of AET after MI improves the LV end-diastolic volume index [198], while as little as three months of AET post-infarction can improve early diastolic function [204, 205]. A recent meta-analysis indicates that EF is improved by aerobic cardiac rehabilitation programs and clearly demonstrated that the sooner the program begins, and the longer it lasts, the greater the improvement in EF [201]. Though limited, these data highlight exercise as a potential first-line therapy for preventing cardiac remodeling post-MI. There is a clear need for additional research to identify the ideal mode and duration of exercise for preventing cardiac remodeling and improving EF after MI.

Diabetic cardiomyopathy

Type 2 diabetes mellitus (T2DM) is associated with a distinct syndrome of cardiac hypertrophy and diastolic dysfunction known as diabetic cardiomyopathy (DCM) [17, 206, 207]. The existence of a distinct DCM in human patients is reasonably well established and has been extensively reviewed in recent papers [19, 21]. Because insulin signaling is generally required for muscle protein synthesis after exercise [32, 33] and is absolutely necessary for physiological hypertrophy in cardiomyocytes [34, 35], it is possible that diabetes-associated hypertrophy is completely distinct from other types of pathological hypertrophy. For example, it has been proposed that DCM results from cardiomyocyte atrophy and apoptosis and cardiac fibrosis, rather than cardiomyocyte
hypertrophy [21]. Indeed, although T2DM is associated with increased ventricular mass [208-211], hypertrophy in diabetic hearts is associated with an increase in echodensity of the ventricular wall [212], suggesting that the increase in mass may be due to fibrosis rather than actual hypertrophy of the myocardium. In general, the molecular mechanisms and phenotype of the diabetic heart are not well understood, partly because translational research studies in this field over the last 30 years have used widely varied methods of inducing diabetes in pre-clinical models and have also reported many different indices of cardiac hypertrophy [42]. However, the clinical characteristics of DCM are well characterized and have been recognized as a distinct entity for several decades [206].

Clinically, DCM is characterized by increased LV wall thickness and mass, independent of other cardiovascular comorbidities [208-211]. T2DM is often comorbid with hypertension; however, in normotensive humans, T2DM has comparable effects to hypertension on myocardial strain and strain rate [213], as well as diastolic dysfunction [22, 214]. The early functional characteristic of DCM is diastolic dysfunction, which occurs in up to 70% of humans with diabetes [215, 216], and although most cases of DCM are asymptomatic, subclinical diastolic dysfunction appears to underlie the development of systolic dysfunction and predisposition to heart failure in humans with diabetes [19]. DCM is not usually associated with systolic dysfunction, but humans with metabolic syndrome and diabetes are more likely to display exercise-induced impaired systolic function; the reason for this exercise-induced dysfunction is currently unclear [217].

The primary therapeutic focus for a patient with DCM is essentially diabetes management: glucose control, reduction of cardiovascular comorbidities such as
hypertension, and preventing organ-specific complications of diabetes [6]. Exercise, therefore, is an ideal potential therapy for DCM because it improves not only the primary cardiac insult – hyperglycemia and insulin insensitivity – but also has a direct effect on the heart, as well as the associated cardiovascular comorbidities.

**Effects of exercise on hypertrophy and function**

To date, there is very limited research on the effects of AET in DCM. In clinical populations, the evidence is mixed; for example, six months of monitored exercise that met current American College of Sports Medicine prescription guidelines did not alter LV function in humans with T2DM [218], but eight weeks of AET reduced total vascular resistance and improved peak exercise cardiac output in humans with metabolic syndrome [219]. Although numerous translational studies have evaluated the effects of exercise on cardiac function in DCM, the conclusions are difficult to synthesize, because pre-clinical studies of DCM have traditionally used highly inconsistent methodology [42]. In our lab, eight weeks of exercise training did not alter the heart weight:tibia length ratio in db/db mice with T2DM [220]. However, others reported that 10 weeks of treadmill exercise enhanced aortic flow in a rat model of type 1 diabetes [221]. There is still very limited evidence regarding the effects of AET in T2DM at this time.

**Effect of exercise on molecular characteristics**

T2DM has unique effects on cardiac metabolism that may underlie functional changes in the diabetic heart [29]. As mentioned above, pathological hypertrophy in response to pressure overload is typically characterized by increased glycolytic metabolism relative to FAO [27, 28]. However, the diabetic heart shows the opposite phenotype in that it is primarily reliant on fatty acid metabolism and develops lipotoxicity [29, 30, 221-223]. While upregulating FAO is probably a compensatory response to insulin resistance and
glucose scarcity, the lipotoxic effects of chronically elevated FAO are associated with apoptosis and contractile dysfunction.

Reducing FAO in the diabetic heart improves the phenotype of DCM [224], suggesting that exercise interventions that reduce FAO may be therapeutic in DCM. In mice with diet-induced obesity, 8-10 weeks of moderate intensity treadmill training reduces FAO, increases glycolytic flux and mitochondrial function, and increases cardiac output in the hearts of mice with diet-induced obesity [225]. In a rat model of type 1 diabetes, 10 weeks of treadmill exercise increased translocation of GLUT4, permitting glucose entry into cardiomyocytes [226]. In a similar model, 10 weeks of treadmill exercise enhanced both glycolytic metabolism and cardiac function [221]. While these studies support the idea that AET improves cardiac function in T2DM, additional studies are needed to confirm this hypothesis.

Conclusion
Current recommendations for exercise in cardiac patients focus on reducing cardiovascular risk factors and accomplishing goals such as blood pressure and glucose management. However, AET induces beneficial, physiological changes in the heart that alter chamber dimensions and function. Therefore, it is possible that different exercise regimens will have specific rehabilitative effects following different types of cardiac events. For example, the beneficial effect of AET in the diabetic heart may be due to enhanced insulin sensitivity and normalization of myocardial metabolism, suggesting that a long duration and moderate intensity exercise prescription may be best for improving cardiac function in this patient population. Conversely, a heart that is concentrically hypertrophied due to chronically high afterload would benefit primarily from reduction in afterload. Therefore, short and repeated intervals of exercise that repeatedly induce
post-exercise hypotension may be the best approach for this patient population. At the present time, however, these speculations are not supported by systematic research, thus preventing more specific guidelines and recommendations for exercise prescription.

It is important to note that a major limitation to such systematic research is patient adherence [227]. There is very limited research on this topic, but recent meta-analyses show that adherence can be improved by reducing individual patients’ barriers to exercise [228], providing extensive personalized follow-up, and providing all-male or all-female exercise groups [229].

In conclusion, this review highlights the need for systematic, controlled research into the effects of exercise mode, intensity, frequency, and duration on the function and morphology of the hypertrophied heart. Because AET is a highly effective and low-cost intervention, has virtually no side effects, and improves almost every comorbidity associated with cardiac hypertrophy, this is a very promising avenue of research for the future of cardiovascular medicine.
CHAPTER 3

A systematic review of fetal genes as biomarkers of cardiac hypertrophy in rodent models of diabetes

Context

This chapter is a published systematic review that addresses two important questions in preclinical research regarding DCM. First, we ask whether all experimental models of diabetes display clinically relevant phenotypes. This is an important question because different diabetogenic methods have significantly different effects on animal phenotypes, some of which are major departures from DCM in humans with T2DM. Second, we ask whether biomarkers of pressure-overload hypertrophy are useful for identifying hypertrophy in rodent models of diabetes. We find no correlations between usual biomarkers and cardiac hypertrophy in diabetic rodents, which supports the hypothesis that hypertrophy in diabetic rodents does not invoke the same signaling mechanisms as pressure-overload hypertrophy. This supports the hypothesis that DCM invokes distinct signaling mechanisms from other types of pathological hypertrophy.

Citation for published paper


Author contributions:

Conceived and designed the experiments: EJC, SAM.

Performed the experiments: EJC.

Analyzed the data: EJC.

Wrote the paper: EJC, SAM.
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A systematic review of fetal genes as biomarkers of cardiac hypertrophy in rodent models of diabetes

Abstract

Pathological cardiac hypertrophy activates a suite of genes called the fetal gene program (FGP). Pathological hypertrophy occurs in diabetic cardiomyopathy (DCM); therefore, the FGP is widely used as a biomarker of DCM in animal studies. However, it is unknown whether the FGP is a consistent marker of hypertrophy in rodent models of diabetes. Therefore, we analyzed this relationship in 93 systematically selected studies. Results showed that diabetes induced with cytotoxic glucose analogs such as streptozotocin was associated with decreased cardiac weight, but genetic or diet-induced models of diabetes were significantly more likely to show cardiac hypertrophy (P<0.05). Animal strain, sex, age, and duration of diabetes did not moderate this effect. There were no correlations between the heart weight:body weight index and mRNA or protein levels of the fetal genes α-myosin heavy chain (α-MHC) or β-MHC, sarco/endoplasmic reticulum Ca^{2+}-ATPase, atrial natriuretic peptide (ANP), or brain natriuretic peptide. The only correlates of non-indexed heart weight were the protein levels of α-MHC (Spearman’s ρ=1, P<0.05) and ANP (ρ=–0.73, P<0.05). These results indicate that the most commonly measured genes in the FGP are confounded by diabetogenic methods, and are not associated with cardiac hypertrophy in rodent models of diabetes.

Introduction

Activation of the fetal gene program (FGP) in the adult heart occurs after cardiac insults and is ubiquitously used as a biomarker of cardiac hypertrophy [40, 230]. Diabetic cardiomyopathy is partly characterized by ventricular hypertrophy [23, 210]; therefore,
the FGP is commonly used as an indicator of diabetic cardiomyopathy in rodent models of diabetes. However, many studies show that fetal genes are unchanged or downregulated in these animal models. For example, rodent models of diabetes show lower circulating serum levels [231-233], lower protein levels in cardiac tissue [105, 220], and lower cardiac transcript levels [100, 104, 105, 220, 234-238] of two commonly measured fetal genes, atrial and brain natriuretic peptide (ANP and BNP). We have also shown previously that the presence of type 2 diabetes in mice blocks cardiac expression of ANP in response to hypertrophic stimuli [239]. Another measure of FGP activation in the adult heart is a decrease in the expression of α- relative to β-myosin heavy chain (MHC); however, it has been reported that this ratio is actually increased in type 2 diabetic db/db mouse hearts, and that this increase depends on the duration of diabetes [104].

To our knowledge it has not been shown that fetal genes are consistent markers of cardiac hypertrophy in rodent models of diabetes. Therefore, this systematic review was performed to determine whether these animals show higher expression of fetal genes in the heart, and whether these genes correlate with cardiac weight. The results of this analysis show that most fetal genes do not correlate with cardiac hypertrophy in diabetic animals, and that methods of diabetogenicity significantly moderate the development of cardiac hypertrophy and the expression of fetal genes.

Overview of fetal genes

The following genes are some of the most commonly measured members of the FGP that are often used as indicators of cardiac hypertrophy.

Serca2
The sarcoplasmic reticulum Ca\(^{2+}\) ATPase 2 (Serca2) is responsible for re-uptake of calcium into the SR following contraction of the sarcomere, thus permitting muscle relaxation. Levels of Serca2 increase throughout fetal development of the mammalian myocardium and are maintained in adulthood [240]. A decrease in Serca2 expression is observed in the diabetic heart [95, 241, 242] and may underlie diastolic dysfunction in diabetic cardiomyopathy [94, 95]. However, the mechanism that underlies Serca2 loss in heart disease is not understood.

**Myofilament proteins**

Myosin filaments in the heart function as complexes composed of α and β subunits. The rodent heart expresses three forms of myosin: an α-MHC form, which has the highest ATPase activity and contractile velocity; an α- and β-MHC form; and a β-MHC form, which has the lowest contractile capability [243]. During fetal development, α-MHC replaces β-MHC as the dominant transcript in cardiac muscle [244], and this difference is maintained perinatally. Therefore, a decrease in the α-MHC/β-MHC ratio is used as a marker of fetal gene reactivation in rodent hearts and is associated with cardiac hypertrophy [245]. It should be noted that the regulation of myosins in the human heart is different; while α-MHC predominates in non-failing adult rodent hearts, the adult human ventricle expresses approximately 95% β-MHC [246-248].

Other non-myosin cytoskeletal proteins which are changed in the hypertrophied heart include actin and titin. Skeletal α-actin is highly expressed in fetal hearts and is not expressed in the adult heart; instead, adult hearts express cardiac α-actin [249]. Therefore, skeletal α-actin is considered a member of the FGP and is used as a marker of hypertrophy, and is associated with cardiac dysfunction [250]. A similar switch is observed in the expression of titin isoforms: embryonic hearts express much higher
levels of the N2BA isoform of titin, which is replaced by the shorter N2B titin isoform in the perinatal and adult heart [251]; therefore, expression of the long-form N2BA in the adult heart is used as a marker of pathological cardiac hypertrophy.

**Peptide hormones**
Atrial and brain natriuretic peptide (ANP and BNP) are small peptide hormones. The prohormone precursors of ANP and BNP are encoded by the *Nppa* and *Nppb* genes, respectively, and are some of the most commonly measured members of the FPG. ANP expression is an early marker of cardiac commitment and is activated by the fetal cardiac transcription factors GATA4 and NKS2-5 [252]. In adult hearts, ANP and BNP are used as markers of heart failure because they are secreted in response to cardiac wall strain [253], although the mechanism of release is only just now being elucidated [254]. While they largely regulate natriuresis and reduce blood pressure, ANP and BNP also antagonize cardiac hypertrophy [255, 256] and fibrosis [257], and stimulate lipolysis [258].

**Transcription factors**
A suite of transcription factors governs the formation of the fetal heart and is used to mark FGP upregulation in the adult heart. The GATA4 transcription factor is expressed at high levels in the fetal myocardium and drives the formation of the fetal heart. It is required for normal valvular development [259, 260], activates a broad group of cardiac-specific genes including ANP [261] and α-MHC [262], and is required for upregulation of β-MHC in pathological hypertrophy after trans-aortic constriction surgery [263].

The NK2 homeobox protein (NKX2-5/CSX) is one of the earliest markers of commitment to the cardiac lineage in embryonic mesoderm [264]. Its expression is confined to the
heart, and is upregulated in fetal development and maintained in the postnatal and adult heart [265]. NKX2-5 expression requires GATA4 [264], and overexpression of these two genes in tandem drives commitment of mesenchymal stem cells to the cardiac lineage [266]. Recapitulation of NKX2-5 and associated transcription factor (GATA4, MEF2, and SP1) expression occurs in animal models of congestive heart failure [37], and is considered a marker of fetal gene reactivation.

Collaborating transcription factors that regulate fetal genes through interactions with GATA4 and CSX include MEF2 and Hand1/2. MEF2 governs a family of transcription factors that regulates fetal gene expression in the adult heart. Hand1/2 (eHand/dHand) are expressed at high levels in the fetal heart, and both Hand1 and Hand2 activate Nppa, via physical associations with MEF2 [267] and NKX2.5 [268].

Although the relationship between fetal genes and pathological hypertrophy is well characterized [39, 41, 230, 269], it is not known how these genes are affected by diabetes. Therefore, the purpose of this systematic review was to determine whether the expression of fetal genes is a consistent marker of cardiac hypertrophy in studies that use rodent models of diabetes. We found no correlations between fetal gene expression and the HW:BW index in rodent models of diabetes, and our results show that methods of inducing experimental diabetes significantly affect the expression of fetal genes in rodent hearts.

**Methods**

**Inputs**
**Diabetogenics**

The search terms for diabetogenic drugs and commonly used animal models of diabetes were generated from a review article [270]. Drug-induced diabetes is most commonly accomplished by injection of cytotoxic glucose analogs, i.e. streptozotocin (STZ) and alloxan, both of which are taken up by glucose transporter 2 into pancreatic β-cells [271]. The diabetogenic drugs that were included in the search parameters were STZ, alloxan, dithizone, or 8-hydroxyquinolone, but it should be noted that only the former two drugs returned results. STZ- and alloxan-induced models of diabetes were categorized as “drug,” spontaneous/genetic models were categorized as “spontaneous,” and diet- or diet/drug-induced models were categorized as “other” for our analyses.

**Fetal genes**

The inputs for the most commonly measured fetal genes were gathered from review articles [39, 230, 269]. Several of these transcription factors were included in the a priori article search, including NFAT, SRF, and the SMAD family, but these did not return any additional results when included with our other search parameters.

**Search**
Table 3.1. Complete search terms used to collect articles from the PubMed database

<table>
<thead>
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<th>Search Terms</th>
</tr>
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</table>

A MeSH search using the previously described inputs (TABLE 3.1) was used to generate an a priori list of 135 articles in PubMed. Of the 136 articles returned by the search, 42 were excluded for containing inapplicable data and one was excluded for having been retracted (FIGURE 3.1). Therefore, 93 articles were included in this review (SUPPLEMENTARY TABLE S1).

We categorized diabetogenic categories as follows: “drug-induced” (STZ or alloxan), “spontaneous” genetic models, or “other.” “Other” included diet-induced, or combination
diet- and low-dose STZ-induced diabetes. Diabetic phenotype was coded as type 1 diabetes mellitus (T1DM) or type 2 (T2DM) based on whether the intervention produced primary insulin deficiency (e.g. STZ models of diabetes) or insulin resistance (e.g. hyperinsulinemic genetic models of diabetes).
Supplementary table 1. Works analyzed in this review.


Cheng, Y.S., et al., Sildenafil and FDP-Sr attenuate diabetic cardiomyopathy by suppressing abnormal expression of myocardial CASQ2.


Sen, S., et al., Preventive effects of North American ginseng (Panax


Vasanji, Z., N.S. Dhalla, and T. Netticadan, Increased inhibition of


Statistics
Specific effect sizes were not reported for most studies; therefore, we categorized changes as up (1), down (-1), or no change (0), and used non-parametric Mann-Whitney, Chi-squared, and Spearman regression analyses as appropriate. Significance was set at P<0.05. Statistics were performed in SigmaPlot 11.0 for Windows.

Results

General description of methods/animal characteristics
Overall, 38 studies (33% of this database) used T2DM models while 78 studies (67%) used T1DM models. By far the most commonly used T1DM model was the Sprague-Dawley rat induced with STZ (TABLE 3.2). The most commonly used model of T2DM was the db/db mouse. Only one study used alloxan as the diabetogenic [272], and none of the studies used dithizone or 8-hydroxyquinolone. Therefore, as alloxan and STZ have very similar mechanisms of action, we grouped these drugs together into a single category of drug-induced diabetes for statistical analyses.
<table>
<thead>
<tr>
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<th>Strain</th>
<th>Species</th>
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<th>Diabetes type</th>
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</tr>
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<td>Spontaneous</td>
<td>OLETF</td>
<td>Rat</td>
<td>3</td>
<td>T2DM</td>
</tr>
<tr>
<td>Spontaneous</td>
<td>NOD</td>
<td>Mouse</td>
<td>2</td>
<td>T2DM</td>
</tr>
<tr>
<td>Spontaneous</td>
<td>UCD-T2DM</td>
<td>Rat</td>
<td>1</td>
<td>T2DM</td>
</tr>
<tr>
<td>Spontaneous</td>
<td>OVE26</td>
<td>Mouse</td>
<td>1</td>
<td>T2DM</td>
</tr>
<tr>
<td>Spontaneous</td>
<td>ob/ob</td>
<td>Mouse</td>
<td>1</td>
<td>T2DM</td>
</tr>
</tbody>
</table>

Dose reporting varied for the studies that used STZ or alloxan. Single-dose STZ was the most common method (n=58); however, the number of doses varied from two to seven (TABLE 3.3). Two studies did not report the number of doses of the diabetogenic agent.
Table 3.3. Summary of dosing regimens used to induce experimental diabetes in rodents.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Sex</th>
<th>Diabetogenic</th>
<th>Doses</th>
<th>N</th>
<th>Diabetes type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>C57BL/6</td>
<td>M</td>
<td>STZ</td>
<td>1</td>
<td>5</td>
<td>T1DM</td>
</tr>
<tr>
<td>Mouse</td>
<td>C57BL/6</td>
<td>M</td>
<td>STZ</td>
<td>2</td>
<td>1</td>
<td>T1DM</td>
</tr>
<tr>
<td>Mouse</td>
<td>C57BL/6</td>
<td>M</td>
<td>STZ</td>
<td>3</td>
<td>3</td>
<td>T1DM</td>
</tr>
<tr>
<td>Mouse</td>
<td>C57BL/6</td>
<td>M</td>
<td>STZ</td>
<td>5</td>
<td>3</td>
<td>T1DM</td>
</tr>
<tr>
<td>Mouse</td>
<td>C57BL/6</td>
<td>M</td>
<td>STZ</td>
<td>7</td>
<td>2</td>
<td>T1DM</td>
</tr>
<tr>
<td>Mouse</td>
<td>C57BL/6</td>
<td>M and F</td>
<td>STZ</td>
<td>7</td>
<td>1</td>
<td>T1DM</td>
</tr>
<tr>
<td>Mouse</td>
<td>CD1</td>
<td>M</td>
<td>STZ</td>
<td>1</td>
<td>1</td>
<td>T1DM</td>
</tr>
<tr>
<td>Mouse</td>
<td>FVB</td>
<td>M</td>
<td>STZ</td>
<td>1</td>
<td>1</td>
<td>T1DM</td>
</tr>
<tr>
<td>Mouse</td>
<td>FVB</td>
<td>M</td>
<td>STZ</td>
<td>5</td>
<td>3</td>
<td>T1DM</td>
</tr>
<tr>
<td>Rat</td>
<td>CR1:W1</td>
<td>Not reported</td>
<td>STZ</td>
<td>1</td>
<td>1</td>
<td>T1DM</td>
</tr>
<tr>
<td>Rat</td>
<td>Not reported</td>
<td>F</td>
<td>STZ</td>
<td>1</td>
<td>1</td>
<td>T1DM</td>
</tr>
<tr>
<td>Rat</td>
<td>Not reported</td>
<td>M</td>
<td>STZ</td>
<td>1</td>
<td>1</td>
<td>T1DM</td>
</tr>
<tr>
<td>Rat</td>
<td>Sprague-Dawley</td>
<td>F</td>
<td>STZ</td>
<td>1</td>
<td>5</td>
<td>T1DM</td>
</tr>
<tr>
<td>Rat</td>
<td>Sprague-Dawley</td>
<td>F</td>
<td>STZ</td>
<td>3</td>
<td>1</td>
<td>T1DM</td>
</tr>
<tr>
<td>Rat</td>
<td>Sprague-Dawley</td>
<td>M</td>
<td>alloxan</td>
<td>1</td>
<td>1</td>
<td>T1DM</td>
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<tr>
<td>Rat</td>
<td>Sprague-Dawley</td>
<td>M</td>
<td>STZ</td>
<td>1</td>
<td>26</td>
<td>T1DM</td>
</tr>
<tr>
<td>Rat</td>
<td>Sprague-Dawley</td>
<td>Not reported</td>
<td>STZ</td>
<td>1</td>
<td>1</td>
<td>T1DM</td>
</tr>
<tr>
<td>Rat</td>
<td>Wistar</td>
<td>M</td>
<td>STZ</td>
<td>1</td>
<td>14</td>
<td>T1DM</td>
</tr>
<tr>
<td>Rat</td>
<td>Wistar</td>
<td>M</td>
<td>STZ</td>
<td>Not reported</td>
<td>2</td>
<td>T1DM</td>
</tr>
<tr>
<td>Rat</td>
<td>Wistar-Kyoto</td>
<td>M</td>
<td>STZ</td>
<td>1</td>
<td>2</td>
<td>T1DM</td>
</tr>
<tr>
<td>Rat</td>
<td>Sprague-Dawley</td>
<td>F</td>
<td>High fructose diet + STZ</td>
<td>1</td>
<td>1</td>
<td>T2DM</td>
</tr>
<tr>
<td>Rat</td>
<td>Wistar</td>
<td>M</td>
<td>High fructose/high sugar diet + STZ</td>
<td>1</td>
<td>2</td>
<td>T2DM</td>
</tr>
</tbody>
</table>

One study used both male and female C57BL/6 mice [273], 16 used female mice and rats, and 96 used male mice and rats. Ninety-six studies reported either a starting age or
weight. Twenty studies did not report the age of the experimental animals. Thirty-three did not report the change in body weight of the animals (i.e. wasting, fat, or no change) after diabetogenic intervention.

Heart weight (HW) was the most frequent method of reporting cardiac hypertrophy (TABLE 3.4). The most common indexing method was normalization to body weight; few studies reported the HW:tibia length (TL) index. Although 33 studies did not report total body weight, seven of these 33 studies reported the HW:BW or HW:TL index.

The type of diabetes/diabetogenic moderates hypertrophy

Neither the age of animals at sacrifice (stratified in 5-week increments) nor animal sex had any effect on absolute final heart weight. Rodent species did not affect absolute final HW; however, diabetic rats were significantly more likely to show an increase in HW:BW compared to diabetic mice (P<0.05). This finding may be confounded by the fact that diabetic rats were significantly more likely to show loss of body weight compared to diabetic mice (P<0.05).

The difference in absolute heart weight from controls was significantly lower in T1DM models than T2DM models (P<0.05) (FIGURE 3.2). The type of diabetes had no significant effect on HW:BW, left ventricular weight:body weight, cardiomyocyte area, or the presence of cardiac dysfunction. The category of diabetogenic agent (drug vs.
spontaneous vs. other) had similar effects on hypertrophy. Diabetogenic category significantly influenced absolute heart weight (P<0.05) but had no significant effect on HW:BW or HW:TL.

![Figure 3.2. Effect of diabetes type and methods of diabetogenicity on indices of cardiac hypertrophy in experimental rodent models of diabetes.]

Because the HW:BW ratio was the most common method of indexing, we also investigated the effects of diabetes type and diabetogenic on body weight (FIGURE 3.3). As would be expected for humans with T1DM versus T2DM, T1DM animals showed significant loss of body weight compared to T2DM animals (P<0.05). Drug-induced diabetic animals also showed significant loss of body weight compared to spontaneous- or diet/drug-induced models of diabetes (P<0.05).
The effect of diabetes duration on cardiac hypertrophy

We coded the duration of diabetes as the time from the final drug administration to the time the animals were sacrificed (for drug-induced diabetes), or as the amount of time a diet was consumed (for diet-induced diabetes). We then stratified the time of the intervention in 5-week increments ranging from 0 to >20 weeks. The HW and the HW:BW index were not affected by rodent age at the start of the intervention, rodent age at the time of analysis, or the duration of the diabetogenic intervention.

There were not sufficient data in each 5-week increment to compare effects of time on fetal genes. Therefore, we re-categorized the shortest duration of diabetes (0-5 weeks) as “acute” and pooled the longer durations as “chronic” (range: 5.1-32 weeks). Acute vs. chronic diabetes had no effect on absolute final heart weight or body weight. However, increased HW:BW was significantly more common in chronic models than acute models. Acute vs. chronic diabetes had no effect on any fetal gene protein or mRNA levels.

*significant effect of diabetes type; # significant effect of diabetogenic from both other categories.

Significance set at P<0.05. T1DM = type 1 diabetes mellitus; T2DM = type 2 diabetes mellitus.
Since some studies used multiple rather than single STZ dosing, we controlled for effects of dose and interactions of dose by duration of diabetes. Dose number (ranging from 1-7) had no effect on HW or the HW:BW index. There were not a sufficient number of studies in each dose category to compare the effect of dose on fetal gene protein or mRNA levels.

*Fetal genes are not correlated with HW:BW or other fetal genes*

**Serca2**

Neither the diabetogenic category nor the diabetes type had any significant effect on Serca2 protein or mRNA levels. Overall, diabetic animals consistently showed lower Serca2 protein levels relative to non-diabetic controls ([FIGURE 3.4](#)). There was no significant effect of species (mouse vs. rat) on Serca2 expression and no interaction of species with diabetogenic method.

---

**Figure 3.4. Effect of diabetes type and methods of diabetogenicity on cardiac Serca2 expression.**

Serca2 is not affected by either diabetes type or by different methods of inducing diabetes in the hearts of experimental rodent models of diabetes. T1DM = type 1 diabetes mellitus; T2DM = type 2 diabetes mellitus.
Myofilament isoforms

The type of diabetes had no effect on α-MHC protein or mRNA levels. However, the type of diabetes moderated the expression of β-MHC. Although protein and transcript levels of β-MHC were upregulated in diabetic animals relative to controls overall, the extent of this upregulation was significantly greater in the T1DM group compared to the T2DM group (P<0.05) (FIGURE 3.5).

Protein and mRNA levels of α-MHC and protein levels of β-MHC were not different between diabetogenic groups. mRNA levels of β-MHC were significantly higher in the drug-induced diabetogenic category compared to the spontaneous diabetogenic category (P<0.05). Interestingly, rodent species (mouse vs. rat) had a significant effect on β-MHC mRNA levels; rats more frequently showed an increase in β-MHC mRNA
compared to mouse models (P<0.05), but there was no significant interaction of species with diabetogenic method.

There were not sufficient data to compare the α-MHC/β-MHC ratio between groups, and the small numbers of studies in our dataset showed conflicting results. The ratio decreased in a spontaneous rat model of T2DM [95] and in a rat model of STZ-induced T1DM [274]. However, it increased in the hearts of female type 2 diabetic db/db mice [104].

**Natriuretic peptides**

Animal models of T1DM tended to show higher transcript levels of ANP relative to controls than T2DM models (P=0.057) (FIGURE 3.6). The diabetogenic category did not have any effect on ANP protein levels; however, drug-induced models also tended to show higher ANP transcript levels relative to spontaneous models (P=0.057). Diabetogenic category did not have any effect on BNP mRNA, and there were not sufficient data to compare BNP protein levels between diabetogenic categories. However, our dataset included three studies that showed increased BNP protein levels in the hearts of type 1 diabetic Akita mice [275], type 1 diabetic STZ-induced diabetic FVB mice [276], and UC Davis type 2 diabetic rats [277]. There was no significant effect of species (mouse vs. rat) on natriuretic peptide expression, and no interaction of species with diabetogenic method.
Correlations of fetal genes with cardiac hypertrophy

Spearman regression was performed to correlate fetal gene mRNA and protein levels with absolute heart weight and the HW:BW index. We report correlations for which $N \geq 3$ studies.

ANP protein levels were negatively correlated with absolute heart weight ($P < 0.05$), but mRNA levels of ANP were not associated with heart weight (TABLE 3.5). Similarly, $\alpha$-MHC protein was directly correlated with increases in absolute heart weight ($P < 0.05$), but transcript levels of $\alpha$-MHC were not.
The HW:BW index did not correlate with the expression of any fetal genes (TABLE 3.6). There were not enough data to correlate fetal genes with the HW:TL index, as only seven studies reported this index.

We then examined these correlations separately within type 1 and type 2 diabetes models. In T1DM models, no fetal genes correlated with heart weight or the HW:BW index (TABLE 3.7, 3.8). However, in T2DM models, BNP mRNA levels were directly correlated to absolute heart weight (P<0.05) (TABLE 3.9), although protein levels of BNP were not.

### Table 3.5. Spearman correlations of fetal genes with absolute heart weight.

<table>
<thead>
<tr>
<th>Correlate</th>
<th>ρ</th>
<th>P</th>
<th>N</th>
<th>P&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANP protein</td>
<td>-0.730</td>
<td>0.03</td>
<td>8</td>
<td>*</td>
</tr>
<tr>
<td>ANP mRNA</td>
<td>-0.003</td>
<td>0.99</td>
<td>15</td>
<td>NS</td>
</tr>
<tr>
<td>BNP mRNA</td>
<td>0.354</td>
<td>0.27</td>
<td>11</td>
<td>NS</td>
</tr>
<tr>
<td>Serca2 protein</td>
<td>0.170</td>
<td>0.60</td>
<td>11</td>
<td>NS</td>
</tr>
<tr>
<td>Serca2 mRNA</td>
<td>-0.411</td>
<td>0.23</td>
<td>10</td>
<td>NS</td>
</tr>
<tr>
<td>α-MHC protein</td>
<td>1.000</td>
<td>0.02</td>
<td>5</td>
<td>*</td>
</tr>
<tr>
<td>α-MHC mRNA</td>
<td>0.362</td>
<td>0.29</td>
<td>10</td>
<td>NS</td>
</tr>
<tr>
<td>β-MHC protein</td>
<td>-1.000</td>
<td>0.08</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>β-MHC mRNA</td>
<td>-0.501</td>
<td>0.09</td>
<td>12</td>
<td>NS</td>
</tr>
</tbody>
</table>

Spearman correlation coefficients; ρ = correlation coefficient, P = p value, N = # studies

### Table 3.6. Spearman correlations of fetal genes with the HW:BW index.

<table>
<thead>
<tr>
<th>Correlate</th>
<th>ρ</th>
<th>P</th>
<th>N</th>
<th>P&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANP protein</td>
<td>0.408</td>
<td>0.45</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>ANP mRNA</td>
<td>0.000</td>
<td>0.98</td>
<td>9</td>
<td>NS</td>
</tr>
<tr>
<td>BNP mRNA</td>
<td>0.612</td>
<td>0.23</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>Serca2 protein</td>
<td>0.234</td>
<td>0.39</td>
<td>15</td>
<td>NS</td>
</tr>
<tr>
<td>Serca2 mRNA</td>
<td>0.310</td>
<td>0.56</td>
<td>6</td>
<td>NS</td>
</tr>
<tr>
<td>α-MHC protein</td>
<td>-0.250</td>
<td>0.68</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>α-MHC mRNA</td>
<td>0.452</td>
<td>0.23</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>β-MHC mRNA</td>
<td>-0.186</td>
<td>0.58</td>
<td>10</td>
<td>NS</td>
</tr>
</tbody>
</table>

Spearman correlation coefficients; ρ = correlation coefficient, P = p value, N = # studies
Table 3.7. Spearman correlations of fetal genes with absolute heart weight in rodent models of type 1 diabetes.

<table>
<thead>
<tr>
<th>Correlate</th>
<th>$\rho$</th>
<th>$P$</th>
<th>N</th>
<th>$P&lt;0.05$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANP protein</td>
<td>-0.707</td>
<td>0.14</td>
<td>6</td>
<td>NS</td>
</tr>
<tr>
<td>ANP mRNA</td>
<td>-0.167</td>
<td>0.66</td>
<td>7</td>
<td>NS</td>
</tr>
<tr>
<td>BNP mRNA</td>
<td>-0.577</td>
<td>0.42</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>Serca2 protein</td>
<td>-0.250</td>
<td>0.49</td>
<td>9</td>
<td>NS</td>
</tr>
<tr>
<td>Serca2 mRNA</td>
<td>-0.816</td>
<td>0.08</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>$\alpha$-MHC protein</td>
<td>1.000</td>
<td>0.08</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>$\alpha$-MHC mRNA</td>
<td>0.632</td>
<td>0.18</td>
<td>6</td>
<td>NS</td>
</tr>
<tr>
<td>$\beta$-MHC protein</td>
<td>1.000</td>
<td>0.33</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>$\beta$-MHC mRNA</td>
<td>-0.571</td>
<td>0.12</td>
<td>8</td>
<td>NS</td>
</tr>
</tbody>
</table>

Spearman correlation coefficients; $\rho$ = correlation coefficient, $P$ = p value, N = # studies

Table 3.8. Spearman correlations of fetal genes with the HW:BW index in rodent models of type 1 diabetes.

<table>
<thead>
<tr>
<th>Correlate</th>
<th>$\rho$</th>
<th>$P$</th>
<th>N</th>
<th>$P&lt;0.05$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANP protein</td>
<td>0.408</td>
<td>0.45</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>ANP mRNA</td>
<td>0.123</td>
<td>0.75</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>BNP mRNA</td>
<td>0.500</td>
<td>1.00</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>Serca2 protein</td>
<td>0.200</td>
<td>0.51</td>
<td>12</td>
<td>NS</td>
</tr>
<tr>
<td>Serca2 mRNA</td>
<td>0.395</td>
<td>0.52</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>$\alpha$-MHC mRNA</td>
<td>0.452</td>
<td>0.23</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>$\beta$-MHC protein</td>
<td>1.000</td>
<td>0.33</td>
<td>3</td>
<td>NS</td>
</tr>
<tr>
<td>$\beta$-MHC mRNA</td>
<td>-0.186</td>
<td>0.58</td>
<td>10</td>
<td>NS</td>
</tr>
</tbody>
</table>

Spearman correlation coefficients; $\rho$ = correlation coefficient, $P$ = p value, N = # studies
Table 3.9. Spearman correlations of fetal genes with absolute heart weight in rodent models of type 2 diabetes.

<table>
<thead>
<tr>
<th>Correlate</th>
<th>$\rho$</th>
<th>$P$</th>
<th>$N$</th>
<th>$P&lt;0.05$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANP mRNA</td>
<td>0.559</td>
<td>0.14</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>BNP mRNA</td>
<td>0.833</td>
<td>0.01</td>
<td>7</td>
<td>*</td>
</tr>
<tr>
<td>Serca2 mRNA</td>
<td>0.000</td>
<td>1.00</td>
<td>6</td>
<td>NS</td>
</tr>
<tr>
<td>$\alpha$-MHC mRNA</td>
<td>-0.577</td>
<td>0.42</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>$\beta$-MHC mRNA</td>
<td>0.333</td>
<td>0.75</td>
<td>4</td>
<td>NS</td>
</tr>
</tbody>
</table>

Spearman correlation coefficients; $\rho = $ correlation coefficient, $P = $ p value, $N = $ # studies

**Correlations of fetal genes with each other**

In the 12 studies that measured both ANP and BNP, ANP protein correlated with BNP mRNA ($P<0.05$). The eight studies that measured Serca2 protein and Serca2 mRNA showed a direct correlation between these two ($P<0.05$). Finally, in nine studies $\alpha$-MHC protein was negatively correlated with $\beta$-MHC protein ($P<0.05$). However, there were no correlations between fetal genes in different categories: the expression of fetal myofilaments did not correlate with Serca2 or the natriuretic peptides, and vice versa.

**Miscellaneous results**

Several of our search terms returned too few studies for statistical analysis; these results are summarized below.

**Transcription factors**

Two studies showed that NCX levels decreased in the hearts of STZ-induced diabetic rats [278, 279]. However, cardiac NCX protein was unchanged in another study with STZ-induced diabetic rats [280] and was increased in the Akita mouse [281]. One study showed that cardiac MEF2 was reduced in STZ-induced diabetic rats [274], but another
study showed an increase [282]. Cardiac mRNA levels of MEF2 were increased in STZ-induced diabetic mice [283]. E-hand and D-hand protein levels were decreased in the hearts of STZ-induced rats [274] but did not change in another study with STZ-induced diabetic rats [282].

Both SMAD2 [284] and SMAD7 [285] protein levels were increased in STZ-induced diabetic rat hearts. Cardiac phospho-SMAD2 and phospho-SMAD3 were also increased in high-fructose-fed diabetic rats [286] and STZ-induced diabetic rats [287]. One study found an increase in phosphorylated GATA4 in STZ-induced diabetic rat hearts [288].

**Cardiac α-actin**

Cardiac α-actin is the form of actin expressed in the postnatal and adult heart; downregulation of cardiac α-actin is indicative of fetal gene activation in cardiomyocytes. In our database, one study showed that transcript levels of cardiac α-actin did not change in STZ-induced rats [236]. Two studies showed that cardiac α-actin was reduced in STZ-induced diabetic rats [289] and db/db type 2 diabetic mice [290].

**Natriuretic peptides**

One study showed that ANP protein was reduced in the atria but increased in the ventricles of STZ-induced diabetic rats [291]. Insulin normalized an increase in left-ventricular transcript levels of ANP in STZ-induced diabetic rats in one study [237]. Both plasma ANP and granular ANP within cardiomyocytes were increased in high-fructose fed C57BL/6 mice [292]. Finally, the mRNA levels of the natriuretic peptide receptors NPR-A and NPR-B were increased in STZ-induced C57BL/6 mouse hearts [293].
Discussion

Results of this study show that diabetogenic methods affect the development of cardiac hypertrophy in diabetic animals and the expression of fetal genes. Models of T2DM showed cardiac hypertrophy relative to controls, while models of T1DM showed significant loss of heart weight. None of the cardiac fetal genes analyzed in this study correlated with the HW:BW index, the most commonly reported estimate of cardiac hypertrophy. The only members of the FGP that were associated with absolute heart weight were α-MHC protein and ANP protein, which had significant positive and negative correlations with heart weight, respectively. However, the mRNA levels of α-MHC and ANP did not correlate with heart weight. When we separated this analysis by the type of diabetes, BNP mRNA levels were significantly positively correlated with heart weight in type 2 models. Results of this analysis suggest that fetal genes are not generally correlates of cardiac hypertrophy in animal models of diabetes, and that fetal gene expression is confounded by animal species, the type of diabetes (type 1 vs. type 2), and the method of inducing experimental diabetes.

We also analyzed the correlations of fetal genes with each other to determine whether they showed similar patterns of expression. Interestingly, there were no correlations in the expression of fetal genes from different functional categories. The expression of genes with similar functions showed some agreement: for example, ANP protein was directly correlated with BNP mRNA levels, Serca2 mRNA and protein levels correlated with each other, and levels of α-MHC and β-MHC protein were negatively correlated. These data suggest that fetal genes within similar functional categories, e.g. the natriuretic peptides or the heavy chain myosins, may be co-regulated in diabetic hearts. However, the natriuretic peptides did not correlate with Serca2 or the myosins, and vice
versa. Collectively, these findings do not support the concept of a cohesively regulated FGP in the diabetic heart.

Most studies reported that cardiac expression of β-MHC was increased in experimental diabetic animals relative to controls. However, this was significantly moderated by the type of diabetes: type 1 animals consistently showed greater upregulation of β-MHC protein and mRNA than type 2 models. β-MHC was also not associated with cardiac hypertrophy in our correlation analysis. Surprisingly, the expression of β-MHC mRNA was significantly higher in rats compared to mice. These results suggest that changes in fetal myofilament isoforms in the adult heart do not strictly indicate cardiac hypertrophy, and are confounded by animal species. It has already been proposed, for example, that β-MHC is a more specific marker of fibrosis than hypertrophy [294]. However, this does not explain the discordant results we found regarding the α-MHC/β-MHC ratio in diabetic hearts. Studies reported that this ratio decreased in spontaneously type 2 diabetic rats [95] and in STZ-induced type 1 diabetic rats [274], but increased in the hearts of female type 2 diabetic db/db mice [104].

**Hypertrophic phenotyping methods**

We found that methods of reporting hypertrophy significantly influence the interpretation of the cardiac phenotype. Absolute heart weight was the most common method of reporting hypertrophy, followed by the HW:BW index. Only seven studies reported the HW:TL index, which is a more reliable correlate of cardiomyocyte area than either HW or HW:BW [295]. Importantly, according to the HW:BW index, there were no significant differences in hypertrophy between animal models of diabetes, and all animals showed hypertrophy. However, both absolute heart weight and body weight were significantly different between type 1 and type 2 animals: type 1 animals generally showed cachexia
and a loss of heart weight, and type 2 models showed obesity and an increase in heart weight. We found that seven studies did not report body weight and only reported either the HW:BW or HW:TL ratio. Until more is understood about hypertrophy in various animal models of diabetes, we suggest that studies report multiple indices of hypertrophy, because the results of this analysis show that simple indexing methods can mask important phenotypic differences.

**Diabetogenic methods**

We propose that the use of toxic glucose analogues for inducing diabetes, which is one of the most common methods for studying diabetic cardiomyopathy at the present time, should be reexamined. At high doses, these diabetogenics induce a model of T1DM that develops cardiac atrophy, cachexia, and primary insulin deficiency. This is a clear departure from the phenotype of humans who develop diabetic cardiomyopathy secondary to T2DM, who are typically hyperinsulinemic, obese, insulin resistant, and show cardiac hypertrophy. The incidence of human diabetes is also overwhelmingly type 2 (approximately 95% of all diabetics); therefore, models of primary insulin deficiency induced with toxic glucose analogues have limited application to the clinical entity of diabetic cardiomyopathy.

These diabetogenic agents also may be toxic to multiple organs and have independent effects on cardiac function. The mechanism of action of STZ and alloxan is inducing cell death secondary to alkylating and oxidative DNA damage and disruption in calcium kinetics [296]. Other mechanisms of β-cell toxicity include inhibition of O-linked β-N-acetylglucosaminidase, which removes O-linked β-N-acetylglucosamine (O-GlcNAc) groups from serine/threonine residues of proteins [297]. This toxicity is relatively selective to pancreatic β-cells; however, animals treated with alloxan or STZ also exhibit
hepatotoxicity and signs of kidney damage [298]. STZ also independently reduces cardiomyocyte contractility [299], and both alloxan and STZ induce cardiomyocyte dysfunction [88, 300].

Many hypotheses have been proposed for the dysfunctional phenotype of the diabetic heart, including disruption in calcium kinetics, increased oxidative stress, energetic disturbances due to glucotoxicity and/or lipotoxicity, inflammation, and cardiomyocyte apoptosis; for recent reviews, see [19] and [27]. It is critical to recognize that the independent effects of toxic glucose analogs on these aspects of cardiomyocyte function that are considered indicative of diabetic cardiomyopathy, such as lipotoxicity and oxidative stress, have not been examined. Therefore, the use of toxic glucose analogues may not produce a physiologically relevant model of diabetic cardiomyopathy.

**Interpretations and proposed mechanisms**

The basic relationship between cardiac hypertrophy and fetal gene activation is unresolved. Although many excellent reviews have been published on the transcriptional mechanisms that regulate these genes [39-41, 269, 301], the pathways that activate these mechanisms are very poorly understood. It is also not yet established whether FGP activation is a cause or a result of hypertrophy, or whether it is beneficial or decompensatory. For example, the natriuretic peptides antagonize hypertrophy and fibrosis [255, 302, 303], suggesting that their action is beneficial. Conversely, the loss of Serca2 expression in lieu of fetal-type calcium handling proteins is clearly detrimental for the adult heart [95, 96, 240, 279, 304, 305].

An emerging hypothesis proposes that fetal gene expression in the adult heart represents compensatory dedifferentiation, or fetal “reprogramming,” of adult
cardiomyocytes. Adult cardiomyocytes show considerable plasticity in their differentiation state [306], and dedifferentiate in response to insults such as myocardial infarction, chronic hypertension, and heart failure [307, 308]. However, the underlying mechanisms are not well understood, and cardiomyocyte plasticity may even be intrinsically different between animal strains [309]. Indeed, the ability to revert from an adult phenotype may be an inherently protective process in the adult heart [308], mimicking the highly cardioprotective phenotype of the fetal heart [310]. This has led to speculation that fetal gene activation in pathological hypertrophy is a protective mechanism [40, 41] and is supported by evidence that the expression of a fetal transcriptome is highly cardioprotective [310, 311]. However, this hypothesis does not yet explain why some fetal genes are regulated differently in fetal vs. diseased hearts. For example, it is not well established why the Nppa gene is reactivated in heart disease [312], and Nppa has distinct regulatory sequences that are activated in the embryonic heart and the adult failing heart [313]. The question is complicated by the fact that the fetal and failing hearts are not the only ones that express fetal genes; an adult heart deprived of afterload also upregulates fetal gene expression [314].

It is also possible that the expression of fetal genes is closely tied to myocardial metabolism. This would explain why the presence of simultaneous metabolic disease and cardiac hypertrophy would have confounding effects on fetal gene expression in diabetic hearts. The adult heart upregulates glycolytic metabolism during pathological hypertrophy, and it has been proposed that this shift toward fetal-like myocardial metabolism underlies fetal gene activation and cardiac dysfunction [27, 28]. The diabetic heart, by contrast, becomes almost exclusively reliant on fatty acid oxidation [29, 30]. Therefore, although the diabetic heart develops pathological hypertrophy, the
fundamental metabolic differences between the pathologically hypertrophied and diabetic heart may confound the expression of fetal genes in diabetic hearts.

Limitations

The specific nature of this systematic review limited the number of results returned by our search parameters. For example, our database did not return a sufficient number of studies to correlate the expression of fetal genes with HW:TL, since this was an uncommon method of measuring cardiac hypertrophy. There were also not sufficient data to compare the α-MHC/β-MHC ratio between groups, or to compare changes in BNP protein between drug-induced and spontaneous models of diabetes. Therefore, the specific nature of our search parameters and the small number of studies it returned limits our conclusions regarding these variables.

The studies we examined also included a wide variety of strains and genetic backgrounds (TABLE 3.2). While we were able to detect some significant effects of species on the HW:BW ratio, BW, and β-MHC mRNA, there were not enough studies to compare the effects of strain within species. Therefore, additional studies are needed to determine whether genetic backgrounds influence these parameters in mice and rats.

Finally, we found that chronic models of diabetes were more likely to show increases in HW:BW. Importantly, however, there was no interaction of diabetes duration and the number of doses of toxic glucose analogues. These data suggest that cardiac gene regulation in diabetes is not the same between mice and rats, and that animal models of diabetes show progressive changes in cardiac hypertrophy independent of the dosing regimen.
Conclusions

In rodent models of diabetes, α-MHC protein and ANP protein levels correlate positively and negatively, respectively, with heart weight. The type of diabetes and the method of diabetogenicity independently moderate body weight and cardiac weight and the expression of β-MHC. We found no correlations between fetal genes and the HW:BW index in animal models of diabetes. These findings indicate that fetal genes are not specific markers of hypertrophy in rodent models of diabetes. In addition, this review finds wide variation in current methods of diabetogenicity as well as methods of reporting cardiac hypertrophy. We suggest that studies using experimental rodent models of diabetes report multiple indices of cardiac hypertrophy to improve the quality of research in this field.
CHAPTER 4

Exponential modeling of VO$_2$ kinetics in endurance-trained diabetic mice

Context

This short report provides reference values for peak exercising oxygen consumption (VO$_2$) and associated parameters for the type 2 diabetic db/db mouse to calibrate our exercise protocols. We also perform preliminary kinetic analyses as proof of the principle that such analyses can be accomplished with reasonable precision on the data obtained from a modular treadmill test in a mouse. The major outcomes of this paper are (1) reference values for peak exercising VO$_2$ in both db/db and non-diabetic C57BL/6J mice, (2) a description of kinetic modeling methods used to evaluate $\tau$ and predict VO$_{2\text{peak}}$ from submaximal exercise data, and (3) verification that cardiorespiratory fitness is improved by a “human” exercise protocol in both db/db and non-diabetic C57BL/6J mice.

Details for manuscript currently in preparation

**Johnson EJ**, Dieter BP, Maricelli J, Medford HM, Rodgers BD, Marsh SA. *Exponential modeling of VO$_2$ kinetics in endurance-trained diabetic mice*

Author contributions

Conceived the study design: EJC, HMM, BDR, SAM.

Planned and performed the experiments: EJC, BD, BDR, JM.

Analyzed the data: EJC, BPD, JM.

Wrote the manuscript: EJC, SAM.
Exponential modeling of VO2 kinetics in endurance-trained diabetic mice

Abstract

Kinetics of oxygen consumption (VO2) are sensitive indicators of cardiorespiratory fitness that can be used to evaluate the effectiveness of exercise training on cardiovascular health in type 2 diabetes mellitus (T2DM). Rodents are the most frequently used preclinical models of T2DM; however, there are no published data on VO2 in type 2 diabetic rodents, and there are no established techniques for studying VO2 kinetics in these models. Therefore, we report peak VO2 values for genetically type 2 diabetic (db/db; DB) mice and non-diabetic background controls (C57BL/6J) before, during, and after 8 weeks of treadmill training at the same relative intensity prescribed for humans with T2DM. After 8 weeks, absolute VO2peak increased significantly in both strains, and a simple exponential modeling approach was used to estimate the time constant (τ) of VO2 and predict VO2peak at exercise termination. Results suggest that a one-component exponential model fit to a submaximal window of exercise estimates τ and predicts VO2peak at end-exercise. These data show that cardiorespiratory fitness in the DB mouse is improved by a “human” exercise prescription and suggest that a simple exponential model can be used to predict VO2 from submaximal data in these mice.

Introduction

Oxygen consumption (VO2) and carbon dioxide production (VCO2) during exercise are sensitive and reliable indicators of cardiorespiratory fitness that are commonly used for the noninvasive evaluation of musculoskeletal, cardiovascular, and pulmonary limitations to exercise [315-318]. Rodent models of cardiopulmonary disease are often used for preclinical research regarding the effects of exercise training in type 2 diabetes (T2DM) [80, 82, 104, 118, 158, 220, 221, 319-326]. However, there are no published values for
exercise capacity (peak work rate, peak VO\(_2\) or peak VO\(_2\)/VCO\(_2\)) in rodent models of T2DM. This makes it impossible to calibrate preclinical exercise protocols to an appropriate level to reflect the exercise prescription for humans with T2DM, which is prescribed to a percentage of VO\(_{2\text{max}}\) [123].

Exercising VO\(_2\) and VCO\(_2\) in humans is measured with airtight masks and high-frequency breath-by-breath gas analysis. In contrast, VO\(_2\) measurement in small animals is measured in airtight chambers that measure net changes in gas composition over time. Although peak values of VO\(_2\) and VCO\(_2\) can be obtained by this method, it is not known whether other important kinetic parameters can be determined from these lower-resolution data. Therefore, this report has two purposes. First, this study provides measured reference values for peak work rate, the respiratory exchange ratio VO\(_2\)/VCO\(_2\) (RER) before and during peak exercise, and peak VO\(_2\) in diabetic and non-diabetic mice before, during, and after an 8-week “human” exercise protocol. Second, this report shows that the kinetic parameter \(\tau\) can be evaluated with reasonable precision from the VO\(_2\) data obtained from a small animal maximal exercise test.

**Materials and methods**

**Ethical approval**

This protocol was approved by the Institutional Animal Care and Use Committee at Washington State University and conformed to the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication number 85-23, revised 2011).

**Animal care and groups**
Five-week-old type 2 diabetic \(db/db\) mice (DB) and age-matched C57BL/6J non-diabetic lean background strain controls (C57) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed singly to control for physical activity and were maintained on a 12:12 light:dark cycle. Due to the quarantine regulations of the vivarium, mice that were exposed to the maximal exercise testing facility had to be sacrificed. Therefore, mice were randomized to three separate cohorts: week 0 (baseline), week 4, and week 8. Additionally the electric shock grid was not used for daily exercise training, and mice who refused to run were excluded from the protocol. Therefore, the sample size for each cohort was as follows: week 0, \(n=6\) C57, \(n=5\) DB; week 4, \(n=5\) C57, \(n=3\) DB; week 8, \(n=5\) C57, \(n=5\) DB.

**Exercise training protocol**

The chronologic exercise training plan is shown in **Figure 4.1**. Daily exercise was performed 5 consecutive days a week on a 6-lane treadmill (Columbus, OH). Before starting the protocol, the week 4 and week 8 groups had one week of accommodation to the treadmill, which consisted of 10 min standing on the belt and 20 min of slow walking at 5 m/min. Every exercise session started and finished with a 5 min warm up or cool down period of slow walking at 5 m/min.

**Figure 4.1. Exercise protocol.**

<table>
<thead>
<tr>
<th>Baseline max test</th>
<th>Week 4 max test</th>
<th>Week 8 max test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 0 Acc. 20 min</td>
<td>Week 1 40% 20 min</td>
<td>Week 6 70% 1 hr</td>
</tr>
<tr>
<td>Week 2 45% 30 min</td>
<td>Week 3 50% 40 min</td>
<td>Week 7 70% 1 hr</td>
</tr>
<tr>
<td>Week 4 60% 1 hr</td>
<td>Week 5 65% 1 hr</td>
<td>Week 8 70% 1 hr</td>
</tr>
<tr>
<td>Ex R Ex R Ex R Ex</td>
<td>Ex R Ex R Ex R Ex</td>
<td>Ex R Ex R Ex R</td>
</tr>
</tbody>
</table>

*Figure 1 legend: 8 week exercise protocol. The intensity level mirrors the clinical exercise prescription for humans with T2DM. Ex = 5 consecutive days of exercise; R = 2 consecutive days of rest. Acc. = acclimation week.*
Mice in the week 0 cohort performed their maximal exercise test at baseline; the results from this test were used to calculate the treadmill speed and grade for the first four weeks of the exercise protocol. Mice in the week 4 cohort performed their maximal exercise test after 4 weeks of exercise; the results from this test were used to set the intensity for the remaining 4 weeks. Mice in the week 8 cohort performed their maximal test after 8 weeks.

The progression of exercise intensity was based on the recommendation for humans with T2DM, which is 3.5 hours per week at a moderate intensity between 40-60% VO\textsubscript{2}\text{max} \[123\]. Work rate was calculated as a function of vertical displacement and treadmill speed (Equation 1): $\text{Work rate} \left( \frac{\text{kg} \cdot \text{m}}{\text{min}} \right) = \text{body mass (kg)} \cdot \text{speed} \left( \frac{\text{m}}{\text{min}} \right) \cdot \text{grade(\%)}$.

Maximal exercise testing

Maximal exercise testing was performed at the timepoints shown in Figure 4.1 on a metabolic modular treadmill (Oxymax FAST Modular; Columbus Instruments, Ohio) using a previously validated ramp protocol [318]. Each lane is a self-contained chamber that performs gas analysis every 10 seconds. C57 mice performed the ramp protocol at 25° incline, and DB mice performed the protocol at 5° incline because DB mice were not capable of running at a higher grade. The electric shock grid was used for the maximal exercise test, and peak exercise was defined as the time point when a mouse rested on the electric shock grid for 10 seconds (i.e. exhaustion).

$\text{VO}_2$ analysis and τ determination
Raw VO$_2$ data from the maximal exercise test were smoothed using locally weighted LOWESS regression with a modest smoothing parameter $f=0.2$ [327]. Iterative nonlinear least squares regression was used to fit the following general exponential equation (Equation 2) to VO$_2$ data: $y = [a(1 - e^{-bx})] + y_0$, where $y$ is VO$_2$, $b$ is a rate constant whose inverse is the time constant $\tau \left( \tau = \frac{1}{b} \right)$ or the time required to reach $1 - \frac{1}{e}$ (~63%) of the plateau value of VO$_2$; and $y_0$ is the VO$_2$ intercept [328]. The parameter $b$ was constrained to $b>0$. Curve fitting was performed using SigmaPlot 11.0 for Windows (SYSTAT Software, San Jose, CA). Models that fit poorly (adjusted $R^2<0.5$) and models with Akaike weights more than 50% greater than the lowest Akaike Information Criterion (AIC) score were eliminated. Because this stipulation eliminated data from some animals, we pooled the values for $\tau$ from all DB and C57 mice for the comparison in Table 4.2.

**Fitting windows**

Equation 2 was fit to four different time windows. Probably due to the slow start of the ramp protocol, we observed approximately a 1 minute delay in the onset of an exponential increase in VO$_2$; therefore, we tested a window that did not include this first minute ($W_{1+}$), as well as a window that included all the data ($W_{total}$). We also tested two submaximal windows that included the first 5 minutes of data ($W_{0-5}$) and a window that included the first 5 minutes of data excluding the first minute ($W_{1-5}$).

**Statistics**

Statistical analysis was conducted using SigmaPlot 11.0 for Windows (SYSTAT Software, San Jose, CA). Effects of genotype and exercise were analyzed using two-factor ANOVA and Bonferroni post-hoc correction. Data that were not normal were log
transformed, and data that failed this transformation were analyzed as ranks. Data are reported as mean ± standard deviation.

**Results**

**Treadmill training increases peak work rate and absolute VO$_2$peak**

The exercise protocol significantly increased peak work rate in C57 mice compared to baseline (Table 4.1). Peak relative VO$_2$ (ml/kg/min) was significantly lower in DB mice in all tests and was not affected by training in DB mice or C57 mice. However, absolute VO$_2$ (ml/min), which controls for the significantly higher body mass in DB mice, was significantly higher in week 8 compared to baseline in both strains. Minimum RER decreased in DB mice over time, and both minimum and maximum exercising RER were lower in DB mice compared to C57 mice in week 8.

**Analyses of VO$_2$ kinetics**

The model that produced the most physiologically appropriate values of $r$ and accurately fit the most data sets was $W_{1.5}$, followed by $W_{0.5}$.

---

**Table 4.1. Reference values of cardiorespiratory parameters in type 2 diabetic DB and C57 mice.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Wk</th>
<th>Weight (g)</th>
<th>Speed (m/min)</th>
<th>Distance (m)</th>
<th>Work rate (kgm)</th>
<th>VO$_2$peak (ml/kg/hr)</th>
<th>Absolute VO$_2$peak (ml/hr)</th>
<th>Minimum RER</th>
<th>Maximum RER</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB</td>
<td>0</td>
<td>32.65 ± 27.9*</td>
<td>16 ± 2*</td>
<td>170.4 ± 144.4*</td>
<td>57.96 ± 329*</td>
<td>18.66 ± 100.7*</td>
<td>0.0448 ± 0.004*</td>
<td>0.75 ± 0.06*</td>
<td>0.91 ± 0.08*</td>
</tr>
<tr>
<td>DB</td>
<td>4</td>
<td>37.92 ± 7.92*</td>
<td>27 ± 6*</td>
<td>40.50 ± 8.54*</td>
<td>32.12 ± 33.0*</td>
<td>20.41 ± 1.10*</td>
<td>0.0669 ± 0.005*</td>
<td>0.67 ± 0.05*</td>
<td>0.94 ± 0.05*</td>
</tr>
<tr>
<td>DB</td>
<td>8</td>
<td>43.36 ± 7.92*</td>
<td>27 ± 6*</td>
<td>40.50 ± 8.54*</td>
<td>32.12 ± 33.0*</td>
<td>20.41 ± 1.10*</td>
<td>0.0669 ± 0.005*</td>
<td>0.67 ± 0.05*</td>
<td>0.94 ± 0.05*</td>
</tr>
<tr>
<td>C57</td>
<td>0</td>
<td>20.41 ± 1.10*</td>
<td>3 ± 3*</td>
<td>85.98 ± 60.9*</td>
<td>32.12 ± 33.0*</td>
<td>20.41 ± 1.10*</td>
<td>0.0669 ± 0.005*</td>
<td>0.67 ± 0.05*</td>
<td>0.94 ± 0.05*</td>
</tr>
<tr>
<td>C57</td>
<td>4</td>
<td>23.67 ± 1.37*</td>
<td>37 ± 17*</td>
<td>65.12 ± 60.9*</td>
<td>32.12 ± 33.0*</td>
<td>20.41 ± 1.10*</td>
<td>0.0669 ± 0.005*</td>
<td>0.67 ± 0.05*</td>
<td>0.94 ± 0.05*</td>
</tr>
<tr>
<td>C57</td>
<td>8</td>
<td>24.83 ± 1.40*</td>
<td>37 ± 17*</td>
<td>65.12 ± 60.9*</td>
<td>32.12 ± 33.0*</td>
<td>20.41 ± 1.10*</td>
<td>0.0669 ± 0.005*</td>
<td>0.67 ± 0.05*</td>
<td>0.94 ± 0.05*</td>
</tr>
</tbody>
</table>

Data are presented as mean plus or minus standard deviation. * = effect of genotype within week. Wk = weight. Minimum RER = lowest RER at the start of exercise. Maximum RER = highest RER achieved during exercise protocol.
None of the models produced \(\text{VO}_{2\text{peak}}\) values that departed significantly from the actual values for that genotype. Models that were fit to the whole data set \((W_{1+}, W_{\text{total}})\) produced slightly closer estimates of VO2max than those fit to stage 1 \((W_{0-5}, W_{1-5})\) (Figure 4.2).

**Table 4.2.** Predicted and observed values of \(\text{VO}_{2\text{peak}}\) and the time constant \(\tau\) in type 2 diabetic DB and C57 mice.

<table>
<thead>
<tr>
<th>Window</th>
<th>Strain</th>
<th>Mean Adj (R^2)</th>
<th>Mean predicted (\text{VO}_{2\text{peak}}) (ml/kg/hr)</th>
<th>(\tau) (min)</th>
<th>COUNT</th>
</tr>
</thead>
<tbody>
<tr>
<td>(W_{0-5})</td>
<td>C57</td>
<td>0.85</td>
<td>8565 ± 1144</td>
<td>6.4 ± 3.6</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>DB</td>
<td>0.87</td>
<td>5645 ± 1030</td>
<td>2.0 ± 1.2</td>
<td>9</td>
</tr>
<tr>
<td>(W_{1-5})</td>
<td>C57</td>
<td>0.93</td>
<td>8106 ± 875</td>
<td>2.1 ± 1.3</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>DB</td>
<td>0.84</td>
<td>5598 ± 1242</td>
<td>2.0 ± 2.5</td>
<td>11</td>
</tr>
<tr>
<td>(W_{1+})</td>
<td>C57</td>
<td>0.76</td>
<td>8981 ± 1242</td>
<td>16.5 ± 21.8</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>DB</td>
<td>0.87</td>
<td>5153 ± 923</td>
<td>5.9 ± 5.7</td>
<td>7</td>
</tr>
<tr>
<td>(W_{\text{total}})</td>
<td>C57</td>
<td>0.83</td>
<td>7844 ± 3010</td>
<td>22.7 ± 26.6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>DB</td>
<td>0.89</td>
<td>4974 ± 959</td>
<td>4.5 ± 7.0</td>
<td>8</td>
</tr>
<tr>
<td>Actual</td>
<td>C57</td>
<td>0.89</td>
<td>8388 ± 806</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DB</td>
<td>0.87</td>
<td>5745 ± 850</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

Parameters \((\text{VO}_{2\text{peak}}\) and \(\tau\)) are presented as mean plus or minus standard deviation. * = significant effect of genotype within the model being analyzed. Count is the N of mice. Equation 2 was fit with data obtained from each set of mice.
Discussion

This study provides values of VO\textsubscript{2peak}, peak RER, and peak workload for C57 and DB mice, and shows that 8 weeks of low- to moderate-intensity treadmill exercise that mirrors the exercise prescription for humans with T2DM increases absolute VO\textsubscript{2peak} (ml/min) in both strains. We also found that minimum RER at the start of exercise decreases over time in the DB mouse, suggesting an increased reliance on fat rather than carbohydrate metabolism [329]. This is a characteristic result of impaired glucose uptake in T2DM and is consistent with previous studies showing a low basal metabolic rate in DB mice compared to controls [330] as well as human studies that show a low resting RER in humans with T2DM [331]. However, we also observed a decreased
minimum RER over the course of the training protocol in C57 mice. This may have resulted from exercise training since a low resting RER is associated with high aerobic fitness in non-diabetic humans [332].

Major barriers to estimating kinetic parameters associated with VO₂ data from a modular treadmill include the kinetics of the equipment (i.e. flow rates and gas volumes) and the resolution of the data (0.1 Hz in this study). These considerations are important and do limit the quality of kinetic data obtained from small animal VO₂ testing. Nevertheless, our results show that a simple monoexponential model fit to a submaximal window of exercise (minutes 1-5) estimates the value of r around minute 2 of exercise, which is a physiologically appropriate value. However, it should be considered that r estimated by \( W_{1-5} \) for DB mice (2.0±2.5 minutes) included zero, suggesting either that the rate of VO₂ increase in mice is highly variable or that this analysis needs to be repeated with a higher sample size. The sample size of this analysis was limited by institutional quarantine regulations of the vivarium, which required mice to be sacrificed after exposure to the treadmill core facility, and by the exclusion criteria, which disqualified mice that refused to exercise or had extremely variable VO₂ data.

**Conclusion**

Results of this study show that cardiorespiratory fitness in both C57 and DB mice is improved by a “human” exercise prescription. We also report reference values for peak VO₂ and peak work rate that can be used to calibrate exercise protocols in these mouse strains. Additionally, data show that some kinetic data can be extracted from the VO₂ data produced by a small animal modular metabolic exercise chamber. This finding suggests that the first five minutes of exercise can be used to evaluate r and predict VO₂peak, which has useful applications for mouse models of diseases that preclude
maximal exercise testing. Although our conclusions are limited by the sample size, these data suggest that simple kinetic modeling techniques can be used for evaluating and predicting cardiorespiratory fitness in mice.
CHAPTER 5
Exercise and diabetes have opposite effects on the assembly and O-GlcNAc modification of the mSin3A/HDAC1/2 complex in the heart

Context
This Chapter describes a study that addresses the connection between cardiac metabolism and hypertrophic signaling. We investigate the effects of chronic exercise training on O-GlcNAc, a byproduct of glucose metabolism that post-translationally modifies nuclear and cytosolic proteins, including key transcription factors involved in cardiac hypertrophy. The key findings show that (1) exercise elevates cardiac O-GlcNAc, and (2) exercise changes the association of O-GlcNAc transferase with histone deacetylases that are critical for hypertrophic growth. These findings suggest that cardiac metabolism affects the activity and associations of transcription factors that regulate cardiac hypertrophy.

Citation for published paper

Author contributions:
Conceived and designed the experiments: EJC, SAM.
Performed the experiments: EJC.
Analyzed the data: EJC.
Wrote the paper: EJC, SAM.
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Exercise and diabetes have opposite effects on the assembly and O-GlcNAc modification of the mSin3A/HDAC1/2 complex in the heart

Abstract

Background: Exercise causes physiological cardiac hypertrophy and benefits the diabetic heart. Mammalian switch-independent 3A (mSin3A) and histone deacetylases (HDACs) 1 and 2 regulate hypertrophic genes through associations with the DNA binding proteins repressor element-1 silencing transcription factor (REST) and O-linked β-N-acetylglucosamine transferase (OGT). O-linked β-N-acetylglucosamine (O-GlcNAc) is a glucose derivative that is chronically elevated in diabetic hearts, and a previous study showed that exercise reduces cardiac O-GlcNAc. We hypothesized that O-GlcNAc and OGT would physically associate with mSin3A/HDAC1/2 in the heart, and that this interaction would be altered by diabetes and exercise.

Methods: Type 2 diabetic db/db (db) and non-diabetic C57 mice were randomized to treadmill exercise or sedentary groups for 1 or 4 weeks.

Results: O-GlcNAc was significantly higher in db hearts and increased with exercise. Db hearts showed lower levels of mSin3A, HDAC1, and HDAC2 protein, but higher levels of HDAC2 mRNA and HDAC1/2 deacetylase activity. Elevated HDAC activity was associated with significantly blunted expression of α-actin and brain natriuretic peptide in db hearts. In sedentary db hearts, co-immunoprecipitation assays showed that mSin3A and OGT were less associated with HDAC1 and HDAC2, respectively, compared to sedentary C57 controls; however, exercise removed these differences.
Conclusions: These data indicate that diabetes and exercise alter interactions between pro-hypertrophic transcription factors, and suggest that an increase in total cardiac O-GlcNAc may be a mechanism by which exercise benefits type 2 diabetic hearts.

Background
Cardiac hypertrophy is the adaptive enlargement of the myocardium in response to physical or neurohormonal stress. Type 2 diabetes is associated with a cardiac syndrome called diabetic cardiomyopathy, which is characterized by pathological hypertrophy, contractile dysfunction [333, 334], and an intractable reliance on fatty acid oxidation [19, 30, 223]. By contrast, chronic endurance exercise training causes physiological hypertrophy that improves contractile mechanics [335, 336] and myocardial metabolism [337, 338]. Exercise benefits the type 2 diabetic heart [221, 339-341], but the underlying mechanisms by which exercise and diabetes control cardiac hypertrophy are not well understood.

In non-diabetic hearts, activation of fetal genes is a protective mechanism [255, 256, 266] that accompanies pathological hypertrophy [37, 41, 256, 269, 342]. These fetal genes include fetal cytoskeletal proteins (skeletal α-actin, β-myosin heavy chain) and the atrial and brain natriuretic peptides (ANP and BNP) [343-345]. Importantly, exercise and diabetes moderate these genes differently. Exercise increases adult cardiac α-actin [346], but does not change fetal gene expression [159], whereas type 2 diabetes actually reduces circulating natriuretic peptides [232, 320], and blocks the activation of fetal genes by hypertrophic stimuli in vitro [239]. This suggests that fetal gene regulation in diabetic hearts is different from that of exercised hearts and non-diabetic hearts, and therefore, may underlie the hypertrophic response to these conditions.
A potential mechanism for the effects of diabetes and exercise on fetal genes is through the post-translational modification of transcription factors by O-linked β-N-acetylgalactosamine (O-GlcNAc). O-GlcNAc is a glucose derivative that post-translationally modifies serine/threonine residues [73, 76]. O-GlcNAcylation modifies signal transduction in a manner analogous to phosphorylation; O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) add and remove the O-GlcNAc moiety, respectively. We have recently shown that O-GlcNAc modifies repressor element-1 silencing transcription factor (REST) [149], a transcription factor that represses fetal genes by recruiting the corepressors mammalian switch-independent 3A (mSin3A) and histone deacetylases (HDACs) 1 and 2 [70]. The HDAC enzymes deacetylate histone tails, thus condensing euchromatin and silencing gene expression; HDAC1 and HDAC2 specifically mediate fetal gene regulation by REST and mSin3A, and have been repeatedly linked to hypertrophic growth of the heart [54].

mSin3A and HDAC1 are O-GlcNAcylated in HepG2 liver carcinoma cells, and are recruited to gene loci by the OGT enzyme [71], which also O-GlcNAcylates itself [76]. The activity of OGT is regulated by cellular concentrations of UDP-GlcNAc substrate [78, 347], which is increased in the diabetic heart [81]. Indeed, total O-GlcNAc and protein O-GlcNAcylation are elevated in the diabetic heart [30, 72], but the functional implications of this finding are unclear as elevated cardiac O-GlcNAc is implicated in heart failure and cardiac dysfunction [72, 75], but also cardioprotection [83, 348]. Nevertheless, two previous studies showed that total protein O-GlcNAcylation and O-GlcNAcylation of the SP1 transcription factor are lowered by swimming exercise in both non-diabetic and streptozotocin-induced type 1 diabetic hearts [80, 82], and OGA overexpression directly ameliorates the cardiovascular complications of type 2 diabetes [81].
We therefore hypothesized that a reduction in O-GlcNAc may be a mechanism by which exercise benefits the type 2 diabetic mouse heart. However, since these interactions have not been studied in the heart, the secondary purpose of this study was to characterize the effects of diabetes and moderate exercise on the mSin3A/HDAC1/2 complex. We used 4 weeks of treadmill exercise training to investigate the early signaling mechanisms in the hypertrophic process. We show that exercise increases total protein O-GlcNAcylation in the type 2 diabetic \( db^+/db^+ \) (\( db \)) mouse heart, and that exercise and diabetes have reciprocal effects on the association of HDAC1 and HDAC2 with fetal gene-regulating transcription factors.

**Methods**

*Animal care and facilities*

The procedures in this study followed the guidelines of the Washington State University Institutional Animal Care and Use Committee and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication no. 85-23, revised 1996). Eight-week-old type 2 diabetic mice (B6.BKS(D)-Lep\(^{db}\)/J, \( db^+/db^+ \) (\( db \)) and age-matched C57BL/6J non-diabetic lean \( db^+/? \) background strain controls (C57) were purchased from Jackson Laboratories (Bar Harbor, ME). To control for activity, mice were singly housed without environmental enrichment in a climate-controlled vivarium on a 12:12 light:dark cycle. Mice consumed water and standard chow ad libitum, except for one overnight fast per week prior to blood glucose measurement.

*Exercise training protocol*

Mice ran on a 6-lane electric treadmill (Columbus Instruments, Columbus, OH) for 5 consecutive days a week with 2 days of rest. In week 0, all mice were acclimated to the
treadmill by standing on the stationary belt for 10 min, then walking at 5 m/min for 20 min. Mice were then randomized to sedentary (n=11) or exercise (n=12) groups for 1 week, or sedentary (n=16) or exercise (n=15) groups for 4 weeks. Human patients with type 2 diabetes are commonly prescribed an exercise intensity of at least 40-60% of their aerobic capacity, but a higher intensity is recommended for maximum health benefits [349]. Therefore, we exercised mice at 10 m/min, which corresponds to approximately 70% of maximal oxygen uptake (VO$_{2\text{max}}$) for the C57 strain [350]. Mice ran at this speed at 0% grade for 10, 20, 30, or 40 min in weeks 1, 2, 3, and 4, respectively. O-GlcNAc is a highly dynamic stress response; therefore, to remove confounding effects of stress, we kept the treadmill covered, and used gentle tactile stimuli rather than the electroshock apparatus to keep mice running. Sedentary groups were handled identically and spent equal time in the same treadmill environment on a stationary belt.

**Blood glucose and body weight measurements**

Blood glucose and body weight were measured weekly after an overnight fast. Blood glucose was measured with a glucometer (ACCU-CHEK® Aviva, Roche Diagnostics, Indianapolis, IN) in a small sample of tail blood. Glucose readings that exceeded the accuracy limit of the calibrated meter (33.3 mmol/L) were imputed this value for statistical analysis.

**Tissue harvesting and morphological measurements**

Following an overnight fast, mice were anesthetized rapidly using an isoflurane vaporizer chamber with 2-4% isoflurane gas in 100% oxygen. Mice were immediately decapitated and blood glucose was measured in neck blood. Whole hearts were excised and the atria were removed. Ventricular tissue was immediately wet-weighed, cut into four equal
tissue aliquots, snap-frozen in liquid nitrogen, and stored at -80° C. The left tibia was dissected from each animal and measured from the tibial plateau to the lateral malleolus.

**Western blotting**

Ventricular tissue was homogenized in Tissue Protein Extraction Reagent (Sigma-Aldrich, St. Louis, MO); 20 mM sodium fluoride; 1 mM sodium orthovanadate; 3% protease inhibitor cocktail (Sigma); and 0.02% PUGNAc, an OGA inhibitor (Sigma), to inhibit O-GlcNAc removal from proteins. Total protein was quantified with a modified Lowry assay (BioRad, Hercules, CA). Proteins were separated by SDS-PAGE and transferred onto PVDF membranes, which were probed overnight at 4° C with primary antibodies (anti-HDAC1 and -HDAC2, Cell Signaling, Beverly, MA; anti-mSin3A, -REST, -OGT, and -calsequestrin, Abcam, Cambridge, MA; anti-NCOAT/OGA, Santa Cruz Biotechnology, Santa Cruz, CA; anti-phospho-HDAC1 (Ser 421/423), Millipore, Billerica, MA), then probed with the appropriate secondary antibodies for 1 hour at room temperature (see Table 5.1 for antibody details). Chemiluminescent substrates (Thermo Fisher Scientific, Rockford, IL) were used to detect horseradish peroxidase activity on a ChemiDoc (BioRad). Protein levels were quantified on duplicate blots with standard densitometry using ImageJ software (National Institutes of Health, Bethesda, MD) and normalized to the loading control calsequestrin.
### Table 5.1. Antibodies

<table>
<thead>
<tr>
<th>Target</th>
<th>Supplier</th>
<th>Cat #</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTD 110.6 (anti-O-GlcNAc)</td>
<td>Gift from Mary-Ann Accavitti, University of Alabama at Birmingham</td>
<td>n/a</td>
</tr>
<tr>
<td>RL2 (anti-O-GlcNAc)</td>
<td>Abcam, Cambridge, MA</td>
<td>2739</td>
</tr>
<tr>
<td>anti-mSin3A</td>
<td>Abcam</td>
<td>3479</td>
</tr>
<tr>
<td>anti-OGT</td>
<td>Abcam</td>
<td>50271</td>
</tr>
<tr>
<td>anti-NCOAT/OGA</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA</td>
<td>sc-66612</td>
</tr>
<tr>
<td>anti-HDAC1</td>
<td>Cell Signaling, Beverly, MA</td>
<td>5356</td>
</tr>
<tr>
<td>anti-HDAC2</td>
<td>Cell Signaling</td>
<td>5113</td>
</tr>
<tr>
<td>anti-Calsequestrin</td>
<td>Abcam</td>
<td>3516</td>
</tr>
<tr>
<td>anti-REST</td>
<td>Abcam</td>
<td>21635</td>
</tr>
<tr>
<td>anti-phospho-HDAC1 (ser421/423)</td>
<td>Millipore, Billerica, MA</td>
<td>07-1575</td>
</tr>
</tbody>
</table>

**O-GlcNAc Western blotting**

Ventricular lysates were separated with SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked overnight at 4° C, probed with 1:5000 anti-O-GlcNAc antibody (CTD 110.6, generous gift of Mary-Ann Accavitti, University of Alabama at Birmingham), then probed with the appropriate secondary antibody. Horseradish peroxidase activity was detected on x-ray film with chemiluminescent substrate (Thermo Scientific) and quantified as described above for Western blotting.

**Co-immunoprecipitation**

Ventricular tissue was homogenized in Tissue Protein Extraction Reagent, 1% phosphatase inhibitor, 2% protease inhibitor (Sigma), and 0.02% PUGNAC. Lysates were assayed for total protein as described for Western blotting. Samples were diluted to equal protein concentrations and precleared over protein A/G agarose beads (Thermo Fisher Scientific) at 4° C for 4 hours. Precleared supernatants were then added to 25 ul
of beads that had been incubated with primary antibody for 4 hours at 4° C. Immunoprecipitation (IP) was performed overnight at 4° C; beads were then washed and eluted at 100° C for 5 min. The eluents were assayed for co-immunoprecipitated proteins using immunoblotting. The positive control was ventricular lysate; the negative control was ventricular lysate that was immunoprecipitated without antibody. Co-immunoprecipitated proteins were normalized to the level of captured target protein for analysis.

**HDAC activity**

HDAC enzyme activity was assayed with a colorimetric kit (Enzo Life Sciences, Farmingdale, NY) per the manufacturer's instructions. The substrate for this assay is predominantly deacetylated by HDAC1/2 and sirtuin 1 (a class III HDAC), but not the class II HDACs. Optical density was read at 415 nm. Results are presented as fold changes from control absorbance.

**RNA isolation and quantitative real-time PCR (qPCR)**

RNA was isolated with an RNA isolation kit (Qiagen, Valencia, CA) and quantified on a NanoPhotometer™ spectrophotometer (Implen, Ontario, NY). Complementary DNA (cDNA) was generated using a cDNA synthesis kit (Thermo Fisher Scientific). Quantitative polymerase chain reaction (qPCR) was performed in triplicate with SYBR green fluorescence chemistry using a qPCR kit (Qiagen). The negative control contained water in place of the cDNA template. Thermal cycling was performed on an iCycler iQTM Real Time PCR Detection System (Biorad) using the following cycle: 95° C for 10 min, and 40 cycles of 95° C for 30 sec and Tm for 10 sec. Primer specificity was confirmed by
melting curve analysis. Amplification data were analyzed with the \(2^{\Delta\Delta Ct}\) method for normalization to the housekeeping gene GAPDH as previously described [351]. See Table 5.2 for primer details.

**Table 5.2. Real-time PCR primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
<th>Amplicon size (kb)</th>
<th>Annealing temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANP</td>
<td>TTCCGGTACCAGAAGAT AACAGCCA</td>
<td>TGACACACCACAAGG GCTTAGGAT</td>
<td>91</td>
<td>60</td>
</tr>
<tr>
<td>BNP</td>
<td>AGACAAGGGAACA CCGCATCAT</td>
<td>ACAGAATCATCTGGGA CACACCT</td>
<td>85</td>
<td>60</td>
</tr>
<tr>
<td>HDAC1</td>
<td>TCCCTGCGTTCTTATC GCCCCAGAT</td>
<td>ACAAAGCATCAAAACA CCGGACAG</td>
<td>98</td>
<td>60</td>
</tr>
<tr>
<td>HDAC2</td>
<td>TACAACAGATCGCTGATGACCGTG</td>
<td>TCCCTTCCAGCACCA ATATCCCT</td>
<td>94</td>
<td>62</td>
</tr>
<tr>
<td>α-skeletal actin</td>
<td>TTGTGCACCGCAATG CTTCTAGG</td>
<td>GCAACCACAGCACGA TTGTCGATT</td>
<td>90</td>
<td>60</td>
</tr>
<tr>
<td>α-cardiac actin</td>
<td>TGTAGGTGATGAAGCC CAGAGCAA</td>
<td>TGGTGCCAGATCTTCT CCATGTCA</td>
<td>105</td>
<td>60</td>
</tr>
<tr>
<td>β-myosin heavy chain</td>
<td>TGGCTGGTGAGGTGCAT TGACAGAA</td>
<td>TGGCTGGTGAGGTCA TTGACAGAA</td>
<td>104</td>
<td>60</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGTGATGGGTGTGAAC CACGAGAA</td>
<td>CATGAGCCCTTCCACA ATGCCAAA</td>
<td>133</td>
<td>Per plate</td>
</tr>
</tbody>
</table>

**Statistics**

Statistics were performed in SigmaPlot 11.0 for Windows. Longitudinal effects of genotype and exercise on body weight and blood glucose were analyzed with two-factor repeated measures ANOVA followed by Bonferroni post-hoc tests. Non-normal data were log transformed prior to analysis. Two-factor ANOVA with Bonferroni post-hoc tests were used to describe genotype and exercise effects on protein levels, HDAC activity, and gene expression. Cardiac hypertrophy, tibia length, and wet heart weight data were resistant to transformations of normality and were analyzed with Kruskal-Wallis analysis of variance followed by Dunn’s post-hoc test. Values are presented as mean ± SEM and significance was accepted at p<0.05.
Results

Db mice show obesity, hyperglycemia, and cardiac hypertrophy despite 4 weeks of exercise

Fasting blood glucose and body weight in db mice were significantly elevated relative to controls and increased over the duration of the protocol (Figure 5.1), as the db mice developed overt diabetes. Exercise did not alter either variable in db mice; this is consistent with previously described work by others [232, 324]. There were no differences in cardiac hypertrophy at 1 week; however, db mice showed overt cardiac hypertrophy (heart weight:tibia length) at the 4 week time point (Table 5.3). Tibia length was also significantly reduced in db mice at the 4 week time point, consistent with previous reports of reduced linear skeletal growth in db mice [352, 353].

Figure 5.1. Effect of 4 weeks of moderate treadmill exercise training on fasting blood glucose and body weight in C57BL/6J and db/db mice.

Ex = exercise (n=15 per group); Sed = sedentary (n=16 per group). * significant effect of genotype within a time point, p<0.05; † significant effect of time between consecutive time points, p<0.05.
Table 5.3: Morphological data

<table>
<thead>
<tr>
<th></th>
<th>Wet heart (mg)</th>
<th>Tibia (mm)</th>
<th>Heart:tibia (mg:mm)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1 Week</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57 Sed</td>
<td>115 ± 4</td>
<td>22.5 ± 0.2</td>
<td>5.13 ± 0.18</td>
<td>11</td>
</tr>
<tr>
<td>C57 Ex</td>
<td>111 ± 4</td>
<td>23.0 ± 0.2</td>
<td>4.83 ± 0.18</td>
<td>12</td>
</tr>
<tr>
<td>Db Sed</td>
<td>114 ± 3</td>
<td>22.4 ± 0.1</td>
<td>5.08 ± 0.14</td>
<td>11</td>
</tr>
<tr>
<td>Db Ex</td>
<td>116 ± 3</td>
<td>22.3 ± 0.2</td>
<td>5.20 ± 0.15</td>
<td>12</td>
</tr>
<tr>
<td><strong>4 Week</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57 Sed</td>
<td>113 ± 2</td>
<td>23.5 ± 0.1</td>
<td>4.82 ± 0.09</td>
<td>16</td>
</tr>
<tr>
<td>C57 Ex</td>
<td>105 ± 2</td>
<td>23.4 ± 0.1</td>
<td>4.50 ± 0.10</td>
<td>15</td>
</tr>
<tr>
<td>Db Sed</td>
<td>123 ± 4*</td>
<td>22.6 ± 0.1*</td>
<td>5.45 ± 0.15*</td>
<td>16</td>
</tr>
<tr>
<td>Db Ex</td>
<td>119 ± 3*</td>
<td>22.4 ± 0.1*</td>
<td>5.30 ± 0.13*</td>
<td>15</td>
</tr>
</tbody>
</table>

*p<0.05, significant effect of genotype

Results are presented as mean ± SEM.

Total protein O-GlcNAcylation is increased by exercise in db mouse hearts

Exercise training increased cardiac O-GlcNAc in db mouse hearts at the 1 week time point; however, at this time point, protein levels of OGT and OGA were not different between groups (Figure 5.2). At 4 weeks, O-GlcNAc was elevated in db hearts relative to controls and was significantly increased by exercise (Figure 5.3). As densitometry of the entire sample lane is dominated by the intense immunoreactive bands at 37 and 82 kDa, the analysis was also performed over the high, mid and low molecular weight ranges, which showed the same exercise-induced increases in db hearts. At this timepoint, levels of OGA and OGT were also significantly increased in db hearts relative to controls, independent of exercise.
We next analyzed changes in O-GlcNAc within each genotype over time. Analysis by weight range revealed a modest but significant decrease in total and low-molecular weight protein O-GlcNAcylation with exercise in C57 hearts (Figure 5.4 B and E). Interestingly, however, there was a significant increase in total and high-molecular weight protein O-GlcNAcylation over time within the exercised C57 mice (Figure 5.4 B and C). By contrast, in db mice, total and high molecular weight O-GlcNAc increased over time (Figure 5.4 G), and O-GlcNAcylation of mid and low molecular weight proteins were increased by exercise (Figure 5.4 I-J).

Diabetes reduces mSin3A/HDAC1/HDAC2 protein levels in the heart

REST protein levels were not different between groups at either time point (Figures 5.5 and 5.6). At the 1 week time point, mSin3A and HDAC1 were significantly lower in db hearts, independent of exercise (Figure 5.5). At the 4 week time point, mSin3A, HDAC1,
as well as HDAC2 protein levels were significantly lower in db hearts, again independent of exercise (Figure 5.6). Exercise training reduced mSin3A levels in non-diabetic control hearts; however, mSin3A levels were even lower in db hearts and did not change with exercise (Figure 5.6B).

**Exercise rescues the mSin3A:HDAC1 association and the OGT:HDAC2 association in db mouse hearts**

As stated above, cardiac hypertrophy as measured by heart weight:tibia length was not apparent at 1 week but was evident in db hearts at 4 weeks (Table 5.3); therefore, we analyzed the association of hypertrophy-regulating transcription factors in the 4 week group only. Co-immunoprecipitation of OGT showed that diabetes reduced the OGT:HDAC2 association (p<0.05), but this difference was removed by exercise training (Figure 5.7). OGT association with mSin3A and
HDAC1 was not different between groups. OGT was also modestly but significantly more associated with REST in sedentary db hearts compared to sedentary controls (p<0.05).

Reciprocal co-immunoprecipitation of mSin3A showed that there were no differences in its association with OGT or HDAC2 (Figure 5.8). However, mSin3A was significantly more associated with REST in sedentary db hearts. Finally, the association of mSin3A with HDAC1 was significantly lower in sedentary db hearts compared to sedentary controls, and this difference was removed by exercise.

It was recently reported that exercise reduces the O-GlcNAcylation of Specificity Protein 1 transcription factor (SP1) [80, 82]. Therefore, we investigated whether exercise would alter the O-GlcNAc modification of mSin3A, a transcription factor that is directly involved in hypertrophic signaling. Although mSin3A immunoprecipitation appeared to show that the O-GlcNAcylation of mSin3A was increased by exercise (Figure 5.8 A-B), reciprocal immunoprecipitation of O-GlcNAc did not confirm this effect (data not shown). Therefore, this effect of exercise should be viewed with caution.

Finally, we performed co-immunoprecipitation of HDAC1 to confirm the results of the mSin3A immunoprecipitation (Figure 5.9 A). Immunoblotting for mSin3A showed the same effect of exercise on the HDAC1:mSin3A interaction; the association of mSin3A with HDAC1 was lower in sedentary db hearts relative to sedentary C57 controls, but exercise removed this difference.
HDAC activity is increased in db mouse hearts

Phosphorylation of HDAC1 at serine 421 and 423 is specifically associated with HDAC1 activation [354]; therefore, we immunoprecipitated HDAC1 and immunoblotted for phospho-HDAC1 (Ser421/423). This showed that HDAC1 phosphorylation was significantly elevated in db hearts independent of exercise (p<0.05) (Figure 5.9 D). Colorimetric assay confirmed that class I HDAC activity was significantly higher in db mice independent of exercise (Figure 5.9 E).

Diabetes blunts fetal gene expression in the heart

We investigated whether changes in these transcription factor interactions were associated with changes in the expression of fetal genes, such as ANP, BNP, and skeletal α-actin, which are regulated by REST and mSin3A [269, 355]. Transcript levels
of ANP and β-myosin heavy chain were not different between groups, but BNP and skeletal α-actin were significantly reduced in db hearts independent of exercise (Figure 5.10A). HDAC2 gene expression was elevated in db hearts independent of exercise, and cardiac α-actin showed a prominent trend to be induced by exercise (p=0.053) (Figure 5.10B).

**Discussion**

While diabetes is a multifactorial cardiac insult, and diabetic cardiomyopathy is associated with multiple factors such as oxidative stress [356], lipotoxicity and mitochondrial dysfunction [27, 357, 358], and impaired calcium signaling [341], O-GlcNAc is emerging as an important signaling mechanism in the development of diabetic cardiomyopathy. Total protein O-GlcNAcylation is chronically elevated in the type 1 and 2 diabetic heart [30, 72]. Reducing protein O-GlcNAcylation by adeno viral overexpression of OGA [81] improves cardiac function, and lowering O-GlcNAc by intensive swim training [80, 82] has been proposed as a mechanism by which exercise
benefits the diabetic heart. Previous work has shown that exercise lowers the O-GlcNAc modification of the SP1 transcription factor [80] and the OGT enzyme [149]. O-GlcNAc directly mediates the expression of fetal genes in response to hypertrophic stimuli [239], and O-GlcNAc modifies mSin3A and HDAC1 [71], which regulate cardiac hypertrophy [57, 269]. Moderate exercise improves cardiac structure and function in humans with type 2 diabetes [359, 360]; we therefore tested the hypothesis that moderate exercise would reduce O-GlcNAc in the type 2 diabetic heart, and would be associated with changes in the O-GlcNAc modification and activity of the mSin3A/HDAC1/2 transcription factor complex, which regulates hypertrophic genes.

**FIGURE 5.6**

Surprisingly, and in contrast with the previous studies, we found that 4 weeks of moderate treadmill exercise increased total O-GlcNAc in type 2 diabetic db mouse hearts. While the previous studies showed that OGT was also reduced by exercise [80, 82], we found that OGT and OGA expression was elevated in db hearts and did not change with exercise. Such parallel regulation of OGT
and OGA expression has been previously reported [83] and may represent a compensatory relationship between these two opposing enzymes. The difference in our findings may be due to the use of type 2 db mice rather than streptozotocin-induced type 1 diabetic mice, and the use of moderate treadmill exercise rather than more intensive swimming exercise. However, other studies have shown that an upregulation of O-GlcNAc is essential in the cardiac stress response [361, 362], is acutely cardioprotective [363, 364], and is part of a constitutively active cardioprotection mechanism in the diabetic myocardium [348]. Therefore, these data suggest that an increase in cardiac O-GlcNAc in the type 2 diabetic heart may be a beneficial effect of exercise.

In our study, mSin3A immunoprecipitation revealed that exercise increased the O-GlcNAc modification of mSin3A; however, this was not supported by reciprocal O-GlcNAc immunoprecipitation. It is possible that the large amount of protein captured in the O-GlcNAc immunoprecipitation masked the changes in mSin3A O-GlcNAcylation, which we observed in the more specific mSin3A immunoprecipitation. However, these data underscore the importance of verifying changes in O-GlcNAcylation of individual proteins with reciprocal assays, and suggest that moderate changes in protein O-GlcNAcylation – including those in the present study – should be interpreted cautiously and confirmed by additional studies.
Nevertheless, our data do suggest an alternate mechanism for the beneficial effect of exercise on the diabetic heart. Db hearts showed lower protein levels of mSin3A, HDAC1, and HDAC2, and an increased association of mSin3A with REST, independent of exercise. Likewise, mRNA transcript levels of BNP and α-skeletal actin, which are typical markers of cardiac hypertrophy activated by HDAC1/2 [58] that are regulated via REST/mSin3A [70], were significantly lower in db hearts independent of exercise. The finding that blunted expression of fetal genes in diabetic hearts is not altered by exercise has been shown in previous studies [232, 320]. Therefore, we suggest that the loss of HDAC1/2 and the increased association of the mSin3A corepressor with REST may underlie the blunted expression of fetal genes in the diabetic heart. Further, since the natriuretic peptides are both anti-hypertrophic and cardioprotective [255, 256, 320], we suggest that this mechanism may be responsible for the increased vulnerability of the diabetic heart to stress and heart failure [365, 366].
While the exercise protocol in this study was of a low intensity and did not elicit large phenotypic benefits in either strain, the exercise stimulus was sufficient to elevate the expression of cardiac α-actin in C57 hearts. Cardiac α-actin is a marker of cardiomyocyte differentiation and hypertrophy [367] and is increased in physiologically hypertrophied hearts after chronic endurance exercise training [346]. Additional transcriptional changes were observed in db hearts, in which the exercise protocol significantly increased the association of mSin3A and OGT with HDAC1 and HDAC2, respectively. Therefore, although the exercise stimulus used in this study did not cause overt changes in cardiac mass, it induced transcriptional events consistent with the early stages of physiological cardiac remodeling.

Finally, these data show a potential interaction between HDAC1 and HDAC2 that has not previously been described in the heart. HDAC1 and HDAC2 regulate cardiac hypertrophy in a similar manner [57], and HDAC1 deficiency induces HDAC2 expression.
in embryonic stem cells [368]. In our study, the loss of HDAC1 protein preceded the loss of HDAC2 protein in db hearts and was similarly associated with an increase in HDAC2 gene expression in db hearts. When HDAC2 deficiency was present at the 4 week time point, we observed an increase in the total activity of class I HDACs in db hearts, which was verified by an increase in the phosphorylation status of HDAC1 at Ser421/423. Phosphorylation at these residues is specifically associated with HDAC1 activity [354]. Therefore, these data suggest that the class I HDACs have compensatory effects on each other’s expression levels and activation by phosphorylation. Further, the reduction in HDAC2 protein levels in db mouse hearts did not occur until 4 weeks and was associated with overt cardiac hypertrophy. Thus, the loss of HDAC2 in the diabetic heart is associated with the progression of hypertrophy in the diabetic heart and may be more specifically involved in hypertrophy than HDAC1.
Conclusions

These data show that exercise increases O-GlcNAc in the type 2 diabetic db mouse heart, and that components of the mSin3A/HDAC1/2 chromatin-modifying complex interact with O-GlcNAc and OGT. Contrary to our hypothesis, exercise increased cardiac O-GlcNAc in the diabetic heart, and it is possible that this may play a role in the beneficial effect of exercise. Finally, we found that diabetes and exercise reciprocally affected the physical associations of mSin3A/HDAC1/2. The effects of exercise observed in this study were generally modest, which suggests that a moderate level of exercise, such as that prescribed for human patients with diabetes, does not have extreme effects on the mSin3A/HDAC1/2 complex. However, since this complex is a key
regulator of cardiac hypertrophy, the results of this study suggest that exercise-induced changes in the association or activity of this complex may underlie the beneficial effect of moderate exercise in the diabetic heart.

List of abbreviations

- **ANP**: atrial natriuretic peptide
- **BNP**: brain natriuretic peptide
- **C57**: C57 background strain control mouse
- **Db**: db/db type 2 diabetic mouse
- **HDAC**: histone deacetylase
- **mSin3A**: mammalian switch-independent 3A
- **O-GlcNAc**: O-linked β-N-acetylglucosamine
- **OGT**: O-GlcNAc transferase
- **OGA**: O-GlcNAcase
- **REST**: repressor element-1 silencing transcription factor
CHAPTER 6

Conclusion

Diabetic cardiomyopathy (DCM) is a syndrome of cardiac fibrosis, diastolic dysfunction, and ventricular hypertrophy that occurs secondary to T2DM. Heart disease is the most frequent comorbidity of T2DM and is often secondary to comorbid risk factors such as obesity and hypertension. However, DCM is a unique type of cardiomyopathy that occurs in patients with T2DM independent of coronary artery disease, hypertension, or other risk factors for heart disease. Because of its independence from classical risk factors for heart disease, the current working hypothesis for the etiology of DCM is that insulin resistance causes energy insufficiency in the heart, predisposing the heart to contractile dysfunction and failure [7]. However, this hypothesis does not explain the full syndrome of DCM (i.e. fibrosis, diastolic dysfunction, and ventricular hypertrophy).

Currently, clinical guidelines for the management of DCM focus on reducing central and peripheral blood pressure, which staves off heart failure but does not address the underlying defect in DCM, which is not load-dependent. The growing prevalence of DCM combined with the lack of understanding of its etiology and clinical management highlight the need to identify underlying mechanisms and therapeutic targets for this condition. Therefore, the purpose of this dissertation was to study the presentation of DCM in preclinical models, identify potential mechanisms for DCM, and hypothesize specific therapeutic targets.

Conclusions support our hypothesis that DCM has a unique etiology from load-dependent hypertrophy and suggest that exercise is a viable treatment option for DCM. Important shortcomings of preclinical models of DCM are highlighted that need to be addressed in future research and suggest that the class 1 HDACs are a therapeutic
target for diabetes and possibly DCM. Cumulatively, the chapters in this dissertation provide insight into the molecular mechanisms of DCM, clinical applications of exercise and HDAC inhibition in T2DM, and important preclinical issues that should inform future research into DCM.

**Diabetic cardiomyopathy has a unique etiology**

The Chapters in this dissertation provide new data in support of the hypothesis that cardiac hypertrophy in DCM has a unique etiology from load-dependent cardiomyopathy and hypertrophy. Chapter 2 highlights the differences between hypertrophy induced by load (e.g. hypertension and exercise) and hypertrophy induced by load-independent (e.g. in T2DM) mechanisms and suggested that DCM invokes distinct mechanisms from load-dependent hypertrophy [117]. Similarly, chapters 3 and 5 showed that fetal gene expression is lower in mice with T2DM compared to controls [42, 220]. This is a significant departure from the phenotype of load-dependent hypertrophy, which is characterized by elevated fetal gene expression [41, 190, 230, 310, 313, 342].

Results from this dissertation open several questions for future research. For example, it is not clear whether fetal gene expression is a cause or an effect of cardiac hypertrophy, or how these genes are regulated by cardiac metabolism (and therefore diabetes). It is also not known whether the expression of these genes in the adult heart is protective or detrimental, and consequently, whether they constitute a therapeutic target for the prevention of hypertrophy and heart failure. However, differential expression patterns between hearts with load-induced hypertrophy and hearts with diabetes clearly shows that these genes are not straightforward markers of hypertrophy and highlights the need for research into the mechanism and purpose of fetal gene recapitulation in the adult
heart. Because of this differential expression, the use of classical load-dependent biomarkers for cardiac hypertrophy should be avoided in preclinical DCM research, except perhaps the expression of brain natriuretic peptide, which may be a positive biomarker of hypertrophy in rodents with type 2 diabetes [42].

**Future clinical exercise research should focus on “precision exercise”**

Aerobic exercise is widely accepted as a beneficial intervention for cardiovascular disease, and it has come to particular prominence in the last decade due to the American College of Sports Medicine’s Exercise as Medicine® movement. Although there are existing procedures and guidelines for improving the accuracy of cardiorespiratory measurements taken during human exercise research [369], there is still substantial variation in the actual protocols used to study exercise. For example, a recent meta-analysis of the validity of the 6-minute-walk test in clinical exercise settings showed that 39 out of 64 studies modified the walking protocol, with substantial confounding effects on their results [370]. Nevertheless, exercise consistently has beneficial effects on different types of cardiovascular diseases, and as argued in chapter 2, there is good reason to expect that exercise can be strategically “dosed” for different therapeutic benefits. For example, combining aerobic and resistance training to increase muscle mass and promote skeletal muscle glucose uptake should help glucose control in patients with type 2 diabetes, and short frequent bouts of exercise could be used to sustain an unloading effect on the heart in hypertensive patients. This is an exciting future direction for exercise research, but it highlights several changes that would need to occur in exercise research as well as the administration of exercise therapy.
First, implementing more precise exercise prescription will require improvements in the precision of exercise research. The previously mentioned systematic review of the validity of the 6-minute walk test by Dunn et al. [370] provides an example of what is currently the major barrier to precise exercise prescription: the lack of systematic methods in exercise research. This problem probably exists because of the relatively recent advent of the Exercise as Medicine® movement, as well as clinical exercise facilities’ focus on monitoring symptoms and retaining patients rather than the precision of exercise intensity. However, there is precedent for a call for more precise exercise research [371], and the ability to precisely target different conditions with a correct exercise “dose” is extremely attractive because of the low cost of exercise, as well as lack of side effects. Second, there are infrastructure barriers to the use of precise exercise prescriptions. For example, it will require research into methods for improving patient adherence, and it will require either advances in remote monitoring of home exercise or an expansion of insurance coverage to include preventative and rehabilitative clinical exercise services. Nevertheless, the idea of “precision exercise” for different conditions is an enticing area for future research, and small increases in the systematicity of current exercise research would make this possible.

**Class 1 HDACs may be therapeutic targets for DCM**

Recent studies have suggested that HDAC enzymes are therapeutic targets for diabetes and its organ-specific complications [49]. HDAC inhibition prevents cardiac hypertrophy in vitro and in vivo [36, 50-58], protects pancreatic β-cell function in response to inflammatory insults [47-49, 59-65], and enhances skeletal muscle insulin sensitivity [66]. However, it has only been tested *in vivo* in one study utilizing a pan-HDAC inhibitor [66].
Chapter 5 describes elevated class 1 HDAC activity in the hearts of type 2 diabetic mice [220], which points to the activity of class 1 HDACs as a potential player in diabetic complications. This may support the hypothesis that class-specific HDAC inhibition has anti-diabetic applications, and suggests that this topic merits further research. Within the last 10 years, a few HDAC inhibitors with class-specific inhibitory properties have been FDA approved, and many others are in clinical trials. Therefore, research into their off-label applications is particularly timely.

Future research into this topic will begin to answer the longstanding question of how epigenetic mechanisms influence the development of type 2 diabetes [372]. HDAC inhibitors may prove anti-diabetic, but their effects may be due to the HDAC's effects on gene expression or direct effects on the acetylation of signaling proteins. Alternatively, the mechanism could involve more intricate events, such as physical interactions between the HDACs and components of the insulin signaling pathway. This may include co-localization of HDAC2 and insulin receptor substrate 1, which was recently reported in postsynaptic glutamatergic neurons [373]. Whether the insulin sensitizing and cytoprotective effects of the HDACs in diabetes are due to epigenetic mechanisms or direct signaling effects will help to elucidate the underlying mechanisms in DCM, as well as specific therapeutic targets for this condition. The recent development of new models for studying DCM in vitro, such as an induced pluripotent stem cell model of diabetic cardiomyopathy that can be used for high-throughput screening of therapeutic agents [374], make this avenue of research increasingly practical and exciting.

Preclinical DCM research requires standardization
Chapter 3 provides an in-depth analysis of the relationship between classical biomarkers of load-dependent hypertrophy (i.e. fetal genes) and cardiac enlargement in rodent models of DCM [42]. Although this study identified many studies from 1980 to 2013 that relied on this association, this is the only study to date that has examined the relationship between these biomarkers and the presence of hypertrophy in diabetic rodents.

The results of this study clearly show no associations between fetal gene expression and hypertrophy in rodent models of diabetes; in fact, brain natriuretic peptide is the only fetal gene exhibiting a positive association with heart size in rodent models of T2DM (not T1DM) [42]. This could be a result of the multifactorial nature of DCM, which includes a metabolic insult and (often) a comorbid hypertension insult. The study also highlights important shortcomings in preclinical DCM research. For example, phenotypes of DCM in preclinical rodent models of diabetes were highly varied and depended on strains, diabetogenic agents, and laboratory methods of measuring hypertrophy. Different species (rat vs. mouse) and different diabetogenic agents (cytotoxic glucose analogues vs. diet or genetic models) had significant confounding effects on heart enlargement. Although the injectable diabetogenic streptozotocin actually induces T1DM, numerous studies used this method to study DCM, and within these studies there were wide variations in the frequency and dose of streptozotocin administration. Finally, studies used widely inconsistent methods of reporting cardiac size. For example, results showed 48 reports of the heart weight to body weight index, which has a biased denominator; 33 studies that did not report body weight; and 32 studies that used diverse measurements, including the left ventricular weight:body weight ratio, heart weight:tibia length, left ventricular weight, right ventricular weight, and the right ventricular weight:body weight
ratio. These indices produce different estimates of the outcome of cardiac enlargement and may have a significant effect on the overall conclusion of a study.

The prevalent use of these indices also suggests that unreliable indexing methods bias a significant portion of DCM research. Therefore, a major conclusion of chapter 3 is that DCM studies should report non-indexed data or use an index that is not biased by body weight changes, such as the heart weight:tibia length ratio. This chapter also underscores the confounding effects of diabetogenic drugs, which may have direct effects on cardiac enlargement. Finally, this study shows that classical biomarkers of load-dependent hypertrophy are very poor correlates of hypertrophy in diabetic rodents, and should not be used as biomarkers of hypertrophy in preclinical rodent models of DCM.

Chapter 4 addresses a different kind of shortcoming in translational DCM research, i.e. that the effects of exercise on the diabetic heart are often studied in rodents, but utilize inconsistent methodologies. This study reports reference values for peak oxygen consumption (VO₂peak), peak VO₂/VCO₂, and work rate in the db/db and C57 mouse, the most commonly used diabetic mouse model and its non-diabetic control, so that future exercise studies can calibrate their protocols to the actual maximum exercise capacity of these mice. Additionally, the study shows that exercising mice at the same percent of maximal exercise capacity that is prescribed for humans with T2DM in clinical settings does improve cardiorespiratory fitness in both DB and C57 mice, which verifies the translational relevance of exercise training studies in these mice. Finally, kinetic analysis suggests that it is possible to extract kinetic parameters from VO₂ data obtained from small-animal VO₂ analysis in a modular exercise chamber. This is an exciting finding because some animal models of disease preclude maximal exercise testing, and our
results suggest kinetic modeling can be used to estimate changes in $r$ or maximal VO$_2$ in lieu of maximal exercise testing. Clinical exercise testing typically uses such predictive kinetic analyses in lieu of maximal exercise testing [375-378]; therefore, applying these approaches to mice may open up a new area of translational research.

**Conclusion**

Over the last 30 years, DCM has evolved from a hypothetical syndrome [379] to a well-recognized clinical entity [380]; however, there is no established treatment course for the management of DCM. Progress in this area is stymied by our lack of understanding about the underlying mechanisms of DCM, as well as inconsistent preclinical research methods for investigating diabetes and DCM.

In this dissertation, several key findings are provided that advance the field of DCM research. First, the chapters in this dissertation repeatedly show that aerobic exercise has beneficial effects on the heart. This leads to the conclusion that aerobic exercise is a viable treatment course for DCM, although additional research is needed to identify the correct “dose” of exercise. Second, this dissertation identifies several distinct areas of preclinical diabetes research that need to be standardized, especially the use of hypertrophic biomarkers, methods of reporting hypertrophy, and dosing of diabetogenic agents. This dissertation also closes an important gap in preclinical exercise research by reporting specific reference values for maximal exercise capacity in trained and untrained type 2 diabetic mice and non-diabetic controls, and provides a protocol for exercise training these mice with a “human” exercise prescription. Finally, this chapter shows that class 1 HDACs are probable therapeutic targets for diabetes and possibly
DCM. Cumulatively, this dissertation provides new strategies for DCM research and suggest a potential therapeutic target for the management of diabetes and possibly DCM.
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