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Insulin Treatment Increases Myocardial Ceramide Accumulation and
Disrupts Cardiometabolic Function

Aimee Elizabeth Hodson

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of
Master of Science

Benjamin T. Bikman, Chair
Paul R. Reynolds
Brian D. Poole

Department of Physiology and Developmental Biology
Brigham Young University
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ABSTRACT

Insulin Treatment Increases Myocardial Ceramide Accumulation and Disrupts Cardiometabolic Function

Aimee Elizabeth Hodson

Department of Physiology and Developmental Biology, BYU
Master of Science

Prevalence of diabetes, especially type 2 diabetes mellitus (T2DM) is increasing worldwide. Millions of people are already affected by T2DM and estimates predict over half a billion people will likely be suffering from the disease by 2030. T2DM is associated with an increased risk of developing cardiovascular disease. Cardiovascular dysfunction is the leading cause of mortality among type 2 diabetics.

Treatment for T2DM has changed over time. Though it was once known as insulin independent, a large portion of type 2 diabetics are now treated with insulin injections. However, type 2 diabetics treated with insulin are more likely to suffer from heart complications. Due to this, we sought to determine the specific effect of insulin and insulin-induced ceramide accrual on heart mitochondrial bioenergetics. To do so we used both *in vitro* and *in vivo* models. H9c2 cardiomyocytes and adult male mice were treated with insulin with or without the ceramide biosynthesis inhibitor myriocin. Mitochondrial bioenergetics were determined in permeabilized cardiomyocytes and myocardium. In this study we demonstrate that insulin induced ceramide accrual in both isolated cardiomyocytes and whole murine myocardium. We further found that insulin treatment is sufficient to disrupt mitochondrial respiration in both models. Inhibition of the ceramide accrual rescued mitochondrial respiration, indicating that ceramide is necessary for the insulin-induced alterations in heart mitochondrial respiration.

These results suggest that insulin has a role in the development of heart complications associated with T2DM due to cardiomyocyte mitochondrial disruption. They also implicate ceramide as a possible mediator in the development of insulin-related heart disorders.

Keywords: type 2 diabetes, ceramide, mitochondria, hyperinsulinemia, insulin

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CHAPTER 1: Introduction and Background

Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) is currently a major public health problem, and incidence is only increasing. In 2014 there were 29.1 million people with diabetes in the United States. Another 86 million were pre-diabetic, and about 15-30% of pre-diabetics were expected to develop T2DM over the next 5 years (Centers for Disease Control and Prevention, 2014). Prediabetes is characterized by blood glucose levels that are higher than normal, but not high enough for a full diagnosis as type 2 diabetes. This is due to the beginning stages of insulin resistance, where insulin levels are abnormally high in order to keep glucose levels from spinning out of control. There are no clearly visible symptoms associated with prediabetes. Consequently, a large portion of those affected by prediabetes are unaware they have the disease, are untreated, and are more likely to develop T2DM.

Estimates predict that in 2030 around 552 million persons worldwide will be affected by T2DM (Whiting, Guariguata, Weil, & Shaw, 2011). T2DM is characterized by insulin resistance, which causes the body to not use the insulin it produces effectively resulting in higher than normal levels of glucose in the blood. It was previously known as non-insulin dependent diabetes mellitus. However, as an estimated 29% of type 2 diabetics use insulin injections in order to regulate their blood glucose levels, this term is no longer accurate (Centers for Disease Control and Prevention, 2014). Another previous name for T2DM, adult-onset diabetes, has also become irrelevant. Correlated with the trend of ever increasing childhood obesity, prevalence of T2DM in children and adolescents has increased greatly over the past decade (Amed, et al., 2010; Pinhas-Hamiel & Zitler, 2005). The dropping of these former

names highlights the growing impact of T2DM as it affects people of all ages and the increasing pervasiveness of insulin treatment.

Cardiovascular Complications

It has long been established that T2DM greatly increases the risk of cardiovascular disease (Kannel & McGee, 1979). The two diseases share many common risk factors such as dyslipidemia, insulin resistance, obesity, pro-inflammatory state, and pro-thrombotic states. The presence of these common risk factors helps explain the correlation between T2DM and cardiovascular dysfunction, however, it has been found that even independent of these risk factors T2DM is associated with about a two-fold risk increase for cardiovascular disease (The Emerging Risk Factors, 2010). Even when controlling for abnormal cholesterol levels, perhaps the most commonly examined risk factor for cardiovascular disease, cardiovascular events have been shown to increase with T2DM (van der Heijden, et al., 2013). Recent studies have also found connections through shared regulator genes involved in the development of both T2DM and cardiovascular disease (Chan, et al., 2014). The impact of these connections, and the search for as of yet unknown pathways is currently a field of pronounced interest amongst researchers examining T2DM.

Not only is T2DM connected with a greater risk of cardiovascular diseases, it increases the risk of recurrence in those who have already experienced cardiovascular events. The increased risk of repetition also escalates cardiovascular related mortality in T2DM (van der Heijden, et al., 2013). It is therefore unsurprising that the major cause of mortality in persons suffering from T2DM is cardiovascular disease (Sowers, Epstein, & Frohlich, 2001).

Due to the prevalence of T2DM, the high correlation between cardiovascular dysfunction and T2DM, the increased risk of recurrence, and the impact of cardiovascular

disease on T2DM-related mortality there is considerable importance in gaining a deeper understanding concerning how these two diseases are connected.

Glucose and Insulin Research Paradigms

A large percentage of past research has focused on the impact of high glucose levels as the link between heart dysfunction and T2DM (Fuentes-Antrás, et al., 2015; Laakso, 1999; Matheus, et al., 2013; Schmidt, Yan, Wautier, & Stern, 1999). Through this paradigm of thought many important discoveries concerning glucose-related mechanisms have been made. Poor glycemic control has been linked to inflammation, receptor for advanced glycation endproducts (RAGE activation, and possible disruption of mitochondrial function (Nelson, et al., 2015a; Schmidt, et al., 1999; Tilton, et al., 1995). It seems possible, however, that there may be an equally, if not more, impactful role for high insulin levels on cardiovascular health in T2DM (Mandavia, Aroor, DeMarco, & Sowers, 2013; Pories & Dohm, 2012). These findings are especially relevant as insulin injections have become a more common medication for controlling glucose levels in T2DM. While such therapies are successful in lowering blood glucose levels in T2DM, they also lead to an increase in mortality (The Action to Control Cardiovascular Risk in Diabetes Study, 2008). It is therefore expedient that further investigations be made to determine the impacts of high insulin levels associated with T2DM on cardiovascular health.

Recent research has suggested that while intensive glucose control may lead to a reduction in microvascular complications, it is not as effective in preventing cardiovascular complications (Laakso, 1999). Other glucose control studies have shown that intensive-therapy, which often includes insulin injections, may result in a decrease in cardiovascular events, but no corresponding increase in patient survival (Hayward, et al., 2015). This is not to say that

glucose control is unimportant in T2DM treatment. Higher risk of cardiovascular events has been seen in conjunction with poor glycemic control due to lack of treatment or delay of treatment (Green, et al., 2015; Paul, Klein, Thorsted, Wolden, & Khunti, 2015). It has also been shown that patients with better controlled glucose experienced a significant reduction in cardiovascular events survival (Holman, Paul, Bethel, Matthews, & Neil, 2008). However, the research is still contradictory. There are contrasting studies showing no significant improvement in cardiovascular dysfunction with better controlled glucose (Prato, 2009; Skyler, et al., 2009).

Due to the inconsistency of the data, some researchers have begun looking for new treatment options with more concrete results. One new direction which has shown promise is the use of medications that control hyperglycemia without increasing insulin to reduce the risk of cardiovascular dysfunction in T2DM (Green, et al., 2015). These hopeful results offer further credence to the importance of insulin in the etiology of T2DM, and offers an area for future research.

Ceramide

Ceramide is a sphingolipid made of a combination of sphingoid base backbone, sphingosine, and a fatty acid. As denoted by their name, given in reference to the mysterious Sphinx, the function of sphingolipids was unknown for many years. Today, these lipids are still under investigation, but roles in cell differentiation, cell membranes, signaling pathways that mediate cell growth, and cell death have been elucidated (Merrill Jr, et al., 1997). Ceramide in particular has been implicated in cellular stress responses and mediation of several inflammatory factors such as TNF- α and interleukin-1 (Merrill Jr, et al., 1997).

There are two pathways through which ceramide is synthesized: the *de novo* pathway and a recycling pathway. In the *de novo* pathway several enzymes -- including serine

palmitoyltransferase, 3-ketosphingosine reductase, ceramide synthase, and ceramide desaturase – are involved in converting palmitoyl-CoA to ceramide (Pralhada Rao, et al., 2013). Ceramide can also be formed through the hydrolysis of more complex sphingolipids, or sphingolipid recycling. This recycling process is an important part of lipid homeostasis (Pralhada Rao, et al., 2013).

In previous studies it has been found that increasing insulin levels also leads to an accumulation of ceramide in skeletal muscle (Hansen, et al., 2014). As understanding of the role ceramide plays has improved, it has become more and more apparent that the accumulation of ceramide is linked with the pathogenesis of several diseases, including Alzheimer's, obesity, metabolic syndrome, type 1 diabetes mellitus, and T2DM (Filippov, et al., 2012; Galadari, Rahman, Pallichankandy, Galadari, & Thayyullathil, 2013; X. Li, Becker, & Zhang, 2010; Yuyama, Mitsutake, & Igarashi, 2014). Ceramides have also recently been increasingly implicated as playing a role in the development of cardiovascular diseases (Di Paola, Cocco, & Lorusso, 2000; X. Li, et al., 2010; Park, et al., 2008; Zhang, et al., 2012). The relationship between ceramide and these diseases, as well as the accumulation of ceramide in the presence of high insulin, implicates a possible role for ceramide as a mediator for cardiovascular dysfunction in type 2 diabetics. It has been postulated that one method through which ceramides are negatively impacting cardiac function is through increasing mitochondrial fission (Smith, et al., 2013).

Mitochondria

Mature cardiomyocytes have a high metabolic demand, and are therefore highly oxidative cells. Most of the energy required for cardiomyocyte contractions is supplied through the oxidative phosphorylation of the mitochondria (Lemieux & Hoppel, 2009). A disruption in

the function of cardiac mitochondria can lead to heart failure (Holmgren, et al., 2003; Lesnefsky, Moghaddas, Tandler, Kerner, & Hoppel, 2001). Mitochondrial function depends greatly on its morphology and connectivity. In the fusion state mitochondria form a reticular network and have increased rates of respiration. In contrast, the fission state is characterized by a disintegration of mitochondrial connectivity and a decrease in respiratory capacity (Ishihara, Jofuku, Eura, & Mihara, 2003). T2DM is associated with an increase in mitochondrial fission (P. Li, et al., 2012), and mitochondrial fission has been connected with an increase in cardiac dysfunction (Pennanen, et al., 2014).

Due to the crucial role of healthy mitochondria to cardiac health, as well as the pathological implications of dysfunctional mitochondria, it is important to understand the factors which affect mitochondrial form and function. Better understanding the connection between T2DM, high insulin, and cardiac dysfunction could lead to better prevention of T2DM mortality and is an important area of research.

In view of the evidence implicating a role for insulin in the etiology of cardiac complications, especially through ceramide mediated mitochondrial disruption, the aim of this study is to better elucidate the role of insulin in cardiac dysfunction and investigate the possibility of ceramide as an intermediate in the pathway of insulin induced disruption of cardiac metabolic activity.

Summary of Research

Previous research conducted in the BYU obesity and metabolism lab has centered on the sphingolipid ceramide and its effects in a wide array of circumstances. The research has reinforced the notion of ceramide as a key player in mediating several of the pathological consequences of obesity and type 2 diabetes mellitus. Our lab has previously demonstrated that

insulin increases ceramides in skeletal muscle (Hansen, et al., 2014). It was shown in our lab that ceramides are secreted from A549 lung cells after exposure to cigarette smoke (Thatcher et al 2014). The lab built on this study in order to show that myocardiocytes incubated with the medium taken from cigarette smoke-exposed lung cells results in a pronounced inhibition of myocardial mitochondrial respiration. This effect could be attenuated through ceramide inhibition (Tippetts, et al., 2014).

These findings complement previous results generated in our lab regarding ceramide and mitochondrial function. As described previously, morphology is extremely important in mitochondrial function. Healthy mitochondria are in the fusion morphology, meaning they form a reticular network throughout the cell (Ishihara, Jofuku, Eura, & Mihara, 2003). In contrast, our lab has found a correlation between ceramides and the disruption of this fusion state. This disruption is known as mitochondrial fission, which means the reticular network is decreased, mitochondrion become more separated from one another, and the mitochondrial respiration is reduced. Our lab found this process stimulates mitochondrial fission through dynamin related protein 1 (Smith, et al., 2013).

Following the previously seen effects of insulin in increasing ceramide accumulation in skeletal muscle, and the effects of ceramide on the heart, we hypothesized that high insulin would produce a similar result in cardiomyocytes, and this presence of ceramides would then disrupt mitochondrial activity.

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CHAPTER 2: Insulin Treatment Increases Myocardial Ceramide Accumulation and
Disrupts Cardiometabolic Function

Aimee E. Hodson, Trevor S. Tippetts and Benjamin T. Bikman*

*Corresponding author: Benjamin T. Bikman: Benjamin_bikman@byu.edu

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Abstract

Background

States of hyperinsulinemia, particularly insulin resistance and type 2 diabetes mellitus, are becoming remarkably common, with roughly half a billion people likely to suffer from the disorder within the next 15 years. Along with this rise has been an associated increased burden of cardiovascular disease. Considering type 2 diabetics treated with insulin are more likely to suffer from heart complications, we sought to determine the specific effect of insulin on ceramide-dependent cardiometabolic risk factors, including insulin resistance and altered heart mitochondrial physiology.

Methods

H9c2 cardiomyocytes and adult mice were treated with insulin with or without myriocin to inhibit ceramide biosynthesis. Insulin and glucose changes were tracked throughout the study and mitochondrial bioenergetics was determined in permeabilized cardiomyocytes and myocardium.

Results

Herein, we demonstrate that insulin is sufficient to disrupt heart mitochondrial respiration in both isolated cardiomyocytes and whole myocardium, possibly by increasing mitochondrial fission. Further, insulin increases ceramide accrual in a time-dependent manner, which is necessary for insulin-induced alterations in heart mitochondrial respiration and insulin resistance.

Conclusions

Collectively, these observations have two implications. First, they indicate a pathological role of insulin in heart complications stemming from mitochondrial disruption. Second, they identify ceramide as a possible mediator of insulin-related heart disorders.

Background

We have known for decades that type 2 diabetes mellitus (T2DM) increases the risk of heart disease [1]. Indeed, the observation is so common that this phenomenon is referred to as “diabetic heart disease”, bringing attention to the fact that cardiovascular complications are the most common cause of mortality in those suffering with T2DM [2,3]. Considering the increasing incidence of T2DM worldwide [4], and the remarkable number of undiagnosed cases, at least in early stages [5], understanding the nature of the relationship between these two pathologies may prevent heart disease and prolong healthy living among those with T2DM.

Reflective of the prevailing understanding of the etiology of T2DM, a great deal of research efforts have focused on glucose and glycemic control as the causal factors between T2DM and heart disease [6–10]. This focus has elucidated several glucose-related mechanisms, such as the reduction of glucose to sorbitol [11], and especially, the formation of advanced glycation end-products (AGE) and activation of its receptor (RAGE) [12–14]. Moreover, whether a consequence of RAGE activation or a distinct mechanism, hyperglycemia is known to induce inflammation [15]. Similarly, poor glycemic control may disrupt mitochondrial function and increase production of reactive oxygen species [16].

However, while the focus on glucose as a mediating mechanism linking T2DM to cardiovascular complications has yielded valuable insight, it nevertheless ignores what may be at least an equally relevant etiological factor of T2DM etiology—insulin. Pories and Dohm

recently posited that excess insulin, not glucose, is the essential factor in T2DM onset [17], a position supported by considerable evidence [18]. T2DM is a progressive spectrum of insulin resistance, with overt T2DM representing a state where insulin secretion, despite being elevated, is no longer sufficient to control blood glucose. As some have recommended a paradigm shift from looking at diabetes as a consequence of hyperinsulinemia rather than hyperglycemia, we are prompted to explore the causal relationship between T2DM and heart disease in a similar light.

Previous reports have observed a role for insulin in the etiology of cardiovascular complications [19]. Importantly, insulin therapy, despite adequately controlling blood glucose, has been shown to increase mortality in T2DM [20]. Similar to glucose-induced mechanisms (e.g., AGE formation, etc.), insulin has distinct downstream mediators; one mediator may be the sphingolipid ceramide. Ceramides are increasingly recognized as an injurious mediator of heart pathologies [21–25] and we have recently found that insulin increases ceramide biosynthesis and accrual in skeletal muscle [26, 27]. In light of the evidence suggesting a role for insulin in the etiology of heart complications, the purpose of these experiments was to determine the effect of insulin on heart ceramides, as well as possible ceramide-induced alterations in mitochondrial function.

Methods

Cell Culture

H9c2 cardiomyocytes were maintained in DMEM +10 % FBS. For differentiation into myotubes, cells were grown to confluency and the medium was replaced with DMEM +10 % horse serum (Invitrogen, Grand Island, NY). Myotubes were used for experiments on day 3 of differentiation. Cells were treated with insulin (50 nM; Actrapid; Novo Nordisk, Plainsboro,

NJ) and myriocin (10 μ M; Sigma), an inhibitor of serine palmitoyltransferase, at the times indicated.

Animals

Sixteen-week-old male C57Bl/6 mice were separated into one of four groups (six per group) to receive morning injections of saline (PBS), insulin (daily; 0.75 U/kg/BW; Actrapid; Novo Nordisk, Plainsboro, NJ), myriocin (thrice weekly; 0.3 mg/kg; Sigma) or both for 28 days with free access to water and chow (Harlan 8604) throughout the length of the study. After the 28-d treatment, mice underwent intraperitoneal glucose (G7021; Sigma-Aldrich, St. Louis, MO) and insulin (Actrapid; Novo Nordisk, Plainsboro, NJ) tolerance tests. For both tests, mice were fasted for 6 h and received an injection of either glucose (1 g/kg body wt) or insulin (0.75 U/kg body wt). These are doses that are above the typical rate of insulin treatment in type 2 diabetics (0.5 U/kg) [28]. Plasma glucose (Bayer Contour glucose meter), insulin (ELISA; Crystal Chem Inc.), and adiponectin (Crystal Chem Inc.) levels were determined. Studies were conducted in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the IACUC (Institutional Animal Care and Use Committee) at Brigham Young University.

Lipid Isolation Analysis

Lipids were extracted and quantified as described previously [29]. Briefly, lipids were isolated with chloroform–methanol (1:2), and after addition of water, the organic phase was collected and dried. After resuspension, lipids were quantified using a shotgun lipidomics technique on a Thermo Scientific LTQ Orbitrap XL mass spectrometer.

Protein Analysis

Cell and tissue proteins were analyzed via western blot as described previously [29].

Cell and Myocardium Permeabilization

For cells, H9c2 cardiomyocytes were detached in culture dishes with 0.05 % trypsin–EDTA (Sigma) and growth medium was added to the culture. Contents were transferred to a tube and centrifuged for 10 min at 1000×g at RT. After removal of supernatant, cells were lifted in MiR05 [0.5 mM EGTA, 3 mM MgCl₂, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM sucrose, and g/l BSA (Sigma; A3803) adjusted to pH 7.1] plus 1 mg/ml digitonin and gently rocked at RT for 5 min before centrifugation at 1000×g for 5 min. After discarding supernatant, cells were then suspended in 2.2 ml warm MiR05 and transferred to chambers in the O2K (Oroboros Instruments, Innsbruck, Austria). Following respiration protocol (outlined below), cells were removed from the chambers and used for protein quantification. For myocardial mitochondrial respiration, left ventricle was quickly removed from euthanized mice and immediately placed in ice-cold buffer X (60 mM K-MES, 35 mM KCl, 7.23 mM K₂EGTA, 2.77 mM CaK₂EGTA, 20 mM imidazole, 20 mM taurine, 5.7 mM ATP, 15 mM PCr, 6.56 mM MgCl₂–6H₂O, pH 7.1) and trimmed of connective tissue. Small fiber bundles were prepared and gently separated along their longitudinal axis under a surgical scope (Olympus, ST) to 1–2 mg. Bundles were then transferred to a tube with chilled buffer X and 50 µg/ml saponin and rocked at 4 °C for 30 min, then washed in buffer Z (105 mM K-MES, 30 mM KCl, 10 mM KH₂PO₄, 5 mM MgCl₂–6H₂O, 0.5 mg/ml BSA, pH 7.1) at 4 °C for at least 15 min. Samples were then blotted dry and weighed.

Mitochondrial Respiration Protocol

High-resolution O₂ consumption was determined at 37 °C in permeabilized cells and

fiber bundles using the Oroboros O2 K Oxygraph with MiR05 respiration buffer. Before addition of sample into respiration chambers, a baseline respiration rate was determined. After addition of sample, the chambers were hyperoxygenated to ~300 nmol/ml. Following this, respiration was determined by all or parts of the following substrate-uncoupler-inhibitor-titration (SUIT) protocol: electron flow through complex I was supported by glutamate + malate (10 and 2 mM, respectively) to determine leak oxygen consumption (GM_L). Following stabilization, ADP (2.5 mM) was added to determine oxidative phosphorylation capacity (GM_D). Succinate was added (GMS_D) for complex I + II electron flow into the Q-junction. To determine full electron transport system capacity in cells over oxidative phosphorylation, the chemical uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was added (0.05 μ M, followed by 0.025 μ M steps until maximal O_2 flux was reached). Mitochondrial membrane integrity was tested in all experiments by adding cytochrome *c* (not shown; 10 μ M). Lastly, residual oxygen consumption was measured by adding antimycin A (2.5 μ M) to block complex III action, effectively stopping any electron flow, which provides a baseline rate of respiration.

Statistics

Data are presented as the mean \pm SEM. Data were compared by ANOVA with Tukey's post hoc analysis (Graphpad Prism; La Jolla, CA). Significance was set at $p < 0.05$.

Results

Insulin Increases Cardiomyocyte Ceramide Accrual, Which is Necessary for Mitochondrial Disruption

We observed a significant time-dependent increase in ceramide accrual in cardiomyocytes with insulin treatment (Fig. 2.1a), which was supported with an increase in

heart levels of serine palmitoyltransferase 2 (SPT2) and dihydroceramides desaturase 1 (Des1) with insulin treatment (Fig. 2.1b) at 24 h. Whereas 1 h of insulin had no effect on ceramides, 6 h of treatment roughly doubled ceramide levels, a level maintained, but not significantly increased, at 12 and 24 h. With previous mitochondrial-specific effects of ceramides in mind [23, 25], we next determined whether this ceramide accrual had any effect on cardiomyocyte mitochondrial bioenergetics. Insulin altered mitochondrial respiration in a contrasting and time-dependent manner. At 1 h of insulin treatment, respiration was increased in cardiomyocytes, but significantly decreased at 6 and 24 h (Fig. 2.2a). Insulin-induced alterations in respiration were also evident in the reduced respiratory control ratio, an overall indication of mitochondrial health [30], at longer time points (Fig. 2.2b). Moreover, distinct function of complex II-mediated respiration, defined as the CII factor, revealed acutely increased (at 1 h) then decreased (at 6 and 24 h) respiration rates (Fig. 2.2c). Overall, uncoupling control ratio, calculated as maximal uncoupled respiration by FCCP relative to ADP-stimulated state, was comparable among all conditions (Fig. 2.2d). Importantly, inhibition of ceramide accrual with myriocin abolished the insulin-induced decrement in respiration (Fig. 2.2a–c).

Additionally, we found that insulin altered mitochondrial morphology, appearing to increase mitochondrial fission (Fig. 2.3a), which was prevented with ceramide inhibition. Drp1 levels were similar among all treatments (Fig. 2.3b). Further, 1 h of insulin treatment was associated with an increase in mitochondrial complex III levels, but this was lost with 24 h (Fig. 2.3c).

Insulin Treatment Increases Body Mass and Causes Hyperinsulinemia and Insulin Resistance in Mice

To determine whether an in vivo correlate exists to substantiate our in vitro findings, we injected adult male mice (16 week old) with insulin daily (0.75 mg/kg) for 28 days. At the

conclusion of the 28 days treatment period, mice injected with insulin (INS) gained significantly more body mass than PBS-injected mice (Fig. 2.4a); however, those injected with insulin (daily) and myriocin (thrice weekly; INS + MYR) did not gain such mass. Moreover, those injected with myriocin alone (MYR) weighed less than PBS-injected mice. While heart mass tended to increase with INS injection (Fig. 4b; $P = 0.072$), the change was not significant, and was even less remarkable when controlled for by body mass (Fig. 2.4c). INS-injected mice also had higher fasting insulin (Fig. 2.5a), but not glucose (Fig. 2.5b) over the course of the treatment. Further, INS treatment caused compromised glucose and insulin tolerance (Fig. 2.5a, b, respectively), but not with ceramide inhibition.

Insulin Treatment in Mice Increases Muscle Ceramides and Alters Mitochondrial Bioenergetics and Morphology

28 days of insulin elicited a roughly two-fold increase in myocardial ceramide content (Fig. 2.6a), though myriocin co-treatment prevented this effect. This effect was supported with an increase in heart SPT2 levels (Fig. 2.6b). Moreover, blood adiponectin was robustly inhibited with INS and moderately protected with MYR injections (Fig. 2.6c). Functionally, the increased ceramide accrual had a demonstrable and deleterious effect on myocardial mitochondrial respiration. In particular, overall respiration and RCR was reduced with INS treatment (Fig. 2.7a, b), though CII factor was not significantly changed (Fig. 2.7c). Lastly, we found that myocardial mitochondria were smaller with INS treatment compared with all other conditions (Fig. 2.8a, b), though this was not reflected in any change in levels of mitochondrial complex proteins (Fig. 2.8c). This effect may be a result of INS-induced increased Drp1-levels

in the heart (Fig. 2.8d).

Discussion

Type 2 diabetes carries an increased risk of developing a surprising and increasing number of pathologies. Multiple lines of evidence reveal its hand in diseases stemming from cognitive [31], reproductive [32], musculoskeletal [33], and cardiovascular disorders [34]. However, type 2 diabetes is typified by two key characteristics—hyperglycemia and hyperinsulinemia [35, 36]—and while the disease has historically been defined by blood glucose levels, insulin may be a more sensitive and relevant diagnostic [37]. Indeed, a very recent study found that higher insulin exposure in type 2 diabetics is associated with a threefold increase in cardiovascular events [38]. Herein, we demonstrate that chronic insulin injections exert a time-specific and ceramide-dependent effect on cardiometabolic function, including insulin resistance and heart mitochondrial changes.

These studies provide additional insight into the etiology of type 2 diabetes-related heart complications. In particular, these results suggest that insulin is an important pathogenic mediator and highlight the need to regularly measure insulin when evaluating heart disease risk. Our findings of insulin impacting mitochondrial physiology are not new—Parra et al. [39] found that insulin increased mitochondrial respiration. However, while we tended to see an overall dampening effect of insulin on mitochondrial respiration, a notable difference between our studies is the length of time; this previous report used a 3-h incubation, while we used several time points in our in vitro model. Indeed, our data corroborate those of Parra et al. [39]

when we analyzed mitochondrial respiration at 1 h, but not at periods over 6 h. Combined with our observations following a 4-week insulin treatment in mice, these data collectively suggest the clinical relevance of prolonged increases in insulin.

In mice, we found that prolonged insulin treatment resulted in reduced glucose and insulin tolerance, suggesting that insulin alone, independent of other variables, is capable of inducing insulin resistance. This observation corroborates evidence from several previous reports in humans and rodents wherein hyperinsulinemia from endogenous (e.g., insulinoma) [40] and exogenous (e.g., injections) [26, 41, 42] sources causes insulin resistance. This insulin-desensitizing effect of prolonged hyperinsulinemia is likely at least partially mediated via ceramide accrual [26]. While it is possible that the insulin-resistant state caused by the insulin treatment in our study exerts some confounding effect on altering heart mitochondrial function independent of insulin-induced heart ceramide accrual, we nonetheless consider this an apparent feature of the prolonged hyperinsulinemia. Nevertheless, insulin resistance per se, in the absence of the often-accompanying hyperinsulinemia, may be the responsible lesion.

In light of the observations by Dohm and Pories [17], who implicate hyperinsulinemia in the etiology of T2DM, we submit an alternative hypothesis as to the origins of diabetic heart disease that should be considered. As opposed to heart disease being a consequence of the potentially harmful milieu associated with T2DM, perhaps heart disease and T2DM are each consequences of one pathology—hyperinsulinemia. Such a theory is supported by multiple reports that implicate insulin alone in the etiology of both heart disease [43–45] and T2DM [46, 47]. Over two decades ago, Haffner et al. [48] wondered about the role of insulin and queried whether heart disease started before diabetes onset, when insulin, but not glucose, is elevated. Their results add to the body of evidence that insulin is an important etiological factor

in heart disease. A significant strength of measuring insulin is that it is elevated earlier than glucose in the progression to frank T2DM [46, 47] allowing not only an earlier diagnosis, but also an earlier, and thus more effective, intervention. Collectively, these observations emphasize the need to measure insulin in routine health screenings.

Altogether, these results highlight the pathogenicity of hyperinsulinemia on cardiometabolic function, including insulin resistance and heart mitochondria. These findings are corroborated by recent work by Marciniak et al. [49] who found reduced cardiac mitochondrial function in a mouse model of type 2 diabetes, with concomitant hyperinsulinemia. Interestingly, cardiac mitochondrial function was largely unaffected in the streptozotocin-induced model of type 1 diabetes, which strengthens the insulin-centric paradigm of altered cardiometabolic health with type 2 diabetes. Another finding from Marciniak et al. [49] was that adiponectin was reduced in their model of type 2 diabetes, but not type 1, which is a common finding in humans [35]. Considering the actions of adiponectin signaling on ceramide metabolism [50] and cardiovascular function [51], the reduced adiponectin that accompanies most insulin-resistant conditions may provide additional explanation into the increased heart ceramide accrual and reduced adiponectin we observed in our model of directly induced hyperinsulinemia [52].

The purpose of these studies was to explore the effect of insulin in altering cardiometabolic function, with a focus on two main components: insulin resistance and heart mitochondrial dynamics and physiology. However, while our findings shed light on the role of insulin in cardiometabolic pathologies, they nevertheless fall short of allowing firm conclusions concerning cardiovascular health. Thus, a significant weakness that will need to be addressed in

future studies is the lack of analyses to determine a *functional* impairment with the heart in this same context.

Conclusions

Our data suggest two potential therapeutic strategies for mitigating the heart disease burden associated with states of elevated insulin (e.g., pre-diabetes or T2DM). First, drugs to induce insulin sensitization (e.g., metformin) should take priority over drugs that induce insulin secretion (e.g., sulfonylurea), which is associated with a reduction [53] and increase [54–56] in heart disease risk, respectively. Second, ceramide inhibition may prove to be an effective deterrent to heart disease risk in various conditions, including hyperinsulinemia, as mounting evidence suggests inhibition of ceramide biosynthesis is effective at protecting cardiovascular health [57–59].

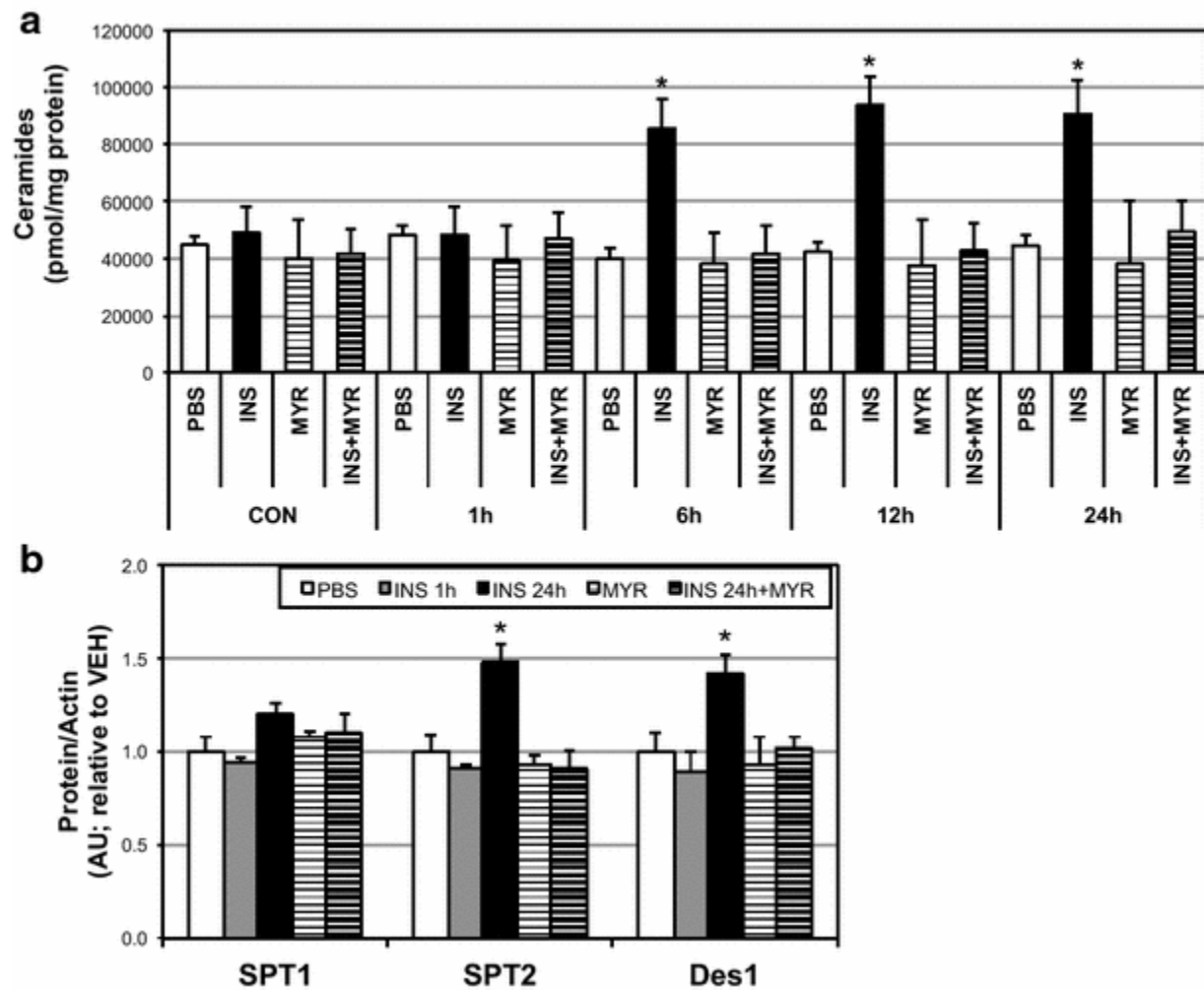


Figure 2.1: Insulin Increases Ceramide in Cardiomyocytes. H9C2 cardiomyocytes were treated with insulin (INS; 50 nM) with or without myriocin (MYR; 10 μ M), an inhibitor of ceramide biosynthesis, for the times indicated (n = 6). Following treatment time, lipids were isolated for analysis of sphingolipids via LCMS (a; n = 6) and protein levels of ceramide biosynthetic enzymes determined (b; n = 4).

*P < 0.05 for INS vs. other treatments.

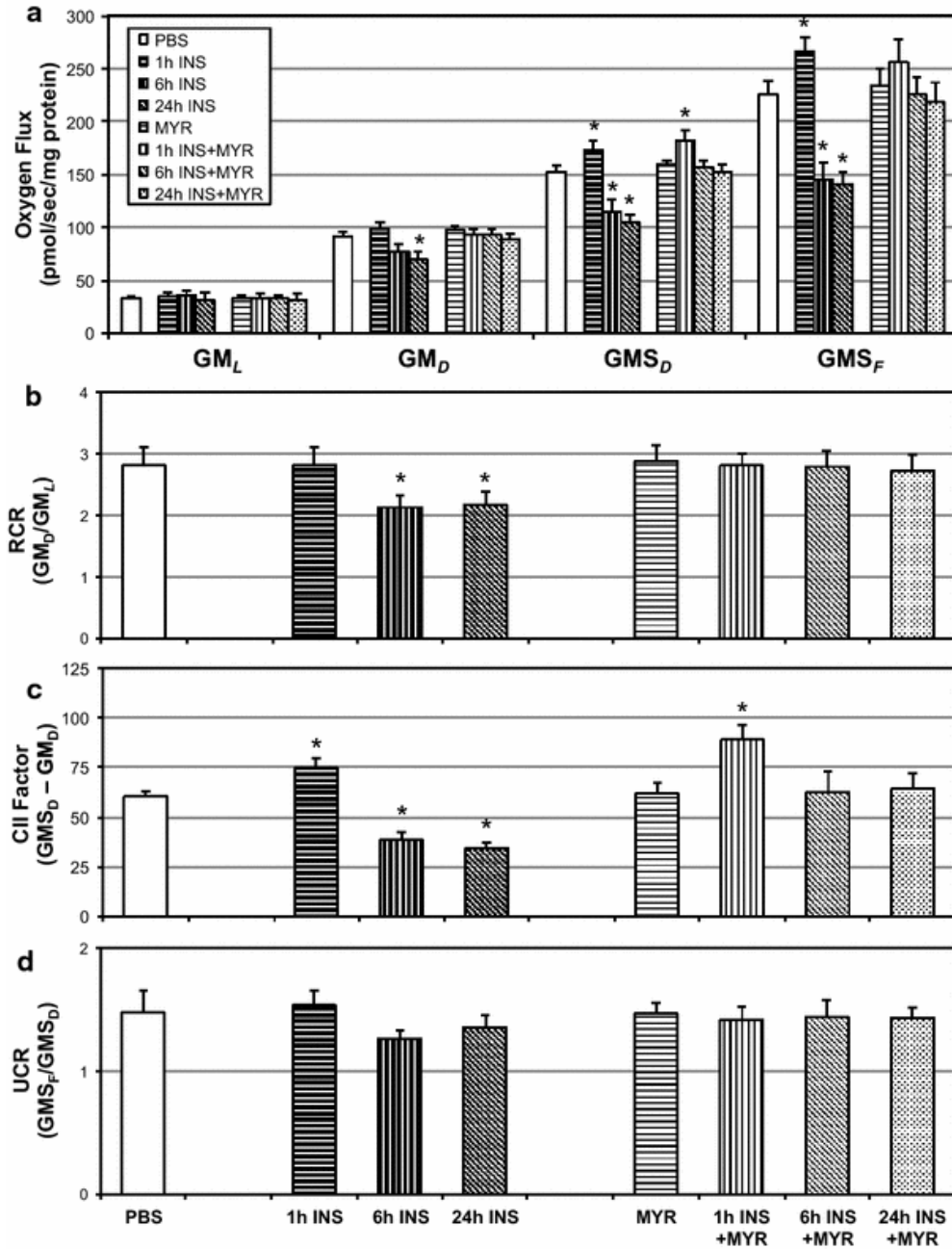


Figure 2.2: Ceramide Inhibition Prevents Insulin-Induced Mitochondrial Disruption. H9C2 cardiomyocytes were treated with insulin (INS; 50 nM) with or without myriocin (MYR; 10 μ M), an inhibitor of ceramide biosynthesis, for the times indicated (n = 6). To measure mitochondrial respiration (a), cells were treated with: GM_L , Glutamate (10 mM) + Malate (2 mM); GM_D , + ADP (2.5 mM); GMS_D , + Succinate (10 mM); GMS_F , + FCCP (0.05 μ M). Respiratory control ratio (RCR; b), Complex II Factor (c), and uncoupling control ratio (UCR; d) were determined by the analysis indicated. *P < 0.05 for condition vs. control (PBS).

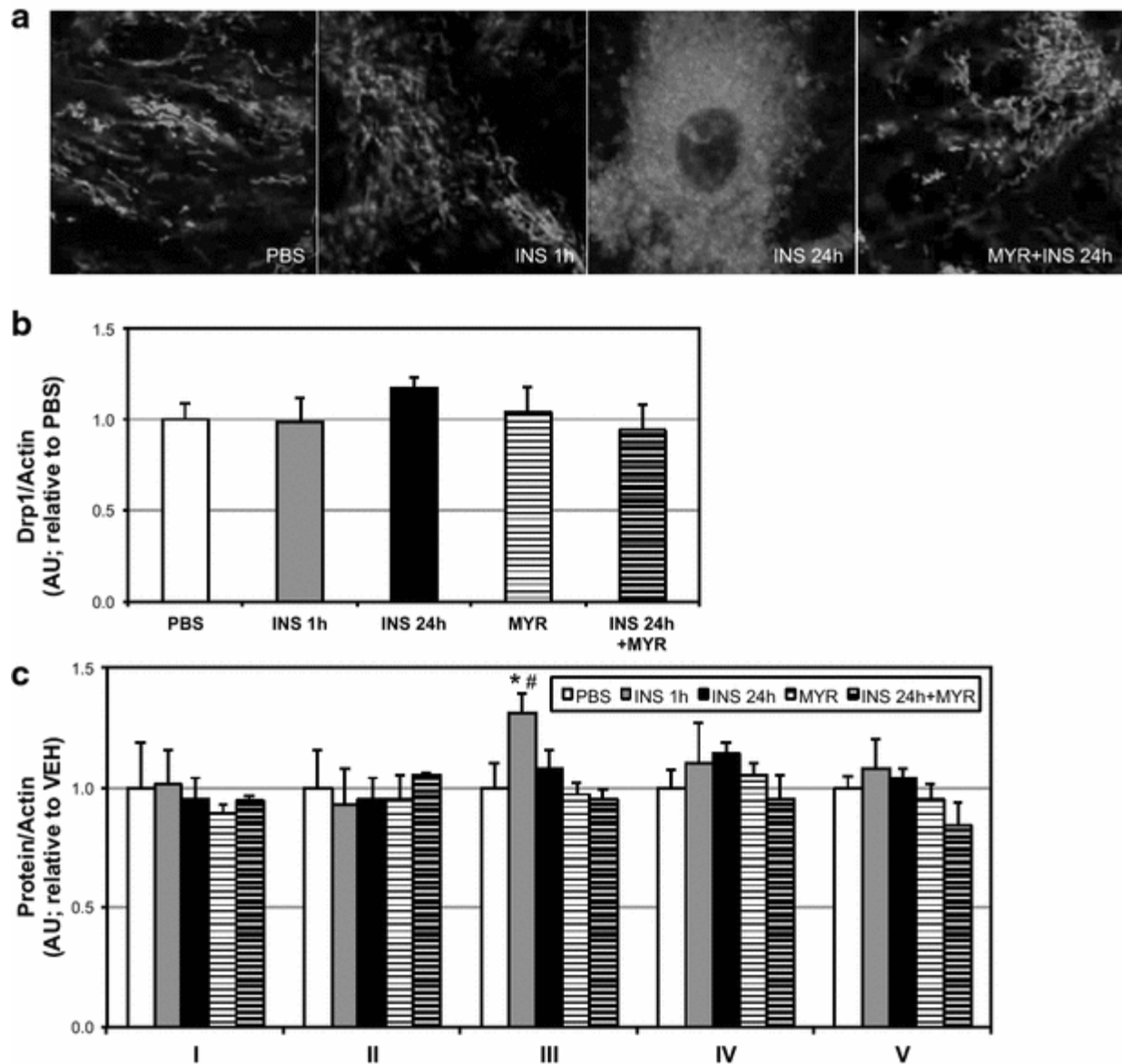


Figure 2.3: Insulin Treatment Affects Cardiomyocyte Mitochondrial Physiology. H9C2 cardiomyocytes were treated with insulin (INS; 50 nM) with or without myriocin (MYR; 10 μ M), an inhibitor of ceramide biosynthesis, for the times indicated. Following treatment, cells were imaged to determine mitochondrial morphology (a; $n = 3$), and analyzed for Drp1 protein levels (b; $n = 4$), and mitochondrial complex proteins (c; $n = 4$). * $P < 0.05$ for INS vs. PBS; # $P < 0.05$ for INS + MYR vs. INS alone.

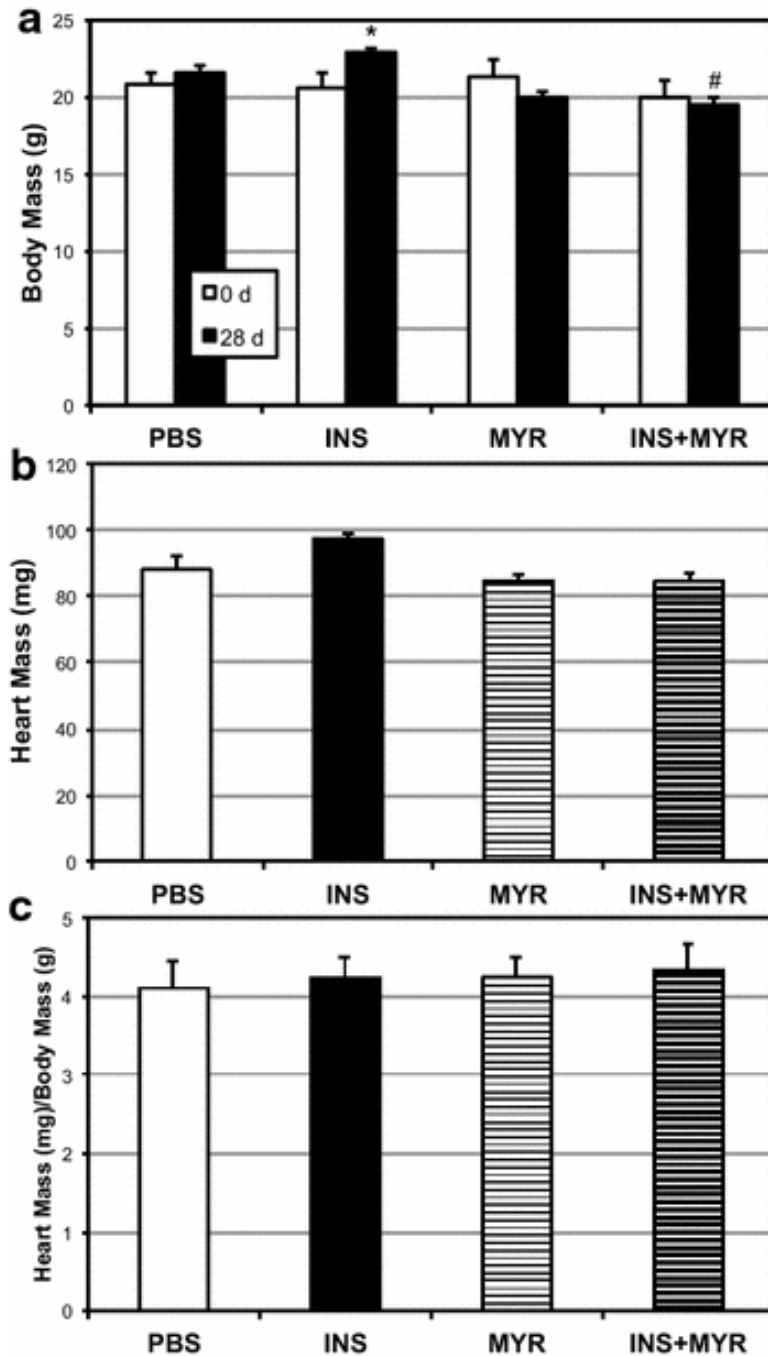


Figure 2.4: Insulin Injections Increase Body Mass, but not Heart Mass in Mice. 16-week-old male mice received injections of PBS (daily), insulin (INS; daily; 0.75 mg/kg), myriocin (MYR, thrice weekly; 3 mg/kg), or INS + MYR for 28 d. Body mass increased in the INS-treated mice only (a; $n = 6$). Heart mass was measured in all mice (b, c; $n = 6$). * $P < 0.05$ for INS vs. PBS; # $P < 0.05$ for INS + MYR vs. INS alone.

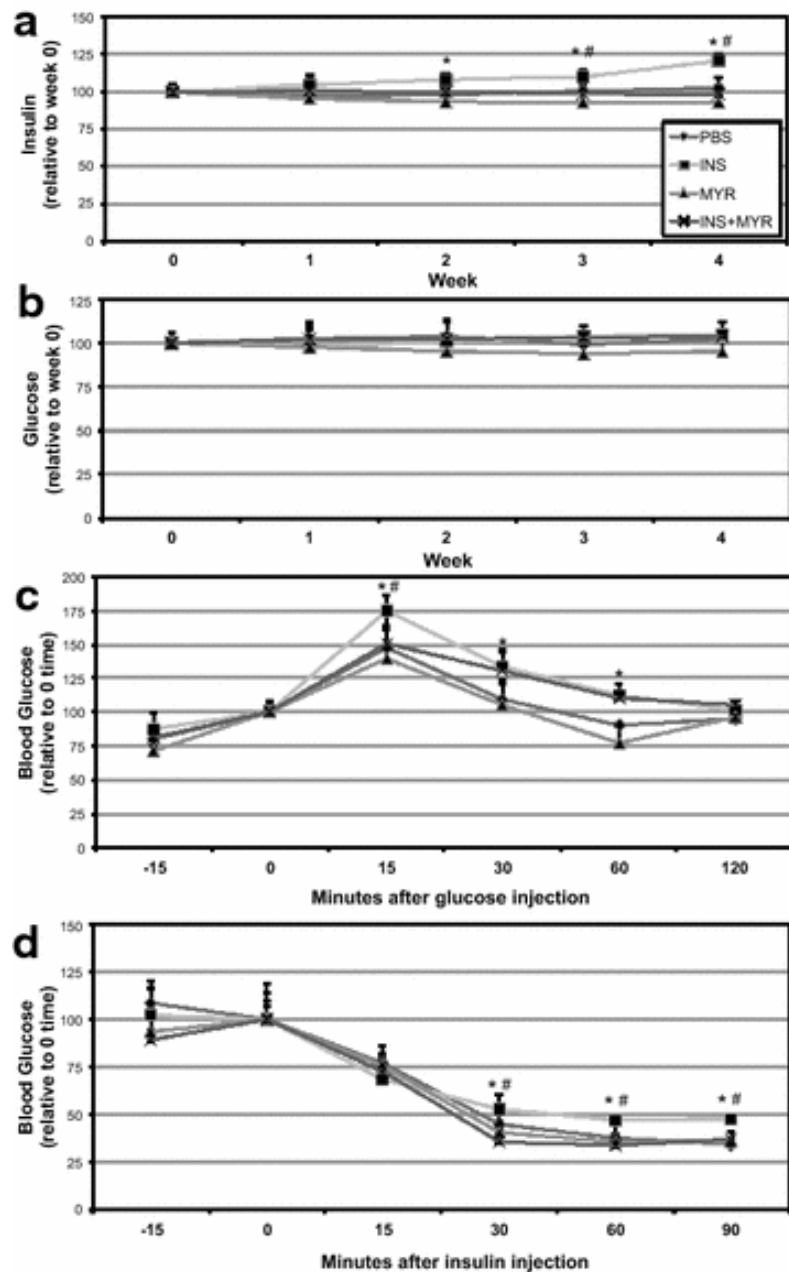


Figure 2.5: Chronic Insulin Injections Increase Blood Insulin and Induce Glucose and Insulin Intolerance. 16-week-old male mice received injections of PBS (daily), insulin (INS; daily; 0.75 mg/kg), myriocin (MYR, thrice weekly; 3 mg/kg), or INS + MYR. Blood insulin (a) and glucose (b) was tracked weekly. At the conclusion of the study, IP glucose (c) and insulin (d) tolerance tests were performed. * $P < 0.05$ for INS vs. PBS; # $P < 0.05$ for INS + MYR vs. INS alone.

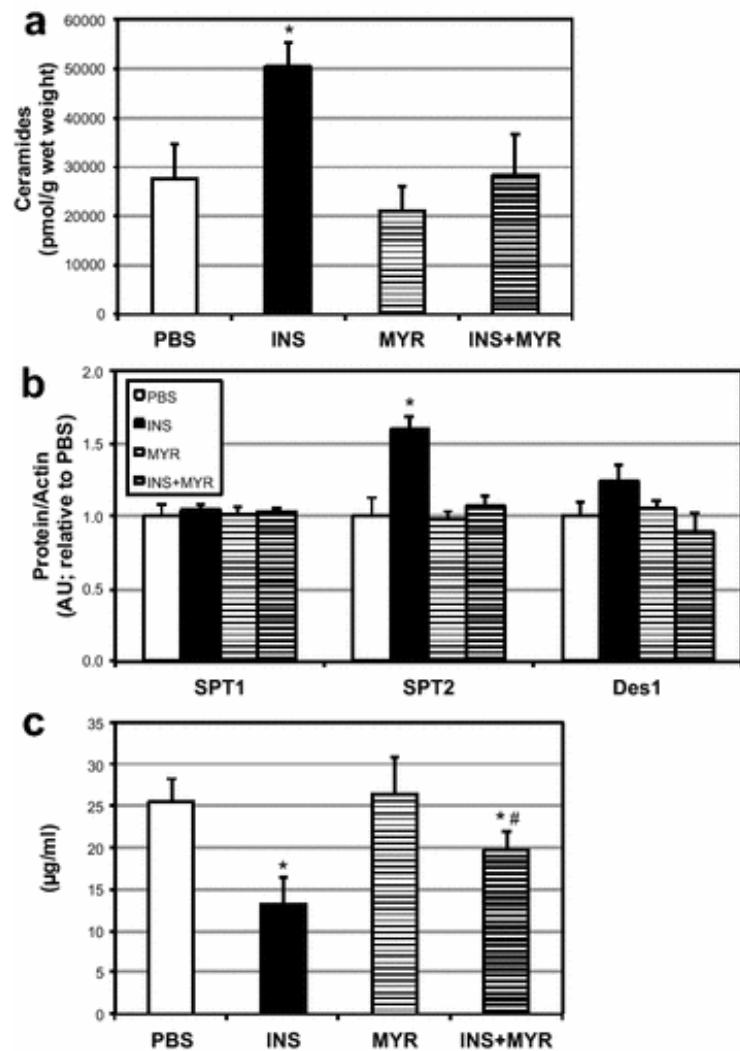


Figure 2.6: Insulin Injection Increases Heart Ceramides. 16-week-old male mice received injections of PBS (daily), insulin (INS; daily; 0.75 mg/kg), myriocin (MYR, thrice weekly; 3 mg/kg), or INS + MYR. INS treatment increased myocardial ceramide accrual (a) and SPT2 (b). Serum adiponectin was also measured (c). *P < 0.05 for condition vs. PBS. #P < 0.05 for INS + MYR vs. INS alone.

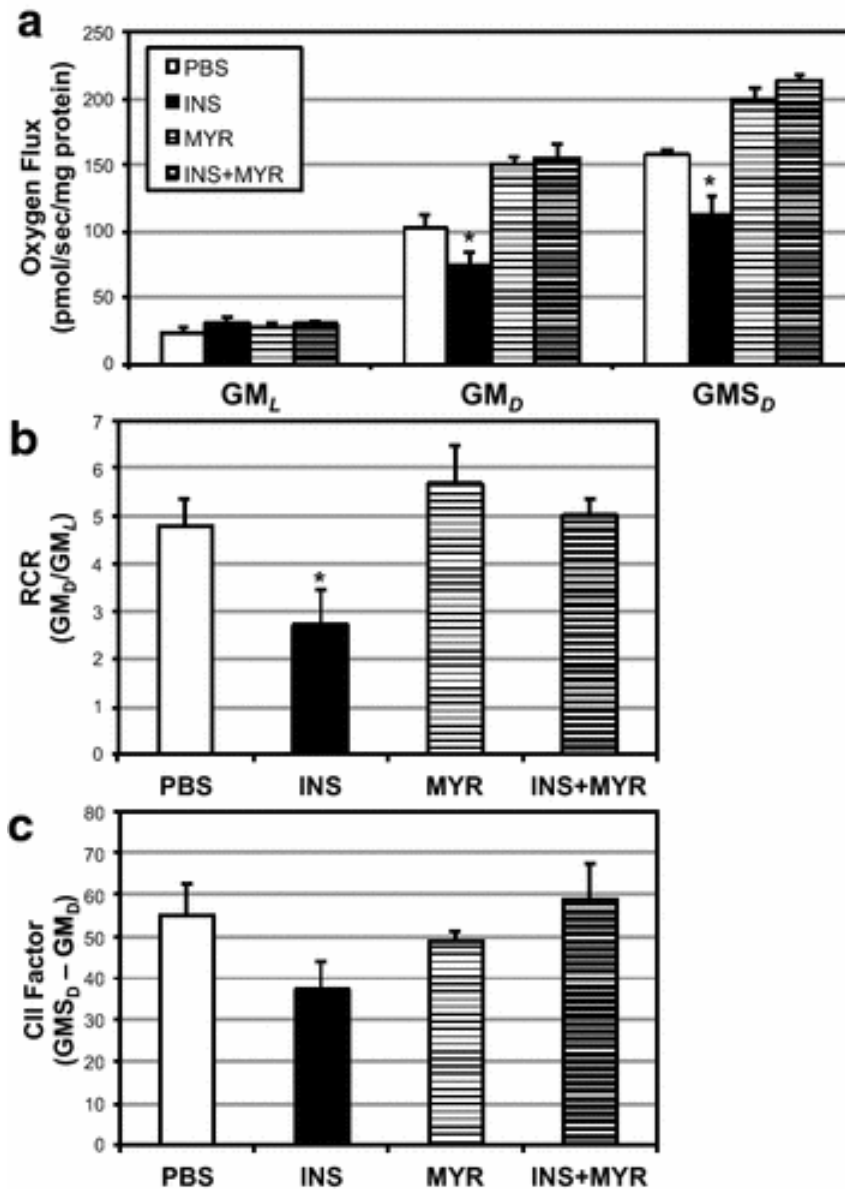


Figure 2.7: Chronic Insulin Injections Disrupt Mitochondrial Function. 16-week-old male mice received injections of PBS (daily), insulin (INS; daily; 0.75 mg/kg), myriocin (MYR, thrice weekly; 3 mg/kg), or INS + MYR. Mitochondrial assessments were determined in permeabilized (saponin, 50 μ g/ml) myocardium. To measure mitochondrial respiration (a), samples were treated with: GM_L, Glutamate (10 mM) + Malate (2 mM); GM_D, + ADP (2.5 mM); GMS_D, + Succinate (10 mM). Respiratory control ratio (RCR; b) and Complex II Factor (c) were determined by the analysis indicated. *P < 0.05 for condition vs. PBS.

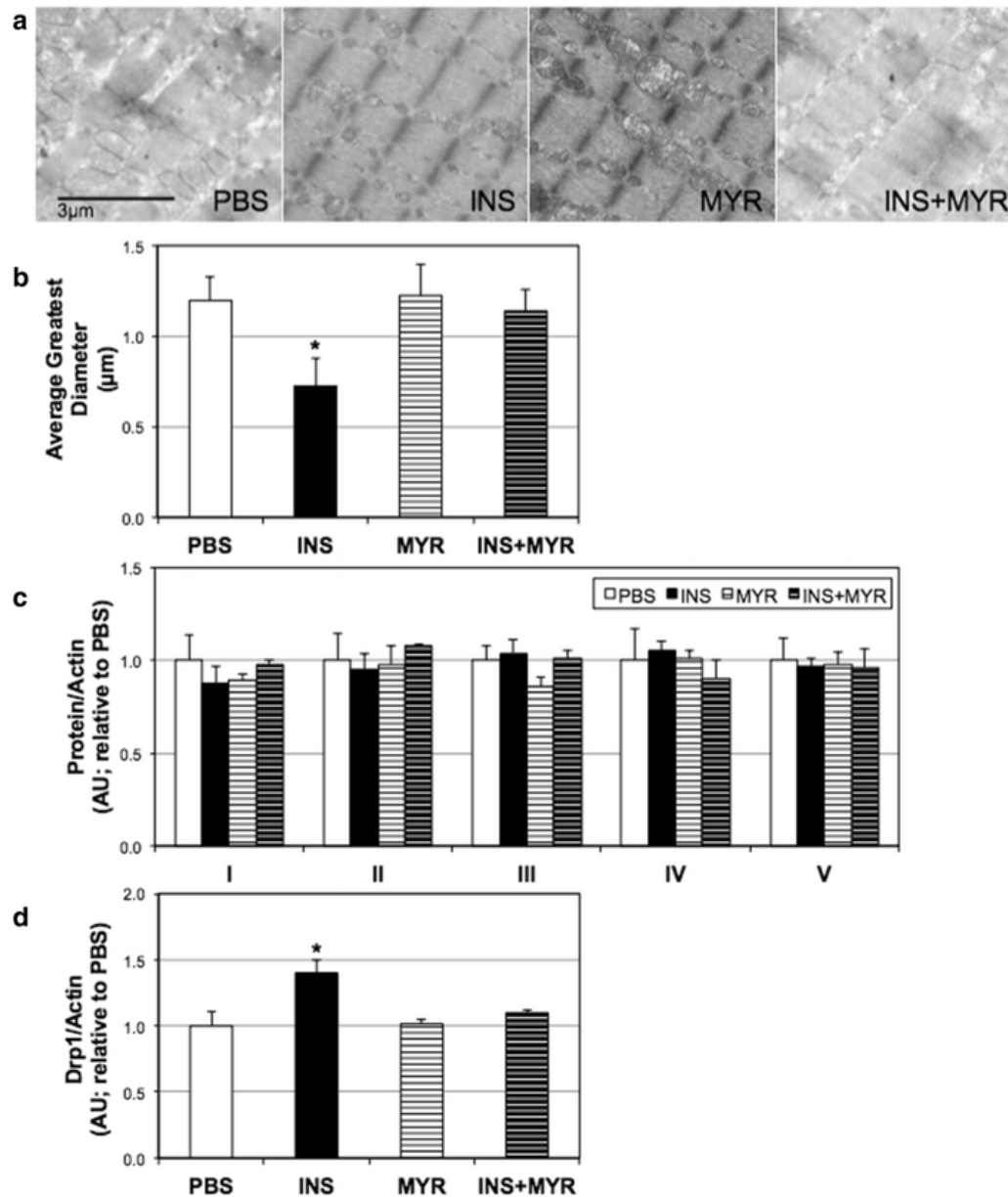


Figure 2.8: Chronic Insulin Injections Disrupt Myocardial Mitochondrial Function. 16-week-old male mice received injections of PBS (daily), insulin (INS; daily; 0.75 mg/kg), myriocin (MYR, thrice weekly; 3 mg/kg), or INS + MYR. Heart samples were processed for imaging via electron microscopy (a) and quantified based on average greatest mitochondrial diameter (b; $n = 3$). A portion of samples was used to probe for mitochondrial complexes (c; $n = 3$) and Drp1 (D; $n = 3$). * $P < 0.05$ for condition vs. PBS.

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CHAPTER 3: General Discussion and Future Directions

Our data from the preceding chapter enhances the findings of previous studies implicating ceramide as an intermediate between hyperinsulinemia and cardiovascular disease. It builds on the earlier study done by our lab showing ceramide accrual in cardiomyocytes contributes to a decrease in mitochondrial respiration and increase in mitochondrial fission, both of which indicate mitochondrial dysfunction (Smith, et al., 2013). Mitochondrial dysfunction has been linked to cardiac dysfunction (Lesnefsky, et al., 2001). Indeed, the highly oxidative character of cardiomyocytes makes mitochondrial function essential for normally functioning cardiomyocytes. In chapter 2 we found that insulin leads to an increase in mitochondrial dysfunction and ceramide is necessary for those observed detrimental effects. Taken in context with these previous studies this provides strong evidence of a link between high insulin levels and cardiac dysfunction.

The first point examined by this study was confirming that high insulin levels will increase ceramide accrual in cardiac tissue. Investigations of insulin and ceramide interactions have become increasingly popular over recent years. However, ceramides have proved somewhat difficult to investigate as there are no membrane receptors for ceramides and ceramides with long chains of fatty acids cannot pass through the cell plasma membrane. This leaves two possible means of study, stimulate cells to increase intracellular content or use short chain ceramides that can diffuse across membranes (Górski, 2012). Intracellular ceramide content can be manipulated by affecting the physiological conditions which regulate amounts of *de novo* synthesis. For example, increased obesity, cigarette smoke, and gene manipulation have all been used to stimulate ceramide accumulation (Alayoubi, et al., 2013; Haus, et al., 2009; Nelson, et al., 2015b; Mikayla O. Thatcher, et al., 2014). Our lab has previously shown that

high insulin levels also cause ceramide accrual in skeletal muscle (Hansen, et al., 2014). In the current study we expanded on this finding by using insulin to increase ceramide accrual in the cardiomyocytes.

We then explored how ceramide accumulation impacted mitochondrial function in the cardiomyocytes. It has previously found that ceramide negatively influences mitochondrial function in a wide variety of cells (Smith, et al., 2013; M. O. Thatcher, et al., 2014; Tippetts, et al., 2014; Wang, et al., 2009). In this study we found that increased insulin levels lead to mitochondrial dysfunction and that ceramide is necessary for this to occur. Prior findings showed an increase, rather than a decrease, in mitochondrial respiration after insulin treatment (Parra, et al., 2014). At first this finding may seem to be at odds with our data; however, the Parra et. al. study used a 3-hour incubation period. In our study we used several different time periods: 1-hour, 6-hour, and 24-hour treatments. The 1-hour study showed an increase in respiration rates, while longer incubation periods resulted in a decrease in mitochondrial respiration.

As mitochondrial function has previously been shown to be linked to mitochondrial morphology (Westermann, 2012) we also looked at insulin effects on mitochondrial morphology. Our findings suggest an increase in mitochondrial fission following insulin treatment, results which can be reversed through ceramide inhibition. Mitochondrial fission and decreased mitochondrial function have been linked to increased risk of heart failure (Chen & Knowlton, 2011; Knowlton, Chen, & Malik, 2014; Marín-García, Akhmedov, & Moe, 2012; Ong & Hausenloy, 2010). While our current study did not measure heart failure risk, it is reasonable to hypothesize the observed changes in mitochondrial function and morphology would have detrimental effects on cardiac health. Future research into the exact effects of high

insulin and consequent mitochondrial morphology changes on cardiac function would be important in better understanding the physiological effects of high insulin.

Historically T2DM has been mainly defined through blood glucose levels. This viewpoint has provided important information relative to the etiology of T2DM (Matheus, et al., 2013; Nelson, et al., 2015a; Schmidt, et al., 1999). The results from this study suggest that insulin, as well as glucose, is an important pathogenic mediator in T2DM. We found that chronic insulin injections exert a time-specific effect on insulin resistance and glucose tolerance, and this effect is dependent on ceramide. These results indicate that insulin alone is able to induce insulin resistance. Insulin induced insulin resistance has been found previously as well (Hansen, et al., 2014; Prato, et al.). Our results corroborate the findings in these previous studies. We also found the insulin resistance development could be mitigated through ceramide inhibition. This implicates ceramide accrual as an intermediate in the development of insulin resistance from increased insulin levels.

Taken together these findings point towards the possibility that heart disease may not be a side effect of the pathological effects of T2DM. Instead, it seems possible both T2DM and heart disease are developed concurrently due to the effects of hyperinsulinemia developed in those who are insulin resistant. It also indicates a potential reason why insulin injections lead to increased mortality in T2DM through cardiac dysfunction. These results suggest increased investigation is needed to better characterize the relationship between high insulin levels, ceramide accrual, and cardiac dysfunction. Such studies may help lead to better treatment options for T2DM.

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CURRICULUM VITAE

Aimee Elizabeth Hodson

2041 Windsor Oak Cove
Sandy, Utah 84092
(801) 918-7072
aimhod@gmail.com

Education

M.S., Physiology and Developmental Biology
Brigham Young University, Provo, Utah 84602

Expected June 2016

Thesis: Insulin Treatment Increases Myocardial Ceramide Accumulation and Disrupts
Cardiometabolic Function
Advisor: Dr. Benjamin T. Bikman

B.S., Physiology and Developmental Biology
Department of Physiology and Developmental Biology
Brigham Young University, Provo, Utah 84602

April 2015

Awards

Graduate Studies School Employment Scholarship
Undergraduate Academic Scholarship

August 2015 – June 2016
August 2010 – April 2015

Professional Experience

Graduate Research Assistant
Mentor: Dr. Benjamin T. Bikman
Brigham Young University, Provo, Utah 84602

January 2016 – Present

Undergraduate Mentored Research
Mentor: Dr. Benjamin T. Bikman
Brigham Young University, Provo, Utah 84602

October 2011 – April 2015

Responsibilities:

- Conduct hypothesis-based research on ceramide in the context of mitochondrial respiratory capacity in cardiomyocytes
- Maintain and operate laboratory research equipment
- Assist, mentor, and train undergraduate research assistants in laboratory protocols and procedures

Endocrinology Teacher's Assistant
Brigham Young University, Provo, Utah 84602

January 2016 - Present

Responsibilities:

- Teach reviews and lectures to undergraduate students on Endocrinology
- Review and correct examinations and other works
- Provide assistance to the Endocrinology Professor, Dr. Thomson

MCAT Preparation Tutor
Altius Test Prep, 560 S. Orem, UT 84058

April 2014 - Present

Responsibilities

- Prepare and deliver classes in the subjects of Biology, Biochemistry, Chemistry, Organic Chemistry, Physics, Psychology, and Sociology to both individuals and groups
- Advise students on writing, study schedules, and applications for medical schools
- Recruit students and increase awareness of company

Service

Volunteer Spanish Interpreter
Central Utah Surgical Clinic

August 2014 – April 2015

- Translation between health care providers and patients receiving various surgical treatments
- Provide assistance to nurses and staff as needed

Missionary
The Church of Jesus Christ of Latter-day Saints
Malaga, Spain

June 2012 – December 2013

- Full time proselytizing missionary
- Taught weekly English classes
- Performed humanitarian work

Publications

Hodson, A. E., Tippetts, T. S., Bikman, B. T. (2015). Insulin Treatment Increases Myocardial Ceramide Accumulation and Disrupts Cardiometabolic Function. *Cardiovascular Dabetology*, 14, 153. doi: 10.1186/12933-015-0316-y

Erickson, K. A., Smith, M. E., Anthonymuthu, T. S., Evanson, M. J., Brassfield, E. S., Hodson, A. E., . . . Bikman, B. T. (2012). AICAR inhibits ceramide biosynthesis in skeletal muscle. *Diabetology and Metabolic Syndrome*, 4, 1. doi: 10.1186/1758-5996-4-45

Meeting Abstracts

Hodson, A. E., Tippetts, T. S., Bikman, B. T. (2015). Insulin Treatment Increases Myocardial Ceramide Accumulation and Disrupts Cardiometabolic Function. Experimental Biology Meeting, 2016

References

Dr. Benjamin T. Bikman, Ph.D.
Department of Physiology and Developmental Biology
Brigham Young University, 3017 LSB
Provo, UT 84602
(801) 422-1798
benjamin_bikman@byu.edu

Dr. Jonathan Wisco, Ph.D.
Department of Physiology and Developmental Biology
Brigham Young University, 2028 LSB
Provo, UT 84602
(801) 422-2402
jjwisco@byu.edu

Todd McPhie
Manager,
Altius Test Prep
560 S. State Street, Suite E2
Orem, UT 84058
(435) 671-5783
todd@altiuustestprep.com