The Influence of Exercise on Inflammation and IGF-1 Signaling in Diabetes

(Spine Title: Exercise, IGF-1 & Inflammation in Diabetes)

(Thesis Format: Integrated Article)

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Submitted in partial fulfillment of the requirements for the degree

Doctor of Philosophy

School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario
April 2009

Your file Votre référence
ISBN: 978-0-494-50224-2
Our file Notre référence
ISBN: 978-0-494-50224-2

THE UNIVERSITY OF WESTERN ONTARIO
SCHOOL OF GRADUATE AND POSTDOCTORAL STUDIES

CERTIFICATE OF EXAMINATION

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The thesis by

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entitled:

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is accepted in partial fulfilment of the
requirements for the degree of
Doctor of Philosophy

Date_____

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Chair of the Thesis Examination Board

ABSTRACT

Diabetes mellitus (DM) is a hyperglycemic condition resulting from impaired insulin signaling which alters metabolism, increases inflammation and leads to progressive complications, including myocardial dysfunction, increased oxidative stress, altered organ mass, bone mineral content (BMC) and skeletal muscle mass. Exercise is commonly prescribed to reduce the progress of DM-related complications, but its ability to normalize intracellular signaling and restore anatomical structure and myocardial function in animals devoid of insulin is not well characterized. Exercise was prescribed either at the onset of, or 4-weeks following, DM induction. Sixty Sprague-Dawley rats were divided into DM and Non-DM groups. Animals from each group (n=10) were assigned to 1 of 3 treatments: (1) sedentary (2) 4-weeks sedentary followed by 4-weeks of exercise (Ex4); or (3) 8-weeks of exercise (Ex8). Exercise training consisted of treadmill running for 1-hour per day, 5-days per week at 27m/min (~70% VO_{2max}). DM was induced by a low-dose injection of streptozotocin (20mg/kg; i.p.) for 5 consecutive days. We measured organ, BMC and muscle mass changes, whole body oxidative stress, tissue-specific inflammatory signaling via NF-κB-DNA binding, delineated myocardial signaling pathways and the expression of structural and functional proteins regulated through insulin-like growth factor-1 (IGF-1), and acquired measures of systolic and diastolic function by echocardiography. Systolic and diastolic function were impaired in DM animals and were not improved by exercise. Skeletal and myocardial mass were reduced, whereas kidney and adrenal mass were increased by DM and not normalized by exercise. In the myocardium, NF-κB-DNA binding was increased by DM, exercise, or a combination of both. However, DM increased contents of the inhibitor specific for the pathological p50-p65 heterodimer, IκB-α, indicating that DM may increase pathology-related target-gene transcription, which was not improved by exercise. In DM skeletal muscle, NF-κB-DNA binding was decreased in the red gastrocnemius muscle (type 1 myosin fibres), but unchanged in the soleus muscle (type 1 myosin fibres), demonstrating a muscle-specific, rather than fibre-type specific inflammatory regulation. Myocardial cellular signaling mediated through the IGF-1-receptor (IGF-1R) was altered by DM measured by increased protein contents of IGF-1R, activated/unphosphorylated GSK-3β, increased β-myosin heavy chain, collagen type III, heat shock factor-1, which were not restored by exercise. In contrast, oxidative stress (serum isoprostanes), elevated in DM animals, was normalized by Ex4 and reduced to below normal levels with Ex8 in DM animals only. In addition, exercise increased the cardio-protective heat shock protein 70 (HSP70) in both DM and Non-DM myocardia. In summary, DM induced systolic and diastolic dysfunction, increased kidney and adrenal gland hypertrophy, oxidative stress, and reduced myocardial and skeletal muscle mass and BMC. DM was associated with specific changes to intracellular signaling pathways via IGF-1R and induced increased contents of pathologically associated structural and functional proteins that were not improved or prevented by exercise. These results indicate that DM negatively affects anatomical structure and myocardial function and its progress is unimpeded by exercise; however, exercise reduces mitochondrial oxidative stress and may provide protection against deleterious myocardial events through the protective functions associated with a significant induction of HSP70.

Acknowledgements

Thank you to everyone for devoting your care and encouragement over the years as I worked towards completing this part of my life.

To Earl Noble: thank you for taking me into your lab and graciously willing to be my supervisor. You effortlessly comfort those around you and ease the burden of daunting tasks with exuberance for life and an ability to see the humor in all things.

To Marius Locke: Once again, thank you for being instrumental in guiding and shaping the direction of my graduate work over the last ten years. You've been tremendously influential and have always been a guiding hand.

To Bert Taylor: Thank you for bringing me to Western and for letting me find my own direction, all the while being ready to help me every time I needed it.

To my Family: Thank you for all that you've done to help me every day. Thank you for providing endless financial, emotional and spiritual support and encouragement.

To my Mom: your dedication to the truth and to the things that truly enrich our lives continues to guide me every day. Your energy and strength of character is the foundation of my true goals.

To my Dad: you are my model of tireless work. Your ability to endure adversity with honesty, fairness and humor makes light work of my days. Your belief that the best is yet to come makes our lives easier, and so I look to the future with anticipation and a smile.

To my brother, Ken: your desire to improve yourself, your hope for the future and your ability to find cheer and meaning every day helps me to appreciate my days no matter where or how far away I am.

To my sister, Lynn: your devotion to helping others with little regard to yourself helps me believe that my efforts over the years may also be directed to helping those in need.

To my nephew, Jonathan: you have a strong and impressive desire to reach the limits of your talents. You bring a peace and joy in our lives and have always been inspirational to us all.

To Pearl: There is nothing you do that is not motivated by your desire to improve the life of your friends and family. You have an honesty and integrity that is unsurpassed and unfailing. I was able to complete this thesis because of your help and care over the years. More importantly, the last several years have been a pivotal time in my life not because of graduate work, scientific research or academic pursuits, but because of the time I got to spend with you.

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CHAPTER 1: Introduction

LIST OF ABBREVIATIONS

A1c	Glycated hemoglobin
AKT	AK-transforming kinase or protein kinase B
ANOVA	Analysis of variance
AWT	Anterior wall thickness
Bcl-3	B-Cell Lymphoma-3
BMC	Bone mineral content of hydroxyapetite
β-MHC I	Beta-myosin heavy chain
B-mode	Brightness mode setting on echocardiogram
Collagen III	Extracellular matrix collagen type III
DM	Diabetes Mellitus
DNA	Deoxyribonucleaic acid
EDL	Extensor digitorum longus
EF%	Ejection fraction
ELISA	Enzyme-linked Immunosorbent assay
EMSA	Electrophoretic mobility shift assay
ERK-1	Extracellular-regulated protein kinase-1
FS%	Fractional Shortening
GSK-3β	Glycogen synthase kinase-3 beta
HSF-1	Heat shock factor-1
HSP	Heat Shock Protein
HSP60	Heat shock protein 60
HSP70	Heat shock protein 70

HSP90	Heat shock protein 90
IGF-1	Insulin-like Growth Factor-1
IκB-α	Inhibitor of NF-κB -alpha
IκK-β	Inhibitor kinase of kappa B
LPS	Lipopolysaccharide
LV	Left ventricle
M-mode	Movement mode setting on echocardiogram
MMP-3	Matrix metalloproteinase-3
MVA	Mitral Velocity of A-wave (Late LV filling - atrial contraction)
MVE	Mitral Velocity of E-wave (Early LV filling)
NF-κB	Nuclear factor of kappa-B
Phospho	Phosphorylated
P_i	Inorganic phosphate
PIP_3	Phosphatidylinositol phosphate-3
PL	Plantaris muscle
PTEN	Phosphatase and tensin homolog
PWT	Posterior wall thickness
RG	Red gastrocnemius muscle
ROS	Reactive oxygen species
RWT	relative oxygen species
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SOL	Soleus muscle
STZ	Streptozotocin

TA	Tibialis Anterior
TBS	Tris buffered saline
TNF-α	Tumor necrosis factor-alpha
TTBS	Tween-20 tris buffered saline
UCP-3	Uncoupling protein-3
V_{cf}	Circumferential fibre shortening velocity
VO_2	Volume of oxygen
WG	White gastrocnemius muscle

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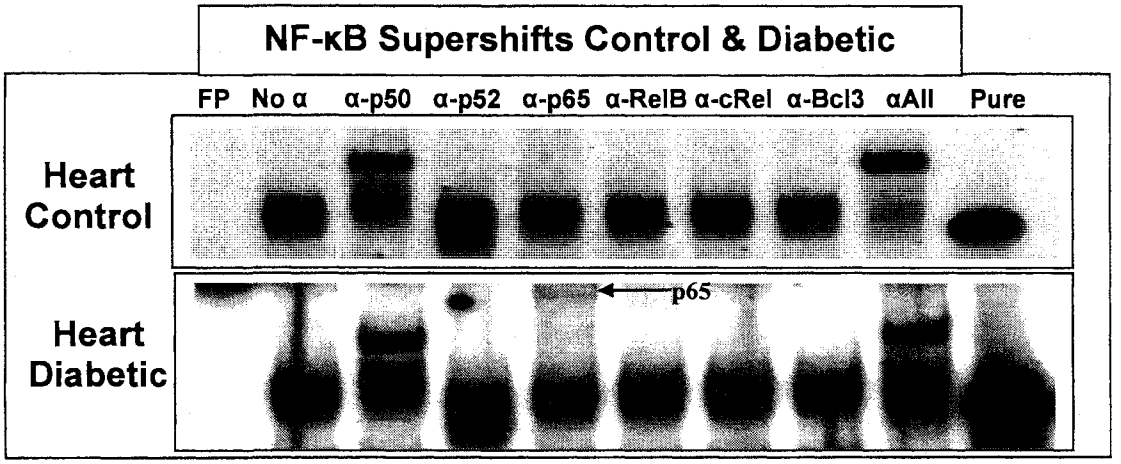
1.0 PREFACE

The following thesis attempted to demonstrate that diabetes mellitus (DM) changes insulin-like growth factor-1 receptor (IGF-1R) signaling and alters structural and functional proteins. Altered production of various stress proteins, collagen and myosin are mediated through the activation of transcriptional regulators downstream of IGF-1R, such as glycogen synthase kinase-3β, heat shock factor-1 and nuclear factor-kappa-B (NF-κB), culminating in impaired heart function. This study also intended to uncover potential mechanisms and delineate the extent to which exercise can prevent and ameliorate the changes to biochemical signaling and cellular structure within the diabetic myocardium.

The motivating observation that led to the development of this thesis was a simple yet interesting finding following a preliminary experiment: we were interested in finding an association between stress proteins and muscle hypertrophy and atrophy. After observing that hypertrophy induced by certain stressors, such as functional overload, may be inhibited by other stressors, such as heat shock, we looked for factors and pathological conditions associated with atrophy. Atrophy is commonly reported in DM, as is a chronic, low-grade inflammatory state. The transcription factor, NF-κB is responsible for initiating the expression of numerous proteins intimately associated with inflammation, and is also associated with hypertrophy, atrophy and DM. We determined whether NF-κB binding and protein composition was tissue-specific and whether it could be correlated to atrophy in DM hindlimb muscles (Chapter 3). We did find muscle-specific differences in the binding quantity, but no changes in the protein members that make up the NF-κB transcription factor. While performing mobility shift and supershift assays on heart tissue

(techniques designed to quantify transcription factor binding and identify protein constituents of the transcription factor, respectively) we observed that p65 (a member of the NF-κB family) became part of the transcription factor in the rat myocardium following 4 weeks of uncontrolled DM (see figure below).



Under normal conditions, p65 is only rapidly and transiently activated. We speculated that the changing transcription factor may represent an early stage in the progression of diabetic cardiomyopathy. We thought it would be interesting to determine whether exercise could reverse or restore this trend. However, we needed to ascertain what might change NF-κB, and what are the physiological consequences; we wanted to find something that could connect the change in NF-κB protein members that may also influence muscle mass. The only regulator in the literature that could affect both hypertrophy and atrophy, and influence the binding and change the composition of NF-κB was IGF-1 (also previously reported to be disrupted by DM). Further, the literature demonstrated that IGF-1R signaling regulates proteins that may ultimately manifest as altered structure and function in the diabetic myocardium.

Very quickly our singular interest moved from determining whether exercise has an ability to restore NF-κB protein composition to also determining whether exercise could influence IGF-1R signaling and its regulation of proteins that may affect myocardial structure and function. Therefore, using exercise as a treatment modality to improve glucose control (a conventional imperative to conferring physiological improvements in diabetic patients) was not a motivating factor in the development of this thesis. Nonetheless, determining the ability of exercise to influence traditional risk factors associated with DM (levels of glucose, glycation products, triglycerides, cholesterol, cortisol, organ mass and bone mineral content) became important measures to establish whether exercise can selectively influence intracellular pathways, restore normal protein production and myocardial function independent of glucose and insulin signaling.

In summary, the present study will demonstrate the effect of diabetes on the activation of the central inflammatory regulator, NF-κB, and its downstream effects on cardiomyopathic protein expression implicated in impaired heart function (see Table 1 for categories of proteins). Moreover, the present study will demonstrate potential mechanisms responsible for cellular signaling within the diabetic myocardium attributable to IGF-1R signaling. The value of prescribing exercise as a treatment for DM may be determined not only by its capacity to prevent or improve intracellular signaling and effectors of protein expression, but, in more dire circumstances, to restore function and structural changes to the myocardium suffered under the sovereignty of unimpeded DM.

Signaling Molecules	Proteins & Products	Structural Change & Function
IGF-1R	UCP-3	Altered Collagen Content
PI-3K	Reactive Oxygen Species	Reduced Ejection Fraction
IκK-β	HSP70	Ventricular Hypertrophy
IκB-α	Hsp60	Increased β-MHC I
NF-κB		
HSF-1		
GSK-3β		

Table 1.1: SIGNALS, PROTEINS & FUNCTION. Responsibilities and division of proteins in pathophysiological sequence.

1.1 INTRODUCTION

Diabetes mellitus (DM) is a metabolic pathology characterized by systemic circulatory glucose accrual, accompanied by diminishing cellular glucose uptake and metabolism. Diabetic metabolism is also evidenced by elevated counter-regulatory hormones, which alter lipid metabolism and increase protein catabolism (Fried et al., 2004). DM effectively removes the primary substrate of cellular ATP production, imposing stress upon the organism to maintain energy provision by re-assigning supportive and alternative energy pathways. Although alternative metabolic pathways exist, the highly conserved utilization of glucose and insulin signaling for regulating multiple functions and transcriptional activity results in a myriad of complications attributable to its metabolic disruption.

Reduced insulin, the massive accumulation of systemic glucose and elevated FFA contribute to altered cell signaling and to the activation of inappropriate pathways, adding to the DM sequelae. Sequelae of DM may include microvascular complications involving retinopathy, nephropathy, and neuropathy (distal symmetric polyneuropathy and autonomic neuropathy), and macrovascular complications culminating in coronary artery disease. All-cause mortality is three to four times greater in subjects with DM, with CVD accounting for the majority of deaths (Soedamah-Muthu et al., 2006). Insulin treatment typically results in normalized glucose levels in Type I (insulin-dependent) diabetic subjects. Coincidentally, normal or better than average blood-borne lipid profiles are also typical. In spite of the lack of pathological levels of traditional risk factors in this cohort, coronary heart disease remains the majority cause of death. Hence, it seems likely that

improved risk factors resulting subsequent to a regimented bolus injection of insulin may not be sufficient to restore the biochemical balance in the diabetic myocardium.

1.2 Conventional Treatment

Established treatments aimed at glycemic control and the interruption of hyperglycemia-induced damage pathways have long been recognized as the most important goals of disease management; there is convincing evidence that controlling hyperglycemia does confer benefit and reduces micro- and macrovascular complications (reviewed by Olansky, 2004 and Davidson, 2004). For example, Selvin et al. (2005) reported that a high level of A1C (glycated hemoglobin) was associated with carotid intima-media thickness and other CVD risk factors (e.g., lipid profile and waist circumference). In addition, the joint Canadian–American Diabetes Control and Complications Trial (DCCT: the effect of intensive diabetes therapy, 1995) and the United Kingdom Prospective Diabetes Study (Manley, 2003) have demonstrated that glucose control leads to significant reduction in micro- and macrovascular (heart disease) complications (~30% reduction per 1% reduction in A1C) (DCCT Research Group, 1993, DCCT Research Group, 1995). Moreover, the ability to control DM-associated high blood pressure has also demonstrated macrovascular/cardiovascular benefit (UKPDS Group, 1998). Several investigations have reported specific biochemical reactions associated with hyperglycemia that may be central to vascular complications in DM (reviewed by Olansky, 2004). Nonetheless, treatment efficacy is typically judged by complication reduction, which invariably has been associated with a reduction in

traditional risk factors with a general disregard for obtaining comprehensive evidence of intracellular biochemical effects.

1.3 NF-kB Regulation

Cellular complications manifest in DM through the varied effects of glycemia and insulinemia on levels of pro-inflammatory factors, cytokines, increased free radical production, increased advanced glycation end-products, and are generally coupled with a chronic inflammatory state in diabetic subjects (reviewed by Engstrom et al 2003). Specifically, DM-induced inflammation appears to require the activation of nuclear factor kappa-B (NF-κB), a dimerized transcription factor composed from its 5 member proteins, including p50, p52, p65, Rel-B, and c-Rel. Under normal conditions, NF-κB remains sequestered in the cytosol by an inhibitor (IκB). When IκB is phosphorylated by an IκB kinase, I Kappa Kinase (IκK), and subsequently degraded, NF-κB is released, allowing for nuclear translocation and subsequent DNA binding (reviewed by Hoffmann et al 2006). This can lead to the expression of gene products involved in inflammation, immune responses, and growth and development (see Figure 1.1).

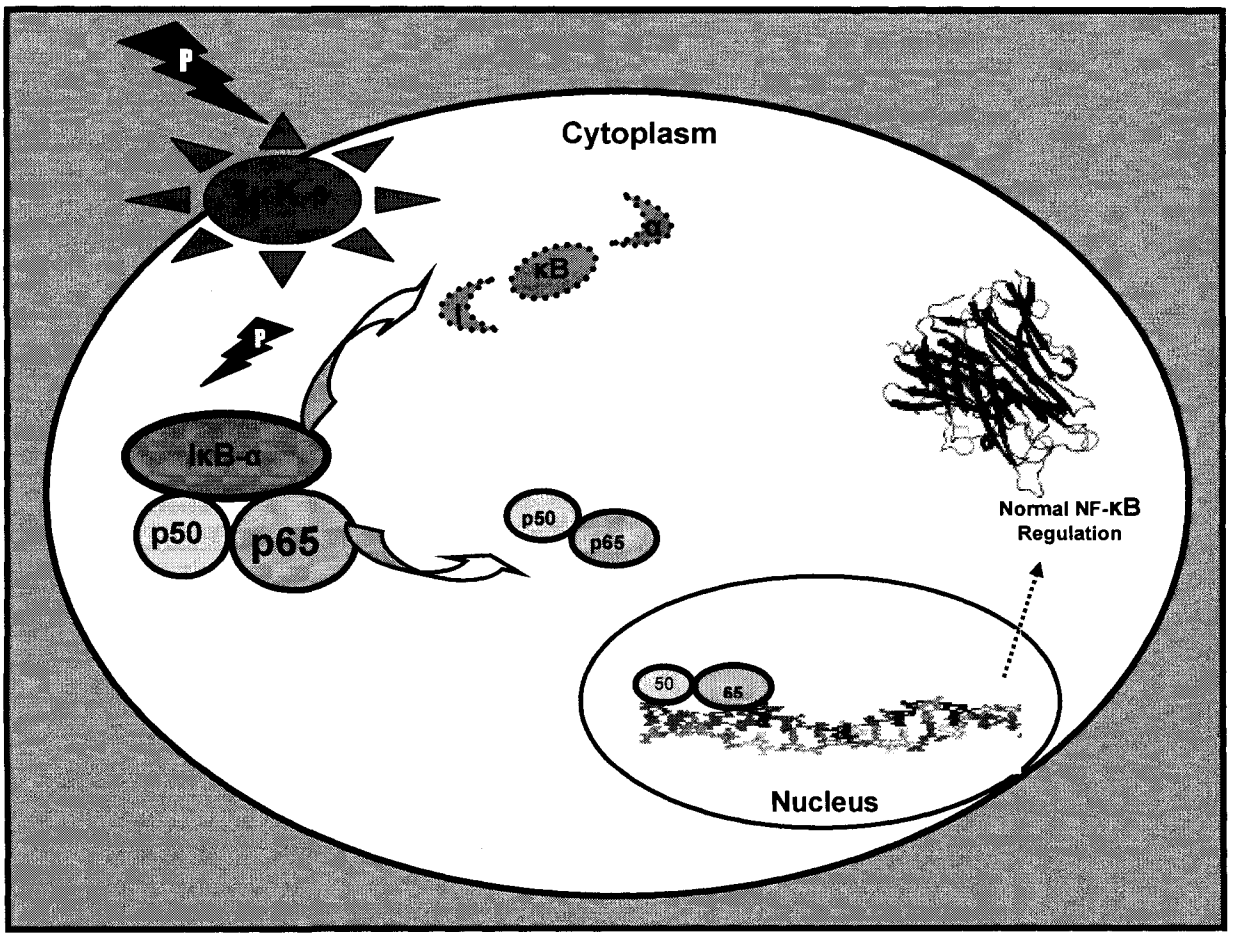


Figure 1.1 Normal Activation of NF-κB (p50-p65). Cytosolic NF-κB is activated & released from its inhibitor via series of IκK-β & IκB-α phosphorylations leading to nuclear translocation, DNA binding, transcription and protein expression.

The most typically observed activated dimer (the *canonical or classical* pathway) appears to be the p50-p65 heterodimer, whereas the *non-canonical* or *alternative* pathway is composed of the p52-RelB subunits, although other subunit combinations are theoretically possible (reviewed by Gilmore 2006). An additional dimer composition is the p50-p50 homodimer, which, by virtue of lacking inhibition and a trans-activation domain, reportedly serves the role of repressing transcriptional activation by binding to a promoter sequence and blocking other factors from binding to target genes (Hoffmann et al., 2006). Activation of the p50-65 pathway leads to a rapid and transient transcription of

normal regulatory and homeostatic genes (See Figure 1.1). The known complement of genes transcribed by the various NF-κB dimer combinations is continually expanding and may explain mechanisms by which selective pathways are activated in any number of pathologies. Certain pathological conditions may preferentially signal the activation of catabolic pathways within various muscles (Lecker et al 2004). Specifically, muscle remodeling observed in DM, disuse, and cancer cachexia has been linked to NF-κB-mediated inflammatory conditions (Wyke 2005), and appears to activate the canonical p50-p65 NF-κB pathway (Guttridge et al. 2000).

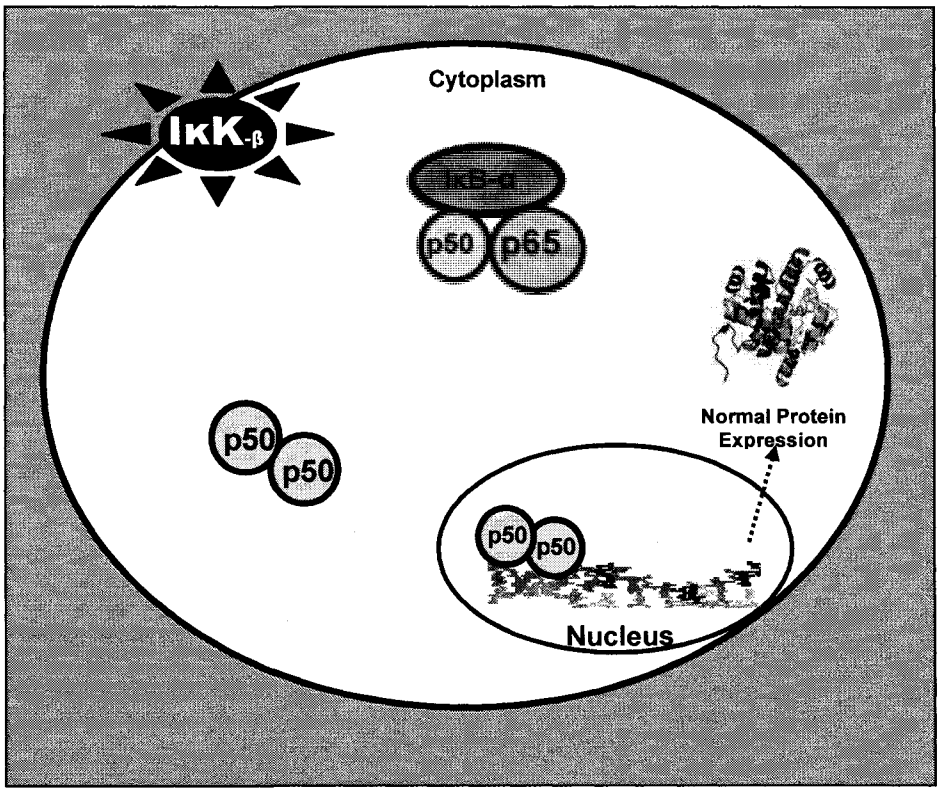


Figure 1.2 Normal Repression of NF-κB (p50-p65) by the p50-p50 homodimer. Cytosolic NF-κB (p50-p65) is inhibited by IκB-α. Unphosphorylated IκB-α sequesters NF-κB until the upstream kinase, IκK-β, is itself phosphorylated. Normally bound p50-p50 allows low levels of transiently activated p50-p65 to transcribe target genes, but under basal conditions, only p50-p50 is detected in a DNA-transcription factor complex.

1.4 NF-kB and DM

Recent work by our laboratory revealed a DM-induced muscle-specific alteration of NF-κB (chapter 3). Although muscles composed predominantly of fast-twitch fibres (gastrocnemius and plantaris) demonstrated no change in dimer composition, they did show reduced DNA binding in the more oxidative portion of the gastrocnemius. However, the slow twitch soleus showed significant DNA binding in Non-DM and DM muscles. Interestingly, it has long been proposed that slow twitch muscles, such as the soleus and heart, are uniquely protected against DM and are similarly better able to maintain phenotype, metabolic profiles, and peptide chain initiation rates (Armstrong et al., 1975; Armstrong and Ianuzzo, 1976; Jefferson et al., 1974). However, the discovery of altered NF-κB within these tissues may provide evidence that the unique activation of specific pathways and signaling molecules are complicit in manifesting long-term complications seen in the diabetic myocardium. Moreover, measuring the activity profiles of NF-κB effectors and the expression of proteins associated with the altered transcription factor and heart disease may provide evidence for potential mechanisms of DM-induced cardiomyopathy and may further identify potential therapeutic targets.

1.5 Mechanisms of Diabetic NF-κB Activation

NF-κB is a cytosolic dimer, sequestered and held inactive by the inhibitor, IκB. Phosphorylation of IκK (the inhibitor's kinase) in turn phosphorylates IκB, leading to its proteolytic dissolution, allowing the now *active* dimer to translocate into the nucleus and bind to target genes. The specific IκB and IκK isoforms involved in the activation of the p50-p65 dimer (and most other dimers) is IκB-α by IκK-β. The normally repressive p50-

p50 homodimer is reportedly not well inhibited by any IκB protein; hence it freely binds to NF-κB target genes in the nucleus, but cannot activate transcription without an activation domain (reviewed by Hoffmann et al., 2006). An activated p50-p65 heterodimer can displace the repressive p50-p50 homodimer following nuclear translocation, initiating genetic transcription. The NF-κB inhibitor, IκB-α, is one of several target genes of p50-p65, which is rapidly synthesized and relocated to the nucleus, where it removes p50-p65 from the DNA, transports and re-sequesters NF-κB in the cytosol. This negative regulation allows for limited and transient transcription of numerous inflammatory and regulatory proteins.

Although DM is commonly characterized with chronic inflammation, our results indicate that the quantity of NF-κB binding in the myocardium is not affected. However, since the active NF-κB composition changes from the p50-p50 homodimer (which requires no activation by upstream effectors) to the p50-p65 heterodimer (which does require upstream effector signaling), it seems likely that DM results in IκB-α phosphorylation via the upstream phosphorylation of IκK-β. Hence, DM is indeed an inflammatory condition induced by phosphorylation of the seminal kinase, IκK-β (see Figure 1.3).

Alternatively, negative regulation, ensured by NF-κB transcription of its inhibitor, IκB-α, may be altered by DM, resulting in a reduction of inhibitor proteins and a chronically activated p50-p65 heterodimer. Indeed, Sriwijitkamol et al. (2006) demonstrated that IκB-α protein content was reduced in diabetic skeletal muscle. However, reduced cellular contents of the inhibitor protein would be expected with chronic p50-p65 heterodimer activation, since either induced proteolysis by upstream

effectors or reduced downstream expression would cause diminished IκB-α protein. Therefore, uncovering alterations to mechanisms that may influence NF-κB activation within or outside the normal activating pathway is critical to understanding specific biochemical targets and actions of DM. Moreover, identifying biochemical targets along the NF-κB pathway may help uncover the specific actions and therapeutic targets of exercise.

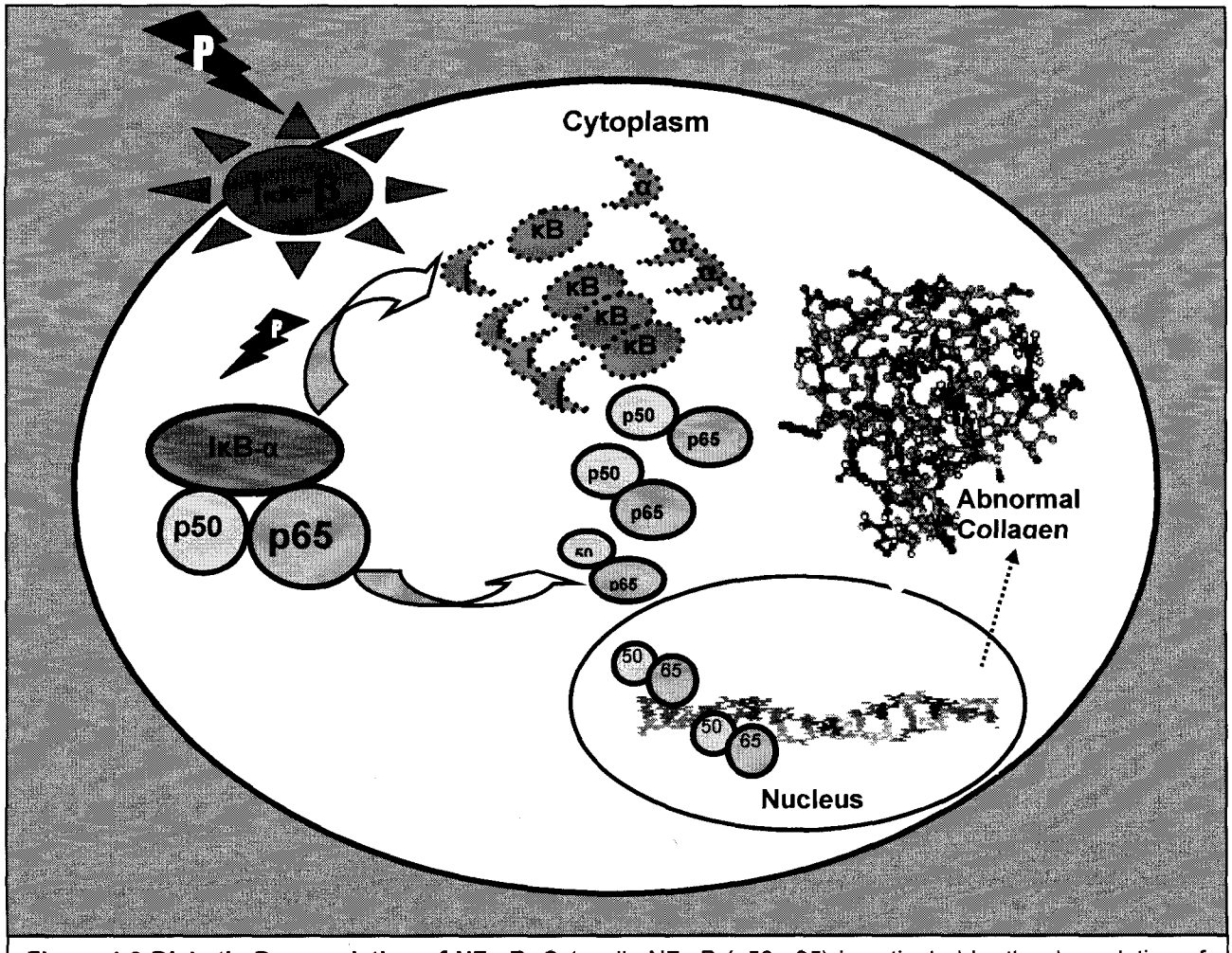


Figure 1.3 Diabetic Dysregulation of NF-κB. Cytosolic NF-κB (p50-p65) is activated by the degradation of phosphorylated IκB-α possibly by activated (phosphorylated) IκK-β. Constitutively active p50-p65 displaces normally bound p50-p50 allowing for large levels of target-gene transcription.

1.6 Modifiers of NF-κB Dimer Binding

Scant information is available with which to delineate intra-cellular mechanisms responsible for the qualitative shift from the normal p50-p50 dimer to p50-p65 activation. Nonetheless, some investigators report that the quantity of bound p50-p50 can be elevated by Insulin-like Growth Factor-1 (IGF-1) by forming p50-p50 dimers and converting p50-p65 dimers into p50-50 dimers, resulting in reduced gene transcription (Knuefermann et al., 2002). Additionally, p50-p65 activation can be inhibited by IGF-1 dephosphorylation of IκB-α (Pons and Torres-Aleman, 2000); hence IGF-1 may be able to influence both transcription factor formation and regulation.

IGF-1 protein (Han and Park, 2006) and receptor signaling (Lai et al. 2007) are reduced in the diabetic rat myocardium. Although IGF-1 has been shown to inhibit NF-κB activation (Knuefermann et al., 2002; Pons and Torres-Aleman, 2000) others have demonstrated that NF-κB activation is required for IGF-1-mediated cellular protection (Heck et al., 1999; Jimenez Del Rio and Velez-Pardo, 2006). Hence, it is not clear whether DM alters NF-κB dimer transformation and activation via reduced IGF-1. Regardless, our preliminary results indicating a shift in NF-κB binding quality, combined with reports of dose-dependent IGF-1-NF-κB alterations and reduced IGF-1 signaling in the diabetic myocardium, might suggest a mechanistic link.

1.7 Complications of Diabetic NF-kB Activation

Measuring changes to NF-kB regulation and subunit composition provides explicit information for the direct consequences of DM on the central regulator of a multitude of other factors and functional proteins. However, even the discovery and

identification of compositional changes does not provide a functional consequence of DM-induced changes within this system. Therefore, the added goal is to unearth changes that may be downstream of DM-altered NF-kB activation that are also associated with specific functional consequences of diabetic cardiomyopathy. Specifically, the need arises to uncover proteins that may directly impact function and may also be expressly influenced by the qualitative shift in NF-kB activation from the p50-p50 homo- to the p50-65 heterodimer.

DM manifests progressively with advancing changes to larger structures within a given organ or target; minute quantities of proteins are initially altered progressing to more blatant phenotypic and functional changes. For instance, the myocardial matrix is composed predominantly of Type I and Type III collagen, and quantities of these proteins (Type III in particular) are increased in the diabetic myocardium. Type III collagen is regulated by matrix metalloproteinase-3 (MMP-3), which is regulated by NF-κB. Collagen deregulation is associated with decreased left ventricular function (Liu et al., 2003; Candido et al., 2003; Avendano et al., 1999; Shimizu et al., 1993), and ventricular dilatation (Tziakas et al., 2005). Approximations of heart function via EKG (Murkofsky et al., 1998) have demonstrated that changes occur early in the streptozotocin (STZ)-treated diabetic rat (Howarth et al., 2005; Van Buren et al., 1998), with some of these alterations able to be attenuated (as measured by echocardiography) with moderate exercise (Smirnova et al., 2006). It is unclear how a DM precisely alters IGF-1R signaling and downstream regulators and proteins, such as NF-κB and Type III collagen, but its deregulation seems grounded in previous reports and the ability to influence their activity may also influence the progression of DM complications.

1.8 Oxidative Stress and Uncoupling Protein-3

DM may affect the structural integrity of the extra-cellular matrix through the dysregulation of collagen and tissue proteinases, but it may also influence metabolism and regulate proteins partly responsible for mitigating reactive oxygen species (ROS). Generation of ROS are known to be elevated in both Type 1- and Type 2 DM (Dave and Kalia, 2007) and may occur by mechanisms other than hyperglycemia (Huang et al., 2003). For instance, increased intracellular contents of free fatty acids (FFA) may cause their accumulation in the mitochondria, leading to elevated fatty acid oxidation and ROS production (St-Pierre et al., 2002), eventually overwhelming mitochondrial FFA oxidative capacity, causing lipotoxicity (Schrauwen and Hesselink, 2004). Under conditions of elevated mitochondrial FFA oxidation and ROS production, there appears to be the up-regulation of uncoupling protein-3 (UCP-3), which can dissipate ROS production (Brand et al., 2004) and reduce the lipotoxic build-up of fatty acids by transporting them back out of the mitochondia (Gustafsson et al., 2004) – although precise mechanisms of UCP-3-mediated protection from FFA oxidation is still equivocal (reviewed by Brand and Esteves, 2005). Nonetheless, UCP-3 expression is increased by FFA, and UCP-3 levels are highly correlated with FFA oxidation and up-regulation of other FFA oxidation-related genes (Schrauwen and Hesselink, 2004). Since DM increases FFA oxidation, UCP-3 may be increased in DM tissue. However, IGF-1R regulates the expression of UCP-3 (Gustafsson et al., 2001) in a dose-dependent manner (Gustafsson et al., 2004), therefore, DM alterations in IGF-1R signaling may impact its regulation of UCP-3. Since attenuated ROS production by UCP-3 appears to be directed by increased

IGF-1 signaling, and increased IGF-1 also decreases NF-κB activity, it's not surprising that attenuated production of ROS has been shown to deactivate NF-κB (Nishikawa et al., 2000).

Taken together, there appears to be a mechanistic link between intracellular IGF-1R signaling NF-κB, and the hallmarks of DM (inflammation, oxidative stress and myocardial remodeling). In the present study, it is our goal to identify the changes to IGF-1R, the keystone mediator of numerous functional regulating proteins in the diabetic myocardium. However, it is also important to identify mechanisms capable of preventing diabetic modification and, perhaps more importantly, identify regulators that may be responsive to therapeutic actions, such as exercise.

1.9 Keystone Regulator

Systemic glucose accretion and the loss of insulin signaling are suspects responsible for cellular alterations leading to diabetic complications, in that glucose control and the restoration of insulin signaling is tantamount to recovery from pathology. Although a semblance of glucose control and restored insulin signaling may be realized with treatments for insulin secretion, sensitizers, and glucose production the disease remains and progresses. Therefore, it seems logical to identify targets of insulin signaling that occur as close to the primary effector of IGF-1 and NF-κB as possible. Further, the utility of identifying one of any number of signaling proteins may be determined not only by its ability to influence the entire signaling cascade, but also by its tendency to be co-operative and persuaded by treatments, such as exercise.

Heat shock protein 60 (Hsp60) is a mitochondrial, cytoplasmic and extracellular molecular chaperone able to transport and alter the conformation of up to 30% of a cell's protein contents (reviewed by Ranford et al., 2000). Along with other HSPs, Hsp60 is important to the cellular stress response, inhibits apoptosis (reviewed by Chi and Karliner, 2004) and confers myocardial protection against normally lethal stressors such as ischemia reperfusion (Lin et al., 2001). A director of cellular trafficking, protein folding and signal transduction, Hsp60 also has the ability to down-regulate NF-κB as evidenced by inhibited nuclear translocation (Zanin-Zhorov et al., 2005) following Hsp60 administration. The inhibition of NF-κB by Hsp60 was also followed by the down-regulation of TNF-α, a powerful activator of NF-κB. Hence, down-regulation of Hsp60 with DM may help to remove the inhibition of inflammatory factors and contribute to the chronic inflammation associated with this disease.

DM, induced by STZ, has been shown to decrease Hsp60 contents in the myocardium (Chen et al., 2005; Shan et al., 2003; Turko and Murad, 2003). Hsp60 has also been shown to regulate the IGF-1 receptor in cardiomyocytes by blocking its ubiquitination and subsequent proteolysis (Lai et al., 2007). Diminished Hsp60 in STZ treated rats was responsible for diminished IGF-1 receptor content, and Hsp60 abundance was related to IGF-1 signaling capacity (Shan et al., 2003). Restoration of Hsp60 was attained by restoring insulin levels, whereas there was no effect on Hsp60 by restoring glucose levels (Chen et al., 2005). Since systemic and local sources of IGF-1 mediate their effects through the membrane receptor (Takigawa et al., 1997; Czech, 1989) the result of Hsp60 down-regulating the IGF-1 receptor (IGF-1R) would be to render the actions of extracellular and intracellular sources of IGF-1 ineffective. Therefore, the

observed ability of IGF-1 to inhibit NF-κB and to promote larger quantities of p50-p50 homodimers at the expense of reducing p50-p65 heterodimers may be lost by DM-induced Hsp60 down-regulation.

Although IGF-1R protein and receptor signaling was restored by insulin, restoration capacity was not limited to the actions of insulin, since receptor content and signaling were recovered by expressing Hsp60 in the absence of insulin. Hence, alternative methods of correcting cellular levels of Hsp60 in the diabetic myocardium may prove effective for reinstating the IGF-1 signaling cascade and mitigating the functional consequences. No direct evidence exists to link the down-regulation of Hsp60 by DM to the dysfunction of a remodeled myocardium; however, there is evidence to draw a direct line between each suspect protein and its closest neighbour (Hsp60, IGF-1, IGF-1R, IκK, IκB, NF-κB, UCP-3, and Type III collagen) in the diabetic environment (see Figure 1.4 for hypothesized pathway). It may be possible to demonstrate that each individual protein is partly culpable for only an aspect of diabetic myocardial dysfunction, but possibly, following experimentation, it may be advocated that all of the suspect proteins, by acting in concert with one another, are liable in large part for the manifest dysfunction (see Table 1.2 for complete list and summary of protein mechanisms).

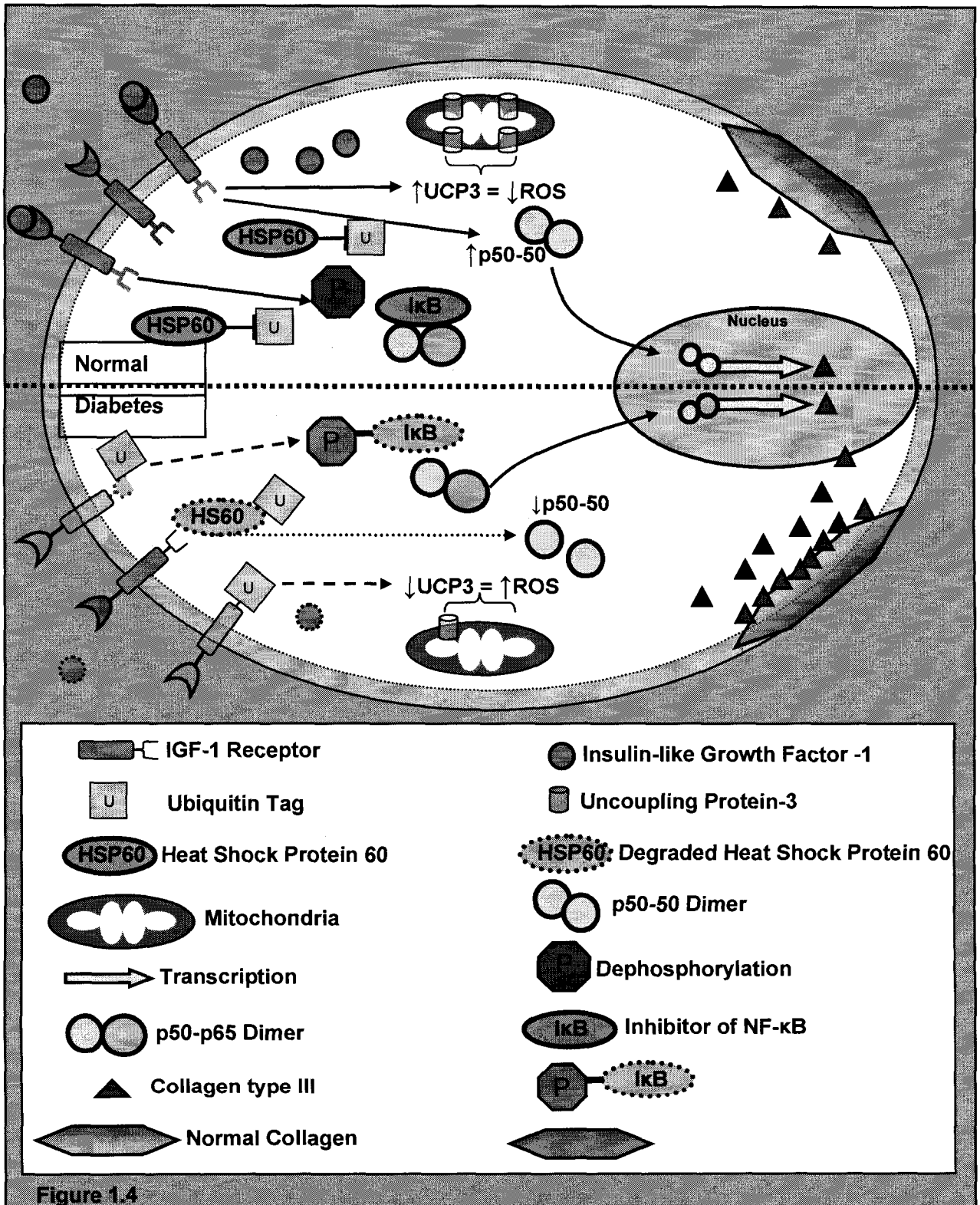


Figure 1.4

Myocardial signaling pathway. Hypothesized signaling pathway within the normal and DM cardiomyocyte.

Protein	Target	Action	Method	Diabetes Impact
Hsp60	IGF-1R	Blocks Degradation	Inhibits Ubiquitination	Hsp60 contents are decreased IGF-1R is degraded
Hsp70	Damaged Proteins	Restores tertiary structures and transports proteins	Hydrophobic interactions and ATP-dependent conformational folding	Tissue dependent expression; generally retained expression capacity
HSF-1	Heat shock element in promoter region of DNA	Enables the binding of additional factors and RNA polymerase to target genes	Nuclear translocation due to cytosolic trimerization following release from inhibitory complex	Equivocal reports; maintained or reduced tissue levels
NF-κB (p50-p50)	Gene promoters containing κB sites	Transcriptional repression	Occupies promoter while remaining inactive	Displaced by activated p50-p65 dimers
NF-κB (p50-p65)	Gene promoters containing κB sites	Transcriptional activation	Rapid & transient displacement of p50-50	Sustained p50-p65 activation Excessive inflammatory product transcription
IκB-α	NF-κB (p50-p65)	Inhibits activation & nuclear translocation	Protein-complex binding	IκB-α is degraded; p50-p65 is released & activated
IκK-β	IκB-α	Promotes or inhibits degradation	Phosphorylation	Increased IκK-β activation; IκB-α degradation increases
IGF-1R	Tyrosine residues	Activates or deactivates proteins	Phosphorylation of ISR-1, PI-3K	Degrades resulting in deregulated kinase activity
GSK-3β	Translational regulators; HSF-1	Activates or deactivates proteins	Phosphorylates serine/threonine residues	Conflicting reports: increased or decreased activity

Table 1.2: List of proteins and their associated factors, and targets of action.

1.10 DM and Exercise

As a treatment modality, exercise is clearly perceived as beneficial for virtually every pathology and confers protection to the organism as evidenced by a reduction of all-cause mortality in a linear dose-response manner in healthy individuals (Kesaniemi et al., 2001) and in CAD and DM patients (Kelley and Goodpaster, 2001). Therefore, it appears that exercise may restore the altered intracellular signaling associated with diseases such as DM. However, the convention of correlating disease complications with reduced risk factors (e.g., lipid profile, blood glucose, insulin serum proteins, BMI, etc.) has resulted in the majority of investigations reporting little data regarding the biochemical effects of exercise on cell signaling in various muscles in favor of reporting the relationship between DM complications and reduced risk factors derived from endurance exercise. For instance, a recent study representative of the risk-factor-complication-complex demonstrated that 1 year of moderate-intensity aerobic exercise led to a decline in BMI, total cholesterol, triglycerides, percent body fat, waist circumference, FPG, A1C, leptin, adiponectin, and C-reactive protein (Hsieh and Wang, 2005). Endurance exercise was also shown to improve exercise tolerance, VO_{2max}, A1C (~1%), in addition to improving baroreceptor sensitivity (autonomic function) (Loimaala et al., 2003). Hersey et al. (1994) reported increased capacity of oxygen consumption in elderly men and women (70–79 years) after 6 months of endurance training at 75–85% VO_{2max} three times per week. The subjects in the study by Hersey et al. (1994) demonstrated a lowered plasma insulin response to an oral glucose tolerance test and decreased body fat compared with control subjects. In support, Wannamethee et al. (2000) reported an inverse relationship between serum insulin levels and physical activity

in a prospective study involving over 5,000 individuals (age 40–59 years, employing a ~17-year follow-up) from 24 towns in England, Scotland, and Wales. These investigators also demonstrated an inverse relationship between moderate physical activities, such as sports, once a week or less and more frequent activities (walking or gardening) with CVD and DM. In addition to traditional blood markers, Rahimi et al. (2005) noted that another CVD marker (C-reactive protein) was inversely related to exercise capacity. Finally, exercise intolerance was reportedly symptomatic of cardiovascular neuropathy in DM patients (Vinik et al., 2003).

In the vast majority of clinically based randomized control trails employing exercise prescription there is an emphasis on measuring blood borne risk factors. As detailed in the previous paragraph and in the section on *conventional treatment*, several risk factors are closely correlated with long term complications. Although risk factors may not cause increased cardiac mortality, exercise-reduced cardiovascular mortality is correlated with a reduction in cardiovascular risk factors. Therefore, it can be appreciated that risk factors and functional outcome may become conceptually interchangeable. Non-insulin dependent DM (type 2) accounts for up to 95% of all DM cases (ADA, 2006), and disease complications are characterized by typical factors. However, a disconnection between risk factors and function is exemplified when values and control of blood glucose and lipids are often better in type 1 diabetics than in normal patients (Wadwa et al., 2005). Hence, the mischaracterization of symptom as outcome exemplifies the long-standing admonishment to assume no cause and effect between factors.

The depth of exercise potential may then be described in terms of its ability to reverse and eliminate the damage rather than simply maintaining function without

fundamental repair. Indeed, metabolic function may improve in STZ-induced diabetic rat muscle following exercise without improvements to metabolic transport proteins (Kainulainen et al., 1994). However, one training effect inherent to exercise (improved metabolic capacity measured by VO_{2peak}) was identified as an independent predictor of all-cause mortality when controlling for lipids, C-reactive protein, anthropomorphic data, family history and medication treatment (Laukkanen et al. 2007), suggesting an intracellular benefit independent of traditional evidence. However, the circulatory marker of inflammation, C-reactive protein, was independently associated with cardiovascular death, further suggesting that the central inflammatory factor NF-κB contributes to diabetic cardiomyopathy. Ergo, the present study proposes to employ exercise as an experimental condition to examine the intracellular effects of DM on upstream regulators of the inflammatory transcription factor NF-κB and other cardiomyopathic proteins associated with DM, NF-κB and impaired heart function. Exercise clearly promotes improved cardiac function and its benevolence may also be extended to restoring intracellular signaling pathways. In fact, it may be that the greatest capacity of exercise to exert its affect on functional outcome is mechanistically derived primarily from the restoration of these signaling pathways.

1.11 Exercise and Diabetic Signaling Cascade

Limited data are available regarding the effects of exercise on the activation of IGF-1R and its downstream targets. For instance, there is limited evidence demonstrating a training-induced increase of IGF-1 in diabetic rats (Gomes et al., 2006). However, since both exercise and IGF-1 have each been shown to promote positive cardiac hypertrophy

and prevent pathological hypertrophy (Wilkins et al., 2004) it is hoped that exercise training may restore damaged pathways in the diabetic myocardium and lead to improved structure and function. Most research seems to indicate NF-κB is transiently activated following an acute bout of moderate or intense exercise (Ji et al., 2004; Ho et al. 2005), with little evidence showing reduced activity after acute exercise (Durham et al., 2004). There is some indication that activation is elevated following aerobic training (Spangenburg et al., 2006), but NF-κB activation was also shown to be reduced following acute exercise (García-López et al., 2007). In addition, aerobic training, owing in part to an increase in the IκB inhibitor, reportedly diminished NF-κB activity in skeletal muscle (Sriwijitkamol et al., 2006). The effect of exercise training on NF-κB in the diabetic myocardium is currently unknown. Similarly, exercise does appear to increase UCP-3 contents in diabetic muscle (Mensink et al., 2007; Fritz et al., 2006); however, these findings are limited to skeletal muscle in Type 2 DM.

Fortunately, training-induced increases in Hsp60 have been demonstrated by various exercise modalities and intensities (Khassaf et al., 2001; Mattson et al., 2000). Exercise uniquely activates Hsp60, as the elevated muscle temperature per se does not solely elevate Hsp60 contents (Morton et al., 2007). Even though acute exercise may induce Hsp60 in skeletal muscle (Morton et al., 2006) and may not induce Hsp60 in the diabetic rat myocardium (Oksala et al., 2006), moderate endurance training does increase Hsp60 in rat cardiac tissue (Samelman 2000). Hence, the prolonged endurance training protocol proposed by the present study may lead to significant increases in myocardial Hsp60. It appears promising that the inciting incident leading to diabetic cardio-dysfunction may be partly mediated by a cardio-protective protein (Hsp60) that is also a

member of an exercise-inducible family of cellular-protective proteins (HSPs). In addition to potentially inducing HSP60 for the purpose of normalizing IGF-1R signaling, another cellular-protective protein, HSP70, is exercise inducible (Locke and Noble, 1990) and displays profound cardio-protective functions (Locke et al., 1995), which lead to restoration of myocardial function following ischemia. Hence, any exercise-induced normalization of myocardial structure and function may reflect IGF-1R normalization, but may also reflect the contribution of additional proteins, only peripherally associated with IGF-1R signaling. It is important to investigate the effects of DM on these proteins and transcription factors in order to delineate the complexity of their relationships and their influence on ideopathic complications. Moreover, observing the effect of exercise on the composition of DM-altered IGF-1R and on the regulating proteins downstream of its activation may help determine not only the capacity of exercise to mitigate pathological stress, but may also identify suspects responsible for the progression of the diabetic sequella, thereby identifying therapeutic targets.

1.12 Rationale for Study

Cardiac disease is the leading cause of death among individuals with DM, and may be directly related to impaired insulin signaling and altered IGF-1 and inflammatory regulators. The ability of exercise to confer improved health in DM may be linked to improved inflammation regulation and normalized protein expression. In particular, modified regulation to the key inflammatory factor, NF-kB, has been linked to cardiomyopathic expression profiles in DM. This project will determine if exercise can prevent alterations and/or restore activation of NF-kB to normal levels in the diabetic myocardium. Also, an intricate pathway flows from IGF-1R that touches several other

downstream protein regulators, such as PI-3K, UCP-3, GSK-3β and HSF-1 that may profoundly affect myocardial hypertrophy, fibre composition, oxidative stress and stress protein production. If DM-induced changes in IGF-1R regulation and related myopathic protein expression profiles can be restored or prevented by exercise then target-specific therapies may be developed and clinically applied to ameliorate DM-induced myocardial complications. While there is little direct evidence demonstrating treatment-induced ultrastructural reversal of extracellular matrix remodeling in the myocardium (Liang et al., 2004; Bruckner et al., 2000; reviewed by Hauptman and Sabbah, 2007), and no reports regarding exercise, there do appear to be several conditions that show the myocardium to obtain some remodeling plasticity (reviewed by Margulies, 2002). The present study is predicated on the observation that DM establishes IGF-1R deregulation, which may mechanistically induce cardiomyopathy, and there is a possibility that exercise may mitigate this process.

1.13 Rationale for Model

A well established model of DM has been thoroughly investigated demonstrating disease progression similar to humans, allowing for a detailed examination of mammalian molecular interactions and physiology. The proposed model of DM induction is obtained via multiple low-dose STZ injections (1 daily 20mg/kg; i.p. injection for 5 consecutive days; Like & Rossini, 1976). This dosing schedule also confers protection against changes to several proteins directly related to our project normally seen to change with the larger dose, single injection regime (Kim et al., 2006). Multiple low-dose injections help to maximize the specific effects of STZ on pancreatic beta cells. A gradual DM induction also leads to a slower onset of hyperglycemia, more closely resembling human

disease progression. The extensively delinated physiology & susceptibility to STZ-induced DM presents the rat as an ideal species to employ. Willingness to exercise, well-characterized physiological responses to exercise and training capacity is also well-suited to this species under diabetic conditions.

1.14 Objectives and Thesis Development

Chapter 2. Anthropomorphic changes to organs, skeletal and cardiac muscle, bone mineral content, alterations to the blood-lipid profile, insulin, glucose and protein glycation due to DM-induction will be determined. An endurance training program will be introduced to determine whether exercise alters the severity of DM, as measured by blood-borne factors, and whether organ, bone and muscle mass can be influenced by exercise.

Chapter 3. Activation of the inflammatory regulator, NF-κB, was measured along with its constituent protein family subunits to determine whether inflammation, as measured by DNA binding and protein expression, is correlated with the effect of DM on mass in a muscle-specific manner.

Chapter 4. Both inflammation and cardiomyopathy are intimated with DM, therefore, myocardial NF-kB transcriptional activation was measured to determine the stimulatory effect of DM on NF-kB activation and whether exercise influences the quantity of activation and the constituent members of the transcription factor. To uncover a

pathological inflammatory profile and to determine the ability of exercise to alter or reverse any changes associated with DM it was necessary to measure the activity and quantity of both the upstream activating kinase and the NF-kB inhibitor, respectively, in addition to measuring NF-kB subunit composition and binding activity.

Chapter 5. Quantification of several proteins provided evidence of an association and correlative relationship between signaling factors, gene products in order to understand potential mediators responsible for influencing myocardial function. Diabetopathic protein expression of IGF-1R, Hsp60, HSP70, HSF-1, UCP-3, β-MHC I, Type III collagen and kinase activity of IGF-1R and GSK-3β, as well as whole body oxidative stress were quantified via western blotting and compared between diabetic and exercise groups. A working model was created and revised from the original pathway hypothesized within this chapter to outline the unexpected biochemical interactions uniquely affected by DM and its response to exercise.

Chapter 6. The value of obtaining a measure of the alterations to risk factors and signaling and structural proteins by either DM or exercise is maximized by demonstrating their effects on function. Therefore, heart function (both systolic and diastolic function, heart rate, and anatomical geometry) was determined by echocardiography.

1.15 Experimental Design

The present study attempted to ascertain the extent to which exercise training prevents and/or reverses DM-related myocardial protein changes. These objectives required exercise training in 1 group at the onset of DM induction and a 2nd group sufficiently exposed to DM prior to exercise training. Proper determination of an exercise effect on muscle proteins in STZ-induced diabetic rats required 4 conditional controls (sedentary control, 4 weeks exercise only, 8 weeks exercise only, and DM only).

All animals were housed for 9 weeks following the 72 hour adjustment period. All tissues were harvested at the end of the 9 week period. All chapters followed the same experimental design with the exception of chapter 3, which use a single control group and 1 DM group to determine tissue-specific regulation of NF-κB. Specific group treatments used in all other chapters will be as follows (see Figure 1.5):

Group 1: Controls = 9 weeks following no intervention.

Group 2: 4 Weeks Exercise (Ex 4) = 5 weeks of no treatment followed by 4 weeks of exercise [1 hour of treadmill running at 27m/min (~75% VO_{2max}) 5 times per week for 4 weeks].

Group 3: 8 Weeks Exercise (Ex 8) = 1 week no intervention followed by 8 weeks of exercise [1 hour of treadmill running at 27m/min (~75% VO_{2max}) 5 times per week for 4 weeks].

Group 4: DM only group (DM) = 1 week of STZ treatment (i.p. injection of 20mg/kg of STZ on 5 consecutive days) + 8 weeks of DM.

Group 5: DM + 4 Weeks Exercise (DM Ex4) = 1 week of STZ treatment + 4 weeks sedentary + 4 weeks of exercise [1 hour of treadmill running at 27m/min (~75% VO_{2max}) 5 times per week for 4 weeks].

Group 6: DM + 8 Weeks Exercise (DM Ex8) = 1 week of STZ treatments + 8 weeks of exercise [1 hour of treadmill running at 27m/min (~75% VO_{2max}) 5 times per week for 4 weeks].

60 Male Sprague Dawley Rats
(@ 220g)

72 hour adjustment period

Exercise Familiarization 1

24 hours

Exercise Familiarization 2

48 hours

Randomization

GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6
n = 10	n = 10	n = 10	n = 10	n = 10	n = 10

Week 1
Week 2
Week 3
Week 4
Week5
Week 6
Week 7
Week 8
Week 9

EX

STZ

CONTROL	EXERCISE	EXERCISE	DIABETES	DM + EX 4	DM + EX 8
No intervention	4 Wks Exercise	8 Wks Exercise	STZ treatment only	Diabetes (8 Wks) + Exercise (4 Wks)	Diabetes + Exercise (8 Wks)

HARVEST ANIMAL TISSUES

Figure 1.5

1.16 Exercise Protocol

Rats were familiarized to the treadmill running at a slow pace (15m/min) for 10 min per day twice (5 & 3 days prior) prior to an acute treadmill run. When the experiment began, rats were run at various intensities progressing from 30 to 75% VO_{2max} for one hour. Exercise training consisted of 1 hour of treadmill running 5 times per week adjusted weekly to greater intensities (15, 19, 23, 27m/min over 4 weeks), the highest intensity corresponding to ~75% VO_{2max}.

1.17 Hypothesis

It was expected that DM would be associated with an abnormal profile of blood-borne factors, organ, bone, muscle mass changes, elevated NF-kB activity in a muscle-specific manner (associated with increased IkK-β phosphorylation and reduced IkB-α protein content). It was anticipated that Hsp60, IGF-1R, UCP-3 would be diminished, but oxidative stress, β-MHC I, collagen Type III would be increased and associated with diminished heart function. Moreover, HSF-1 would be diminished in myocardial tissue and exercise-induced HSP70 would also be reduced by DM. It was expected that exercise would restore IGF-1R signaling and related cardiomyopathic protein content and activity to levels comparable to Non-DM levels observed at rest. It was hypothesized that the restoration of protein profiles would be reflected in echocardiographic measures of improved cardiac function.

1.18 REFERENCES

American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care*. 2006; 29(Suppl 1):S43–S48

Armstrong RB, Gollnick PD, Ianuzzo CD. Histochemical properties of skeletal muscle fibers in streptozotocin-diabetic rats. *Cell Tissue Res.* 1975; 162(3):387-94.

Armstrong RB, Ianuzzo CD. Decay of succinate dehydrogenase activity in rat skeletal muscle following streptozotocin injection. *Horm Metab Res.* 1976; 8(5):392-394.

Avendano GF, Agarwal RK, Bashey RI, Lyons MM, Soni BJ, Jyothirmayi GN, and Regan TJ. Effects of glucose intolerance on myocardial function and collagen-linked glycation. *Diabetes* 1999; 48:1443–1447.

Bassaganya-Riera J, Reynolds K, Martino-Catt S, Cui Y, Hennighausen L, Gonzalez F, Rohrer J, Benninghoff AU, Hontecillas R. Activation of PPAR gamma and delta by conjugated linoleic acid mediates protection from experimental inflammatory bowel disease. *Gastroenterology*. 2004; 127(3):777-791.

Brand MD, Esteves TC. Physiological functions of the mitochondrial uncoupling proteins UCP2 and UCP3. *Cell Metab*. 2005; 2(2):85-93.

Brand, M.D., Affourtit, C., Esteves, T.C., Green, K., Lambert, A.J., Miwa, S., Pakay, J.L., and Parker, N.. Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. *Free Radic. Biol. Med.* 2004a; 37:755–767.

Bruckner BA, Stetson SJ, Farmer JA, Radovancevic B, Frazier OH, Noon GP, Entman ML, Torre-Amione G, Youker KA. The implications for cardiac recovery of left ventricular assist device support on myocardial collagen content. *Am J Surg.* 2000; 180(6):498-501.

Candido R, Forbes JM, Thomas MC, Thallas V, Dean RG, Burns WC *et al.* A breaker of advanced glycation end products attenuates diabetes-induced myocardial structural changes. *Circ Res* 2003; 92:785–792.

Chen HS, Shan YX, Yang TL, Lin HD, Chen JW, Lin SJ, Wang PH. Insulin deficiency downregulated heat shock protein 60 and IGF-1 receptor signaling in diabetic myocardium. *Diabetes*. 2005; 54(1):175-181.

Chi NC, Karliner JS. Molecular determinants of responses to myocardial ischemia/reperfusion injury: focus on hypoxia-inducible and heat shock factors. *Cardiovasc Res*. 2004; 61(3):437-447.

Czech MP. Signal transmission by the insulin-like growth factors. *Cell*. 1989; 59(2):235-8.

Dave GS, Kalia K. Hyperglycemia induced oxidative stress in type-1 and type-2 diabetic patients with and without nephropathy. *Cell Mol Biol (Noisy-le-grand).* 2007; 53(5):68-78.

Davidson JA. Treatment of the patient with diabetes: importance of maintaining target HbA(1c) levels. *Curr Med Res Opin.* 2004; 20:1919–1927.

DCCT Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. *N Engl J Med.* 1993; 329:977–986.

DCCT Research Group Effect of intensive diabetes management on macro-vascular events and risk factors in the Diabetes Control and Complications Trial. *Am J Cardiol* 1995; 75:894–903.

Durham WJ, Li YP, Gerken E, Farid M, Arbogast S, Wolfe RR, Reid MB. Fatiguing exercise reduces DNA binding activity of NF-kB in skeletal muscle nuclei. *J Appl Physiol.* 2004; 97:1740–1745.

Fried LP, Ferrucci L, Darer J, Williamson JD, Anderson G. Untangling the concepts of disability, frailty, and comorbidity: implications for improved targeting and care. *J Gerontol A Biol Sci Med Sci* 2004; 59:255–263.

Frier BC, Noble EG, Locke M. Diabetes-induced atrophy is associated with a muscle-specific alteration in NF-kappaB activation and expression. *Cell Stress Chaperones.* 2008; 13(3):287-296.

Fritz T, Krämer DK, Karlsson HK, Galuska D, Engfeldt P, Zierath JR, Krook A. Low-intensity exercise increases skeletal muscle protein expression of PPARdelta and UCP3 in type 2 diabetic patients. *Diabetes Metab Res Rev.* 2006; 22(6):492-498.

García-López D, Cuevas MJ, Almar M, Lima E, De Paz JA, González-Gallego J. Effects of eccentric exercise on NF-kappaB activation in blood mononuclear cells. *Med Sci Sports Exerc.* 2007; 39(4):653-664.

Gomes RJ, de Mello MA, Caetano FH, Sibuya CY, Anaruma CA, Rogatto GP, Pauli JR, Luciano E. Effects of swimming training on bone mass and the GH/IGF-1 axis in diabetic rats. *Growth Horm IGF Res.* 2006; 16(5-6):326-331.

Gustafsson H, Adamson L, Hedander J, Walum E, Forsby A. Insulin-like growth factor type 1 upregulates uncoupling protein 3. *Biochem Biophys Res Commun.* 2001; 287(5):1105-1111.

Gustafsson H, Soderdahl T, Jonsson G, Bratteng JO, Forsby A. Insulin-like growth factor type 1 prevents hyperglycemia-induced uncoupling protein 3 down-regulation and oxidative stress. *J Neurosci Res.* 2004 15; 77(2):285-291.

Gustafsson H, Tamm C, Forsby A. Signalling pathways for insulin-like growth factor type 1-mediated expression of uncoupling protein 3. *J Neurochem.* 2004a; 88(2):462-468.

Han HJ, Park SH. Alteration of the gene and protein levels of insulin-like growth factors in streptozotocin-induced diabetic male rats. *J Vet Med Sci.* 2006; 68(5):413-419.

Hauptman PJ, Sabbah HN. Reversal of ventricular remodeling: important to establish and difficult to define. *Eur J Heart Fail.* 2007; 9(4):325-328.

Heck S, Lezoualc'h F, Engert S, Behl C. Insulin-like growth factor-1-mediated neuroprotection against oxidative stress is associated with activation of nuclear factor kB. *J Biol Chem.* 1999; 274:9828–9835.

Hersey WC III, Graves JE, Pollock ML, Gingerich R, Shireman RB, Heath GW, Spierto F, McCole SD, Hagberg JM. Endurance exercise training improves body composition and plasma insulin responses in 70- to 79-year-old men and women. *Metabolism.* 1994; 43:847–854.

Ho RC, Hirshman MF, Li Y, Cai D, Farmer JR, Aschenbach WG, Witczak CA, Shoelson SE, Goodyear LJ. Regulation of IkappaB kinase and NF-kappaB in contracting adult rat skeletal muscle. *Am J Physiol Cell Physiol.* 2005;289(4):C794-C801.

Hoffmann A, Natoli G, Ghosh G. Transcriptional regulation via the NF-κB signaling module Oncogene 2006 25: 6706–6716.

Howarth FC, Jacobson M, Shafiullah M, Adeghate E. Long-term effects of streptozotocin-induced diabetes on the electrocardiogram, physical activity and body temperature in rats. *Exp Physiol.* 2005; 90(6):827-835.

Hsieh CJ, Wang PW Effectiveness of weight loss in the elderly with type 2 diabetes mellitus. *J Endocrinol Invest.* 2005; 28:973–977.

Huang TJ, Price SA, Chilton L, Calcutt NA, Tomlinson DR, Verkhratsky A, Fernyhough P. Insulin prevents depolarization of the mitochondrial inner membrane in sensory neurons of type 1 diabetic rats in the presence of sustained hyperglycemia. *Diabetes.* 2003; 52(8):2129-2136.

Jefferson LS, Rannela DE, Munger BL, Morgan HE. Insulin in the regulation of protein turnover in heart and skeletal muscle. *Federation Proc.* 1974; 33:1098-1104.

Ji LL, Gomez-Cabrera MC, Steinhafel N, Vina J. Acute exercise activates nuclear factor (NF)-kappaB signaling pathway in rat skeletal muscle. *FASEB J.* 2004; 18(13):1499-506.

Jimenez Del Rio M, Velez-Pardo C. Insulin-like growth factor-1 prevents Abeta [25-35](H2O2)-induced apoptosis inlymphocytes by reciprocal NF-kappaB activation and p53 inhibition via PI3K-dependent pathway. *Growth Factors.* 2006; 24(1):67-78.

Kainulainen H, Komulainen J, Joost HG, Vihko V. Dissociation of the effects of training on oxidative metabolism, glucose utilisation and GLUT4 levels in skeletal muscle of streptozotocin-diabetic rats. *Pflugers Arch.* 1994; 427(5-6):444-449.

Kelley DE, Goodpaster BH Effects of exercise on glucose homeostasis in type 2 diabetes mellitus. *Med Sci Sports Exerc.* 2001; 33:S495–501; discussion S528–S529.

Kesaniemi YK, Danforth E,Jr, Jensen MD, Kopelman PG, Lefebvre P, Reeder BA Dose–response issues concerning physical activity and health: an evidence-based symposium. *Med Sci Sports Exerc.* 2001; 33:S351–S358.

Khassaf M, Child RB, McArdle A, Brodie DA, Esanu C, Jackson MJ. Time course of responses of human skeletal muscle to oxidative stress induced by nondamaging exercise. *J Appl Physiol.* 2001; 90(3):1031-1035.

Knuefermann P, Chen P, Misra A, Shi SP, Abdellatif M, Sivasubramanian N. Myotrophin/V-1, a protein up-regulated in the failing human heart and in postnatal cerebellum, converts NFkappa B p50-p65 heterodimers to p50-p50 and p65-p65 homodimers. *J Biol Chem.* 2002; 277(26):23888-23897.

Laukkanen JA, Rauramaa R, Salonen JT, Kurl S. The predictive value of cardiorespiratory fitness combined with coronary risk evaluation and the risk of cardiovascular and all-cause death. *J Intern Med.* 2007; 262(2):263-272.

Lai HC, Liu TJ, Ting CT, Yang JY, Huang L, Wallace D, Kaiser P, Wang PH. Regulation of IGF-I receptor signaling in diabetic cardiac muscle: dysregulation of cytosolic and mitochondria HSP60. *Am J Physiol Endocrinol Metab.* 2007; 292(1):E292-E927.

Liang H, Muller J, Weng YG, Wallukat G, Fu P, Lin HS, Bartel S, Knosalla C, Pregla R, Hetzer R. Changes in myocardial collagen content before and after left ventricular assist device application in dilated cardiomyopathy. *Chin Med J.* 2004; 117(3):401 -407.

Lin KM, Lin B, Lian IY, et al. Combined and individual mitochondrial HSP60 and HSP10 expression in cardiac myocytes protects mitochondrial function and prevents apoptotic cell deaths induced by simulated ischemia – reoxygenation. *Circulation* 2001; 103: 1787– 1792.

Liu J, Masurekar MR, Vatner DE, Jyothirmayi GN, Regan TJ, Vatner SF, Meggs LG, Malhotra A. Glycation end-product cross-link breaker reduces collagen and improves cardiac function in aging diabetic heart. *Am J Physiol Heart Circ Physiol.* 2003; 285(6):H2587-H2591.

Locke, M., Tanguay, R.M., Klabunde, R.E., Ianuzzo, C.D. Enhanced postischemic myocardial recovery following exercise induction of HSP 72. *Am J Physiol.* 1995; 269: H320–H325.

Loimaala A, Huikuri HV, Koobi T, Rinne M, Nenonen A, Vuori I Exercise training improves baroreflex sensitivity in type 2 diabetes. *Diabetes.* 2003; 52:1837–1842.

Lupia E, Elliot S, Lenz O, Zheng F, Hattori M, Striker G, Striker L. IGF-1 decreases collagen degradation in diabetic NOD mesangial cells: implications for diabetic nephropathy. *Diabetes* 1999; 48:1638–1644.

Ma XY, Wang H, Ding B, Zhong H, Ghosh S, Lengyel P. The interferon-inducible p202a protein modulates NF-kappaB activity by inhibiting the binding to DNA of p50/p65 heterodimers and p65 homodimers while enhancing the binding of p50 homodimers. J Biol Chem. 2003; 278(25):23008-23019.

Manley S. Haemoglobin A1c—a marker for complications of type 2 diabetes: the experience from the UK Prospective Diabetes Study (UKPDS). *Clin Chem Lab Med* 2003; 41:1182–1190.

Margulies KB. Reversal mechanisms of left ventricular remodeling: lessons from left ventricular assist device experiments. *J Card Fail.* 2002; 8(6 Suppl):S500-S505.

Mattson JP, Ross CR, Kilgore JL, Musch TI. Induction of mitochondrial stress proteins following treadmill running. *Med Sci Sports Exerc.* 2000; 32(2):365-369.

Mensink M, Hesselink MK, Borghouts LB, Keizer H, Moonen-Kornips E, Schaart G, Blaak EE, Schrauwen P. Skeletal muscle uncoupling protein-3 restores upon intervention in the prediabetic and diabetic state: implications for diabetes pathogenesis? *Diabetes Obes Metab.* 2007; 9(4):594-596.

Morton JP, MacLaren DP, Cable NT, Bongers T, Griffiths RD, Campbell IT, Evans L, Kayani A, McArdle A, Drust B. Time course and differential responses of the major heat shock protein families in human skeletal muscle following acute nondamaging treadmill exercise. *J Appl Physiol.* 2006; 101(1):176-182.

Morton JP, Maclaren DP, Cable NT, Campbell IT, Evans L, Bongers T, Griffiths RD, Kayani AC, McArdle A, Drust B. Elevated core and muscle temperature to levels comparable to exercise do not increase heat shock protein content of skeletal muscle of physically active men. *Acta Physiol (Oxf).* 2007; 190(4):319-327.

Mukherjee R, Bruce JA, McClister DM Jr, Allen CM, Sweterlitsch SE, Saul JP. Time-dependent changes in myocardial structure following discrete injury in mice deficient of matrix metalloproteinase-3. *J Mol Cell Cardiol.* 2005; 39(2):259-268.

Murkofsky RL, Dangas G, Diamond JA, Mehta D, Schaffer A, Ambrose JA. A prolonged QRS duration on surface electrocardiogram is a specific indicator of left ventricular dysfunction *J Am Coll Cardiol.* 1998; 32(2):476-482.

Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature*. 2000; 404(6779):787-790.

Oksala NK, Laaksonen DE, Lappalainen J, Khanna S, Nakao C, HÃ¤nninen O, Sen CK, Atalay M. Heat shock protein 60 response to exercise in diabetes: effects of alpha-lipoic acid supplementation. J *Diabetes Complications*. 2006; 20(4):257-261.

Olansky L. Advances in diabetes for the millennium: chronic microvascular complications of diabetes. *MedGenMed*. 2004; 6:14.

Pons S, Torres-Aleman I. Insulin-like growth factor-I stimulates dephosphorylation of ikappa B through the serine phosphatase calcineurin (protein phosphatase 2B). *J Biol Chem*. 2000; 275(49):38620-38625.

Rahimi K, Secknus MA, Adam M, Hayerizadeh BF, Fiedler M, Thiery J, Schuler G Correlation of exercise capacity with high-sensitive C-reactive protein in patients with stable coronary artery disease. *Am Heart J*. 2005; 150:1282–1289.

Ranford JC, Coates AR, Henderson B. Chaperonins are cell-signalling proteins: the unfolding biology of molecular chaperones. *Expert Rev Mol Med*. 2000; 2(8):1-17.

Samelman TR. Heat shock protein expression is increased in cardiac and skeletal muscles of Fischer 344 rats after endurance training. *Exp Physiol*. 2000; 85(1):192-102.

Schoenhagen P, Vince DG, Ziada KM, Kapadia SR, Lauer MA, Crowe TD, Nissen SE, Schrauwen P, Hesselink MK. The role of uncoupling protein 3 in fatty acid metabolism: protection against lipotoxicity? *Proc Nutr Soc*. 2004; 63(2):287-292.

Schoenhagen P, Vince DG, Ziada KM, Kapadia SR, Lauer MA, Crowe TD, Nissen SE, Tuzcu EM. Relation of matrix-metalloproteinase 3 found in coronary lesion samples retrieved by directional coronary atherectomy to intravascular ultrasound observations on coronary remodeling. *Am J Cardiol*. 2002; 89(12):1354-1359.

Selvin E, Coresh J, Golden SH, Boland LL, Brancati FL, Steffes MW Atherosclerosis risk in communities study. Glycemic control, atherosclerosis, and risk factors for cardiovascular disease in individuals with diabetes: the atherosclerosis risk in communities study. *Diabetes Care*. 2005; 28:1965–1973.

Shan YX, Yang TL, Mestril R, Wang PH. Hsp10 and Hsp60 suppress ubiquitination of insulin-like growth factor-1 receptor and augment insulin-like growth factor-1 receptor signaling in cardiac muscle: implications on decreased myocardial protection in diabetic cardiomyopathy. *J Biol Chem*. 2003; 278(46):45492-454928.

Shimizu M, Umeda K, Sugihara N, Yoshio H, Ino H, Takeda R, Okada Y, Nakanishi I. Collagen remodelling in myocardia of patients with diabetes. *J Clin Pathol.* 1993; 46(1):32-36.

Smirnova IV, Kibiryeva N, Vidoni E, Bunag R, Stehno-Bittel L. Abnormal EKG stress test in rats with type 1 diabetes is deterred with low-intensity exercise programme. *Acta Diabetol.* 2006; 43(3):66-74.

Soedamah-Muthu SS, Fuller JH, Mulnier HE, Raleigh VS, Lawrenson RA, Colhoun HM. All-cause mortality rates in patients with type 1 diabetes mellitus compared with a nondiabetic population from the UK general practice research database, 1992–1999. *Diabetologia.* 2006; 49(4):660–666.

Spangenburg EE, Brown DA, Johnson MS, Moore RL. Exercise increases SOCS-3 expression in rat skeletal muscle: potential relationship to IL-6 expression. *J Physiol.* 2006; 572(Pt 3):839-848.

Sriwijitkamol A, Christ-Roberts C, Berria R, Eagan P, Pratipanawatr T, DeFronzo RA, Mandarino LJ, Musi N. Reduced skeletal muscle inhibitor of kappaB beta content is associated with insulin resistance in subjects with type 2 diabetes: reversal by exercise training. *Diabetes.* 2006; 55:760–767.

St-Pierre, J., Buckingham, J.A., Roebuck, S.J., and Brand, M.D. Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J. Biol. Chem.* 2002; 277:44784–44790.

Takigawa M, Okawa T, Pan H, Aoki C, Takahashi K, Zue J, Suzuki F, Kinoshita A. Insulin-like growth factors I and II are autocrine factors in stimulating proteoglycan synthesis, a marker of differentiated chondrocytes, acting through their respective receptors on a clonal human chondrosarcoma-derived chondrocyte cell line, HCS-2/8. *Endocrinology.* 1997; 138(10):4390-4400.

Tayebjee MH, Lip GY, MacFadyen RJ. What role do extracellular matrix changes contribute to the cardiovascular disease burden of diabetes mellitus? *Diabet Med.* 2005; 22(12):1628-1635.

Turko IV, Murad F. Quantitative protein profiling in heart mitochondria from diabetic rats. *J Biol Chem.* 2003; 278(37):35844-35849.

Tziakas DN, Chalikias GK, Papaioakeim M, Hatzinikolaou EI, Stakos DA, Tentes IK, Papanas N, Kortsaris A, Maltezos E, Hatseras DI. Comparison of levels of matrix metalloproteinase-2 and -3 in patients with ischemic cardiomyopathy versus nonischemic cardiomyopathy. *Am J Cardiol.* 2005; 96(10):1449-1451.

UKPDS Group. Tight blood pressure control and risk of macro-vascular and micro-vascular complications in type 2 diabetes: UKPDS 38. UK Prospective Diabetes Study Group. *BMJ.* 1998; 317:703–713.

Van Buren T, Schiereck P, De Ruiter GJ, Gispen WH, De Wildt DJ. Vagal efferent control of electrical properties of the heart in experimental diabetes. *Acta Diabetol.* 1998; 35(1):19-25.

Vinik AI, Freeman R, Erbas T. Diabetic autonomic neuropathy. *Semin Neurol.* 2003; 23:365–372.

Wadwa RP, Kinney GL, Maahs DM, Snell-Bergeon J, Hokanson JE, Garg SK, Eckel RH, Rewers M. Awareness and treatment of dyslipidemia in young adults with type 1 diabetes. *Diabetes Care.* 2005; 28(5):1051-1056.

Wannamethee SG, Shaper AG, Alberti KG. Physical activity, metabolic factors, and the incidence of coronary heart disease and type 2 diabetes. *Arch Intern Med.* 2000; 160:2108–2116.

Westermann D, Rutschow S, Jager S, Linderer A, Anker S, Riad A, Unger T, Schultheiss HP, Pauschinger M, Tschope C. Contributions of inflammation and cardiac matrix metalloproteinase activity to cardiac failure in diabetic cardiomyopathy: the role of angiotensin type 1 receptor antagonism. *Diabetes.* 2007; 56(3):641-646.

Wilkins BJ, Dai YS, Bueno OF, Parsons SA, Xu J, Plank DM, Jones F, Kimball TR, Molkentin JD. Calcineurin/NFAT coupling participates in pathological, but not physiological, cardiac hypertrophy. *Circ Res.* 2004; 94(1):110-118.

Wu TC, Leu HB, Lin WT, Lin CP, Lin SJ, Chen JW. Plasma matrix metalloproteinase-3 level is an independent prognostic factor in stable coronary artery disease. *Eur J Clin Invest.* 2005; 35(9):537-545.

Zanin-Zhorov A, Bruck R, Tal G, Oren S, Aeed H, Hershkoviz R, Cohen IR, Lider O. Heat shock protein 60 inhibits Th1-mediated hepatitis model via innate regulation of Th1/Th2 transcription factors and cytokines. *J Immunol.* 2005; 174(6): 3227-3236.

CHAPTER 2

Anthropomorphic changes with exercise in streptozotocin-induced diabetic animals

2.1 INTRODUCTION

Glucose is typically disposed by the body into metabolic tissues as a function of an immediate, responsive and proportional release of insulin (the potently anabolic, uptake-signaling molecule). A disruption in insulin signaling due to a loss of insulin release (Type 1) or impaired receptor activation (Type 2) leads to a massive accumulation of circulating glucose. Diabetes Mellitus (DM) is defined by chronically high levels of circulating glucose and typically leads to a complex consortium of physiological complications, including micro- and macrovascular disease, retinopathy, nephropathy, and autonomic and peripheral neuropathies (ADA, 2006). In addition to the dangerous accruement of blood glucose, a progressive accumulation of circulating lipids, lipoproteins and compounds is a hallmark of DM (Levy et al., 1988) and may accelerate or uniquely initiate DM complications (Felig et al., 1977).

An altered blood profile of traditional risk factors for cardiovascular disease (elevated LDL, total cholesterol, triglycerides, cortisol, glycated protein and reduced HDL) may not only be evidence of a disordered metabolism, but may also be a general barometer of disease status and overall health. Moreover, DM-induced changes to circulatory constituents may extend beyond the micro- and macrovasculature to include bone status, as well. It has been reported that reduced bone mineral density is markedly evident in DM and may be directly linked to an accumulation of lipid-related compounds (Dennision et al., 2007). Therefore, the control of blood glucose levels and a reduction in additional blood-borne risk factors may also be therapeutically beneficial to improving bone health.

Various tissues may be susceptible to DM to varying degrees by virtue of unique function, reliance on insulin signaling, and dependence on glucose as a metabolic substrate. For instance, increased lipolysis may affect skeletal muscle anatomy differently than adipose tissue, or reduced insulin signaling may affect heart tissue differently than a less insulin sensitive tissue, such as the kidney (Zambotti-Villela et al., 2008). Indeed, previous studies have demonstrated a profound effect of DM on structure and form in a tissue-specific manner. For example, when insulin production and secretion is abolished in an animal model of type 1 DM via streptozotocin (STZ) injection, specific skeletal muscle mass (in particular, fast twitch muscles) was reportedly diminished (Armstrong et al., 1975), whereas the kidney mass was elevated (Pillion et al., 1998). Therefore, a profound change in metabolic priorities and the loss of a principle genetic regulator increases the mass of some structures while decreasing the size of others.

Exercise is an intentional stimulation of the physiological economy by creating metabolic demand through sustained skeletal muscle contraction. At a glance, actively contracting skeletal and heart muscle alters metabolic rate, ATP demand and production, directly and indirectly influences genetic expression in the working muscle and organs, alters circulation, perfusion, secretion and production of numerous signaling molecules. Through these varied effects on numerous tissues and structures, exercise has been exploited precisely to abate the deleterious actions of DM. Exercise is commonly perceived and prescribed to improve cellular function and has been convincingly demonstrated to confer protection against cellular insults and reportedly reduces all-cause mortality in a linear dose-response manner (reviewed by Kesaniemi et al., 2001). Previous reports indicate that exercise positively influences bone mass within at risk

populations, including post-menopausal women (Kerr et al., 2001) and cardiac patients (Braith et al., 1996). Although recommendations regarding exercise as a preventive or reversing agent for DM complications exist (Albright et al., 2000), its specific and mechanistic ability to affect anatomical indicators of DM progression, improve blood-borne factors and maintain or improve skeletal health has not been established.

The present study examined the ability of exercise to alter the development of typical DM disease indicators in an animal model of type 1 diabetes via multiple low-dose STZ injections. DM-induction via STZ injections has been shown to be T-cell mediated in an autoimmune fashion (Pechhold et al., 2001). In addition, previous studies have demonstrated the low dose regime attains the same level of hyperglycemia and hypoinsulinemia as a single high dose injection, but has significantly fewer effects on non-islet cells and peripheral tissues (Like and Rossini, 1976; Kim et al., 2006; Howarth et al., 2005). By determining any measurable exercise-induced change to heart and skeletal muscle anatomy, various blood lipids, stress markers and hyperglycaemia-related factors, as well as skeletal BMC, it was expected that a tissue-specific effect of exercise would be observed. It was hypothesized that bone mass and tissue susceptibility to DM-induced atrophy (highly glycolytic fibres) or hypertrophy (organs) would be associated with an exercise-induced preservation or restoration.

2.2 METHODS AND MATERIALS

Animal Characteristics and Experimental Groups. All procedures were approved by the Animal Care Subcommittee at The University of Western Ontario. Sixty male Sprague-Dawley rats (~220-250g; Charles River Laboratories, Quebec, Canada) were

maintained on a 12-hour dark/light cycle, housed at 20 ±1°C, 50% relative humidity, and provided with standard rat chow and water ad libitum. Animals were divided into DM and non-DM groups. Animals from each group (n=10) were assigned to 1 of 3 treatments: (1) sedentary (SED); (2) 4-weeks sedentary followed by 4-weeks of exercise (EX-4); or (3) 8-weeks of exercise (EX-8).

Diabetes Induction. Animals received a low-dose injection of STZ (20mg/kg; i.p.; Sigma-Aldrich) within 5 minutes of it being dissolved in citrate buffer (0.1 M, pH 4.5) on 5 consecutive days. Non-DM animals were injected with citrate buffer only. DM was confirmed by measuring a blood glucose level >15 mmol. Whole body animal weights and blood glucose values were monitored weekly via blood draw from the saphenous vein using the One Touch Ultra 2 Blood Glucose Monitoring System (Lifescan Canada Ltd, Burnaby, BC, Canada) and One Touch test strips (Lifescan Canada; range = 0–600 mg/dl).

Exercise Training. Rats were familiarized to treadmill running on 2 occasions with brief, 10-minute exposures at 15m/min (5 & 3 days prior to initial training run). At the beginning of the training period, rats were run at progressing intensities with unchanging duration and frequency (one hour/day, 5 days/week) starting at 19m/min for 5 days (week 1), followed by 23m/min for 5 days (week 2), 27m/min for 5 days (week 3), and continued thereafter at this stabilized running intensity of 27m/min (the final intensity corresponding with a work rate of ~75% VO_{2max}). Continuous running during the exercise session was encouraged by an electronically activated puff of compressed air blowing at right angles onto the rat haunches. This air jet served to act as a stimulus to

encourage running and to act as a warning that the end of the belt was approaching. All animals were able to adjust to increasing intensities and completed the training protocol.

Serum Collection and Analysis. All samples and tissues were collected 2 days following the last exercise bout. Animals were anaesthetized with sodium pentobarbitol (65mg/kg) and blood was drawn from the abdominal aorta. Blood samples were allowed to clot at room temperature for at least 30 minutes and were centrifuged at 10,000 rpm for 15 minutes at 4°C. Aliquots of serum were collected and stored at -80°C for subsequent analysis. Values for fructosamine, HDL cholesterol, total cholesterol and triglycerides were obtained using a Hitachi 911 analyzer. Cortisol levels were measured with a chemiluminescent competitive immunoassay using an Immulite machine. Insulin was measured using a sandwich ELISA technique (Catalog # 80-INSRTU-E01) from Alpco Immunoassays.

Tissue Collection. The heart was removed by cardiac excision and the left ventricle was weighed on a digital balance after trimming away the atria and right ventricle. Both the left and right kidney and adrenal glands were removed, weighed and averaged to determine a final mass. Similarly, left and right plantar and dorsi flexors of the hindlimb (gastrocnemius, plantaris, soleus, tibialis anterior and extensor digitorum longus) were removed, weighed and averaged to obtain a final muscle mass. The expression of tissue masses in absolute values are presented as an indication of the direct effects of DM on specific structures. However, a standardizing index is needed to determine whether any differences in tissue mass are proportional to the growth of the animal. DM is expected to have a direct and severe effect on total body mass by markedly elevating lipolysis, as well as limiting muscle growth due to glucose

deregulation. Therefore, tibial length was used as a constant indexing reference to reflect overall growth of the animal rather than body mass, as tibial length has been shown to correlate with normal lean mass development and more accurately reflect true cardiac hypertrophy in disease states that adversely affect body mass (Yin et al., 1982).

Bone Mineral Content, lean and adipose tissue. One day prior to tissue collection animals were also scanned using a cone-beam micro-CT volumetric scanner with flat-panel detector (GE eXplore Locus Ultra, GE healthcare, London, ON). Total bone content of hydroxyapatite, lean and adipose tissue mass was determined by measuring three global thresholds, applied to all imaged volumes. Each threshold value was used to separate CT numbers into adipose tissue, lean tissue and bone. The conversion of adipose tissue, lean tissue, and bone tissue volumes into fractional weights used mean densities of 0.95, 1.06, and 1.94 g/cm^3, respectively, based on average histogram attributes where pronounced peaks in the number of voxels constituted either air, AT, or lean tissue voxels.

Statistical Analysis. A two-way ANOVA (SPSS) was performed using diabetes and exercise as conditions for each group. A Bonferonni post hoc was used to determine significant differences between groups. Differences between groups were considered statistically significant at a level of $P < 0.05$. Data are expressed as the mean ± SEM.

2.3 RESULTS

Insulin and Blood Glucose. Initial glucose values for DM and Non-DM animals were not significantly different (6.45 ± 0.17 mmol/L and 6.92 ± 0.30 mmol/L, respectively), whereas a significant difference ($P < 0.05$) was found 1 week following the

first STZ injection and remained elevated throughout the eight weeks of exercise training, obtaining a final glucose value of 27.14 ± 0.83 mmol/L for DM animals and 6.23 ± 0.25 mmol/L in Non-DM animals (Figuers 2.1*A* and 2.1*B*). There were no differences between exercised and non-exercised animals within either group. Quantification of insulin and glucose showed an evident division between the DM and Non-DM groups (Figures 2.2). As a group, non-DM animals had significantly more ($P < 0.05$) insulin than DM animals (3.41 ng/ml vs.0.23 ng/ml, respectively) 8 weeks following the experimental protocol. Within each group there were no differences between exercised and non-exercised animals. Histochemistry of the pancreas demonstrated degranulation and depleted beta cells in DM animals, as evidenced by reduced insulin staining (Figure 2.3).

Blood Lipids and Fructosamines. Pre- and post-experimental values were collected for blood lipids, cortisol and glycated proteins and are shown in Table 2.1. Total cholesterol and triglycerides showed similar initial values between groups (cholesterol: 1.93 ± 0.11 mmol/L vs 2.13 ± 0.10 mmol/L; triglycerides: 1.03 ± 0.08 mmol/L vs 1.09 ± 0.11 mmol/L, DM vs. Non-DM, respectively). Final values for cholesterol and triglycerides in Non-DM animals were unchanged from initial values, but were significantly increased ($P < 0.05$) in DM vs. Non-DM animals (cholesterol: 2.33 ± 0.10 mmol/L vs 1.87 ± 0.08 mmol/L; triglycerides: 3.57 ± 0.62 mmol/L vs 1.01 ± 0.08 mmol/L, respectively). Initial values for HDL were similar and showed no significant change following the experimental protocol (HDL: pre-intervention values, 1.59 ± 0.09 mmol/L vs 1.77 ± 0.08 mmol/L, post-intervention values, 1.39 ± 0.07 mmol/L vs 1.39 ± 0.06 mmol/L, DM vs. Non-DM, respectively). Similarly, cortisol values showed no difference between DM and Non-DM animals either before or after experimental

treatments (cortisol: pre-intervention values, 35.45 ± 2.68 nmol/L vs 39.04 ± 4.40 nmol/L, post-intervention values, 45.53 ± 2.86 nmol/L vs 44.49 ± 2.58 nmol/L, respectively). Initial fructosamine values were not different between groups (149.7 ± 2.3 μmol/L vs 159.7 ± 2.0 μmol/L, DM vs. Non-DM, respectively), but were significantly greater ($P < 0.05$) in DM animals after 8 weeks of experimental intervention compared to Non-DM animals (266.0 ± 4.0 μmol/L vs 150.9 ± 2.4 μmol/L, respectively). Exercise had no effect on blood lipids, cortisol or fructosamine values.

Anthropomorphic Measures. Whole body, lean tissue and adipose tissue mass are represented in Table 2.2. A clear distinction was demonstrated between DM and Non-DM animals for all anthropomorphic measures. DM resulted in significantly less ($P < 0.05$) total body mass compared to Non-DM animals (352 ± 7.7 g vs. 512 ± 7.6 g, respectively). Similarly, lean tissue mass was reduced significantly ($P < 0.05$) in DM animals (289 ± 6.8 g) compared with Non-DM animals (414 ± 6.1 g), as was adipose tissue mass (16.4 ± 0.8 g v. 47.2 ± 2.5 g, respectively). No significant differences for lean or adipose tissue mass were found between exercised and non-exercised animals within either group.

Muscle and Organ Mass. Left ventricular mass was significantly greater ($P < 0.05$) in Non-DM animals in both absolute values and relative to tibial length (absolute: 0.956 ± 0.02 g vs. 1.22 ± 0.02 g; tibial length index: 0.22 ± 0.01 g/cm vs. 0.27 ± 0.01 g/cm, DM vs Non-DM, respectively) and shown in Figures 2.4*A* and 2.4*B*. Absolute myocardial mass was significantly increased ($P < 0.05$) with 8 weeks of exercise in Non-DM animals. However, exercised animals did not demonstrate a significant change in myocardial mass when values were indexed to tibial length compared to sedentary

control animals. All DM hindlimb plantar flexors range from a predominantly glycolytic fibre type (gastrocnemius, plantaris) to highly oxidative (soleus) (Armstrong and Phelps, 1984). These muscles showed attenuated growth ($P < 0.05$; Figures 2.5, 2.6 and 2.7) in DM compared to Non-DM hindlimbs when adjusted for tibial length (gastrocnemius: 0.34 ± 0.01 g/cm vs. 0.56 ± 0.01 g/cm; plantaris: 0.07 ± 0.002 g/cm vs. 0.11 ± 0.001 g/cm; soleus: 0.04 ± 0.001 g/cm vs. 0.05 ± 0.001 g/cm, respectively). Likewise, the predominantly glycolytic dorsi flexors (shown in Figures 2.8 and 2.9) demonstrated significantly ($P < 0.05$) less mass in DM animals compared to Non-DM animals (extensor digitorum longus: 0.03 ± 0.004 g/cm vs. 0.05 ± 0.001 g/cm; tibialis anterior: 0.12 ± 0.004 g/cm vs. 0.21 ± 0.003 g/cm, respectively). Exercise was not a sufficient stimulus to prevent attenuated growth or restore any DM hindlimb muscle mass to normal levels.

In contrast, DM significantly increased ($P < 0.05$) organ mass (both absolute values and standardized to tibial length) in kidney (Figures 2.10*A* and 2.10*B*) and adrenal gland (Figures 2.11*A* and 2.11*B*) mass (kidney-to-tibial mass ratio: 0.44 ± 0.01 g/cm in DM animals and 0.37 ± 0.01 g/cm in Non-DM animals; adrenal gland-to-tibial mass ratio: 0.008 ± 0.0002 g/cm in DM animals and 0.007 ± 0.0003 g/cm in Non-DM animals).

Bone Mineral Content. Absolute whole body hydroxyapatite mass is shown in Figure 2.12*A* and was significantly lower ($P < 0.05$) in DM animals compared to Non-DM animals (11.4 ± 0.2 g vs. 13.2 ± 0.2 g, respectively). To determine whether this difference simply reflected attenuated whole body growth, mineral content was standardized to tibial length (Figure 2.12*B*). BMC-to-tibial length index demonstrated

significantly reduced ($P < 0.05$) BMC in DM compared to Non-DM animals (2.66 ± 0.04 g/cm vs. 2.94 0.04 ± g/cm, respectively). Exercise did not demonstrate an effect on BMC in either condition.

Figure 2.1. Blood glucose values obtained before and after induction of DM. Blood samples were obtained via lancet puncture from the saphenous vein. Initial blood samples demonstrated no difference between animals divided into Non-Dm and DM groups. DM animals showed significantly greater ($P < 0.05$) blood glucose immediately following DM-induction and remained elevated throughout the entire study duration. Data expressed as mean ± SEM (n = 30). *Significantly greater ($P < 0.05$) than Non-DM animals and pre DM induction.

Figure 2.1

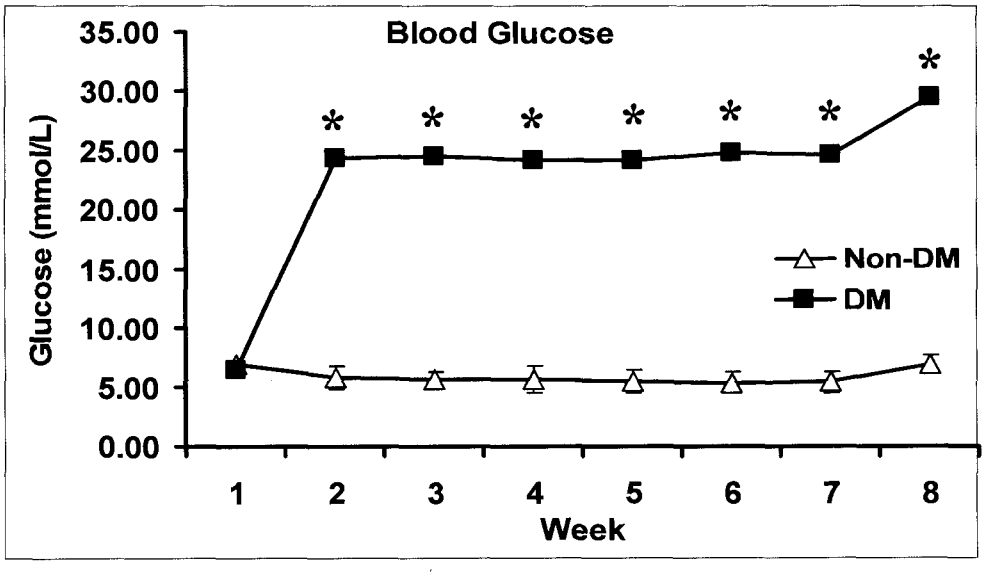

Blood Glucose
Glucose (mmol/L)
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
1
2
3
4
5
6
7
8
Week
Non-DM
DM
*
*
*
*
*
*
*

Figure 2.2. Insulin values following 8 weeks of DM and/or exercise. Insulin was significantly reduced ($P < 0.05$) in DM animals compared to Non-DM animals. Insulin levels in Non-DM and in DM animals were not significantly different between exercise and sedentary groups. Data expressed as mean ± SEM (n = 10). *Significantly less ($P < 0.05$) than Non-DM exercised animals.

Figure 2.2

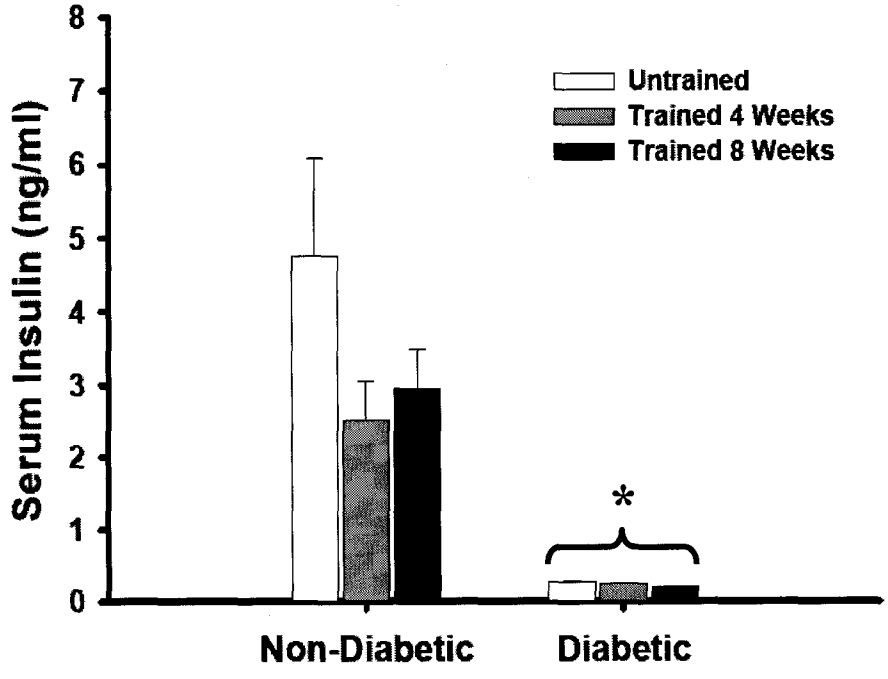

Serum Insulin (ng/ml)
8
7
6
5
4
3
2
1
0
Untrained
Trained 4 Weeks
Trained 8 Weeks
*
Non-Diabetic
Diabetic

Figure 2.3. Immunohistochemical staining for pancreatic insulin in all 6 experimental groups. Brown stain, positive for insulin, is markedly reduced in DM groups. Degranulation of insulin stain in all DM animals suggests an STZ-induced targeting and destruction of insulin producing beta cells. There appeared to be no effect of exercise on insulin production.

Figure 2.3

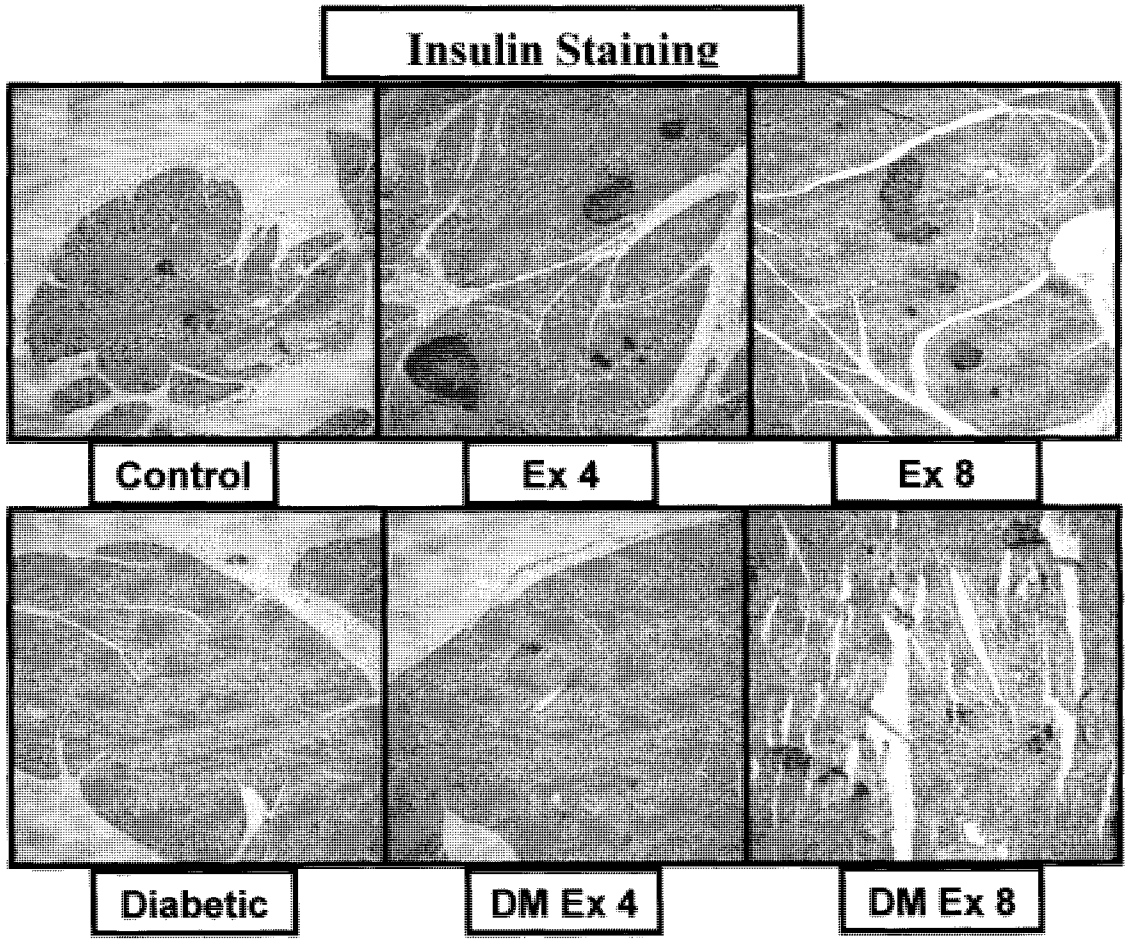


Table 2.1. Blood Lipids, Cortisol and Fructosamines.

Group	Total Cholesterol (mmol/L)	Triglycerides (mmol/L)	HDL (mmol/L)	Cortisol (nmol/L)	Fructosamines (µmol/L)
			Pre-Treatment Values		
Non-DM	2.1 ± 0.1	1.1 ± 0.1	1.8 ± 0.1	39.0 ± 4.4	159.7 ± 2.0
DM	1.9 ± 0.1	1.0 ± 0.1	1.6 ± 0.1	35.5 ± 2.7	149.7 ± 2.3
Control	2.2 ± 0.1	1.0 ± 0.1	1.9 ± 0.1	38.5 ± 10.5	161.0 ± 5.2
Ex 4	2.1 ± 0.2	1.4 ± 0.2	1.7 ± 0.2	38.4 ± 5.5	157.2 ± 3.2
Ex 8	2.1 ± 0.2	0.9 ± 0.2	1.8 ± 0.2	40.3 ± 7.7	161.0 ± 2.0
DM	1.7 ± 0.1	0.9 ± 0.1	1.4 ± 0.1	43.0 ± 6.3	155.6 ± 2.6
DM Ex 4	1.8 ± 0.1	1.2 ± 0.2	1.5 ± 0.1	31.6 ± 2.3	142.8 ± 3.1
DM Ex 8	2.3 ± 0.2	1.0 ± 0.1	1.9 ± 0.2	31.7 ± 3.0	150.8 ± 4.4
			Post-Treatment Values		
Non-DM	1.9 ± 0.1	1.0 ± 0.1	1.4 ± 0.1	44.5 ± 2.6	150.9 ± 2.4
DM	2.3 ± 0.1[a]	3.6 ± 0.6[a]	1.4 ± 0.1	45.5 ± 2.9	266.0 ± 4.0[a]
Control	1.9 ± 0.2	1.1 ± 0.1	1.5 ± 0.1	45.8 ± 4.01	154.5 ± 2.9
Ex 4	1.9 ± 0.2	1.0 ± 0.2	1.3 ± 0.1	45.4 ± 5.1	149.0 ± 5.9
Ex 8	1.8 ± 0.2	0.9 ± 0.1	1.4 ± 0.1	42.0 ± 4.5	149.1 ± 2.9
DM	2.4 ± 0.2[b]	4.6 ± 1.2[b]	1.3 ± 0.1	40.1 ± 2.8	257.7 ± 3.5[b]
DM Ex 4	2.3 ± 0.2[b]	3.1 ± 1.1[b]	1.4 ± 0.1	53.1 ± 6.5	283.0 ± 4.1[b]
DM Ex 8	2.3 ± 0.2[b]	3.0 ± 0.9[b]	1.5 ± 0.1	44.2 ± 4.8	258.0 ± 8.7[b]

Measures of blood-borne factors in all groups. *Non-DM*: combined values for Non-DM control, Ex4 and Ex8 animals (n=30). *DM*: combined values for DM-control, DM-Ex4 and DM-Ex8 animals (n=30). Also represented are the 6 individual groups (n=10). Values are means ± SEM; n=10/group. [a]Significantly different ($P<0.05$) from Non-DM. [b]Significantly different ($P<0.05$) from Non-DM-relative control.

Table 2.2. Body, Adipose and Lean Tissue Mass

Group	Body Mass (g)	Adipose Mass (g)	Lean Mass (g)
Non-DM	512 ± 7.6	47.2 ± 2.5	414 ± 6.1
DM	352 ± 7.7[a]	16.4 ± 0.8[a]	289 ± 6.8[a]
Control	524 ± 15.3	53.7 ± 3.2	419 ± 13.0
Ex 4	497 ± 11.9	43.2 ± 3.4	403 ± 10.2
Ex 8	514 ± 12.1	45.0 ± 5.4	419 ± 8.3
DM	341 ± 14.5[b]	16.4 ± 1.3[b]	279 ± 13.1[b]
DM Ex 4	351 ± 12.7[b]	15.4 ± 1.3[b]	288 ± 10.1[b]
DM Ex 8	366 ± 12.6[b]	17.5 ± 1.7[b]	302 ± 11.3[b]

Measures of body, adipose and lean tissue mass in all groups. *Non-DM*: combined values for Non-DM control, Ex4 and Ex8 animals (n=30). *DM*: combined values for DM control, Ex4 and Ex8 animals (n=30). Also represented are the 6 individual groups (n=10). Values are means ± SEM; n=10/group. [a]Significantly different ($P<0.05$) from Non-DM. [b]Significantly different ($P<0.05$) from Non-DM-relative control.

Figure 2.4*A*. Absolute heart mass following 8 weeks of DM and/or exercise. Heart mass was significantly reduced ($P < 0.05$) in DM animals, regardless of exercise. In Non-DM animals, 8 weeks of exercise increased absolute heart mass compared to controls. Data expressed as mean ± SEM (n = 10). *Significantly less ($P < 0.05$) mass than control and non-DM exercised animals. † Significantly greater ($P < 0.05$) than Non-DM controls.

Figure 2.4*B*. Heart mass expressed relative to tibial length following DM and/or exercise. Heart mass-to-tibial length ratio was significantly reduced ($P < 0.05$) in DM animals, regardless of exercise. Data expressed as mean ± SEM (n = 10). *Significantly less ($P < 0.05$) mass-to-tibial length than Non-DM exercised animals.

Figure 2.4*A*

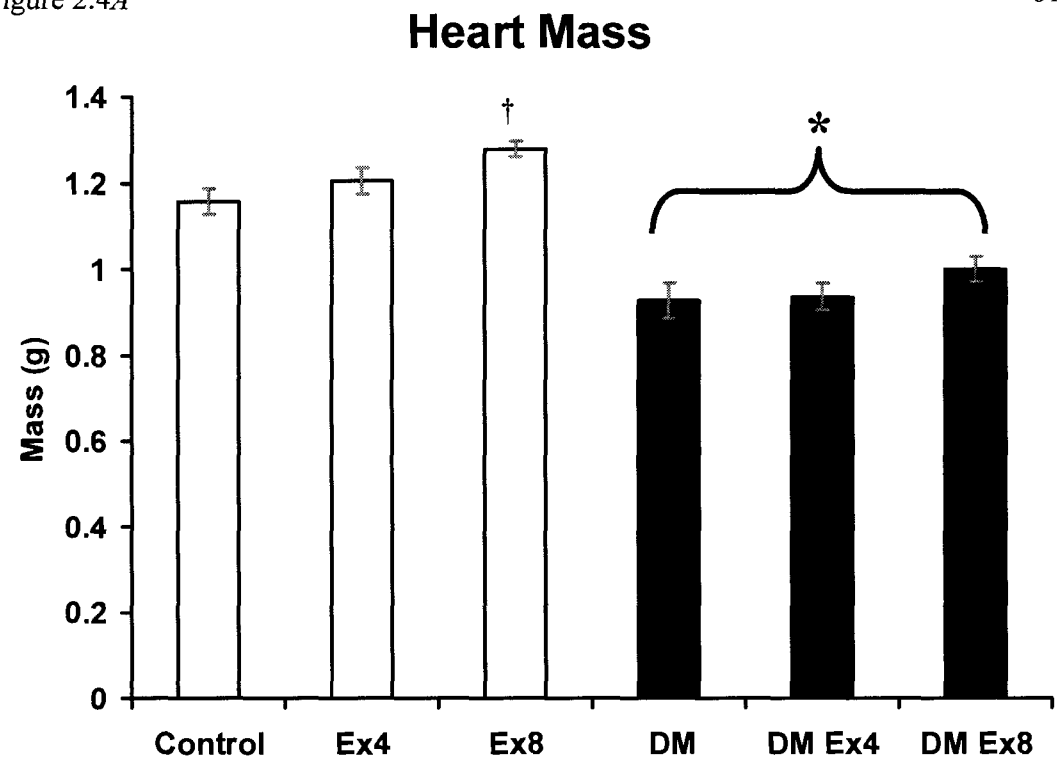


Figure 2.4*B*

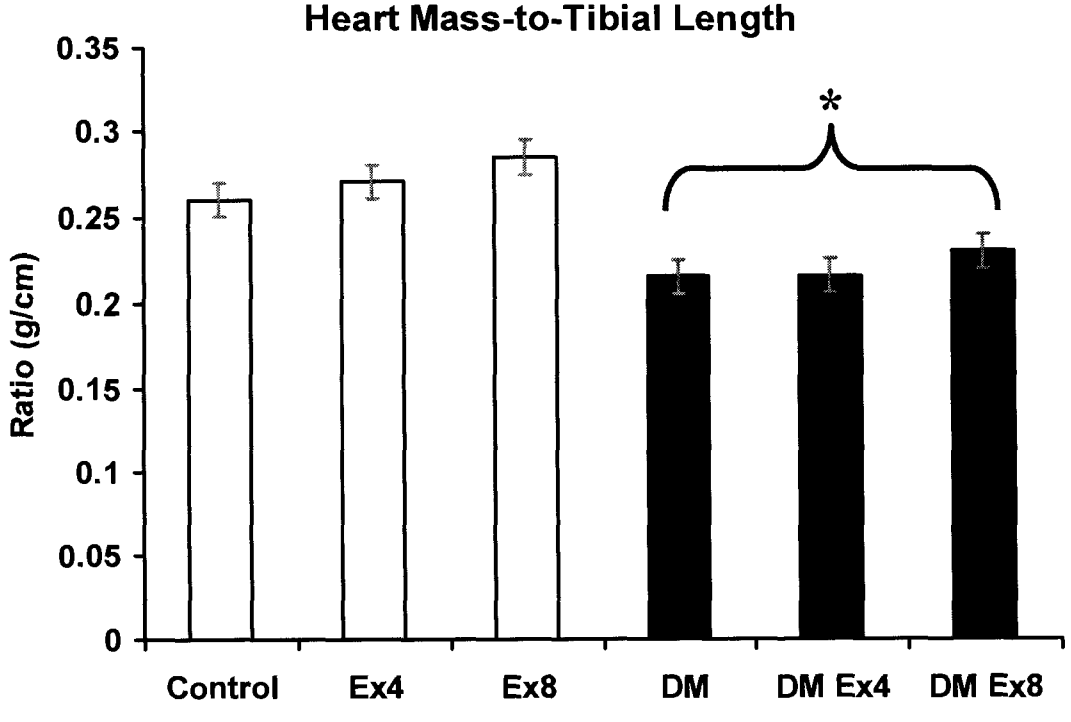


Figure 2.5. Gastrocnemius muscle mass-to-tibial length ratio. Eight weeks following DM induction, growth of gastrocnemius muscles were significantly attenuated ($P < 0.05$). Exercise did not affect muscle growth attenuation. Data expressed as mean ± SEM (n = 10). *Significantly reduced ($P < 0.05$) gastrocnemius mass-to-tibial length ratio than Non-DM exercised animals.

Figure 2.5

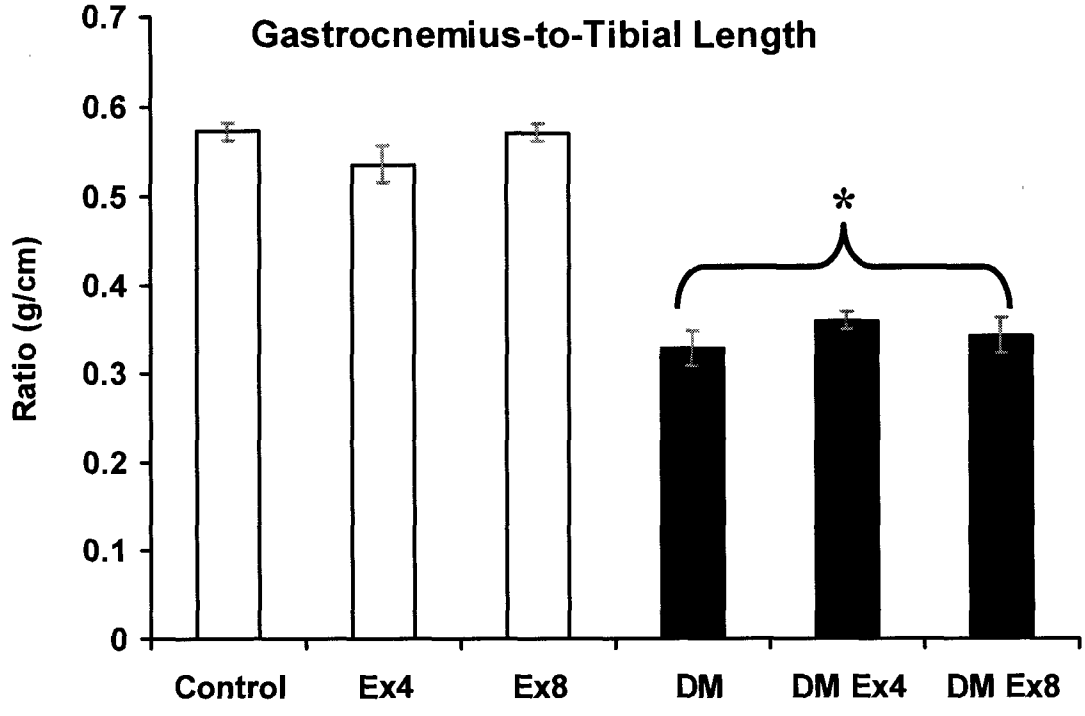

Gastrocnemius-to-Tibial Length
Ratio (g/cm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Control
Ex4
Ex8
DM
DM Ex4
DM Ex8

Figure 2.6. Plantaris muscle mass-to-tibial length ratio. Eight weeks of DM significantly reduced ($P < 0.05$) plantaris muscle mass was when compared to Non-DM animals. Exercise did not increase muscle mass. Data expressed as mean ± SEM (n = 10). *Significantly reduced ($P < 0.05$) plantaris mass-to-tibial length ratio compared to Non-DM exercised animals.

Figure 2.6

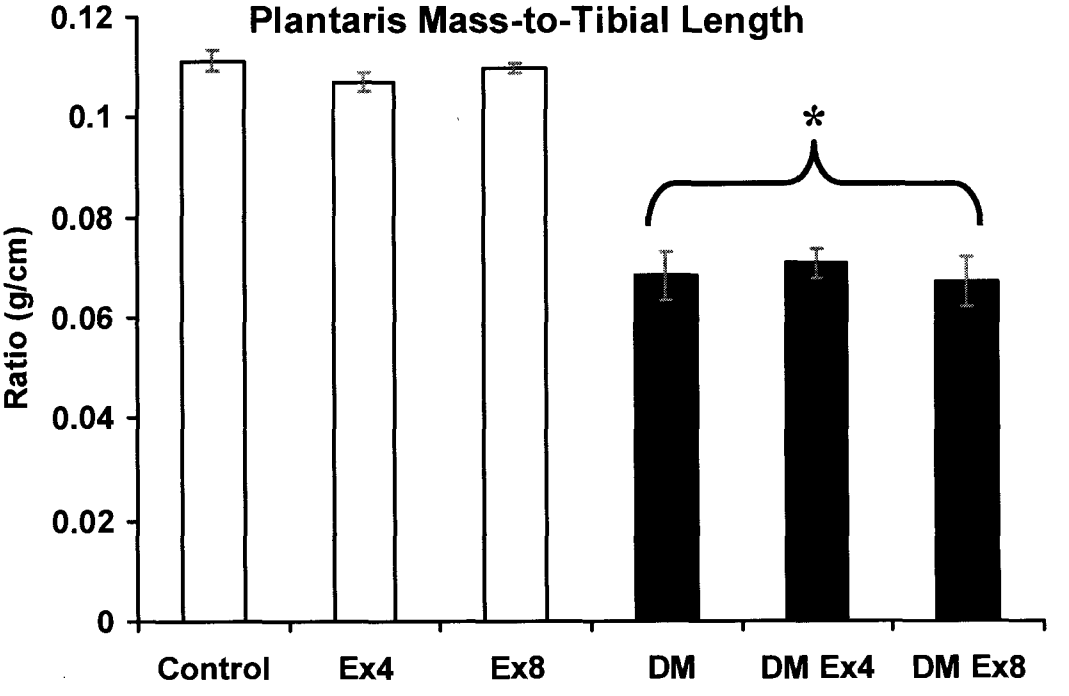

Plantaris Mass-to-Tibial Length
Ratio (g/cm)
0.12
0.1
0.08
0.06
0.04
0.02
0
*
Control
Ex4
Ex8
DM
DM Ex4
DM Ex8

Figure 2.7. Soleus muscle mass-to-tibial length ratio. The highly oxidative soleus muscle demonstrated significantly reduced ($P < 0.05$) mass was when compared to Non-DM animals. Exercise initiated at either the onset or 4 weeks after DM induction did not increase muscle mass. Data expressed as mean ± SEM (n = 10). *Significantly reduced ($P < 0.05$) soleus mass-to-tibial length ratio compared to Non-DM exercised animals.

Figure 2.7

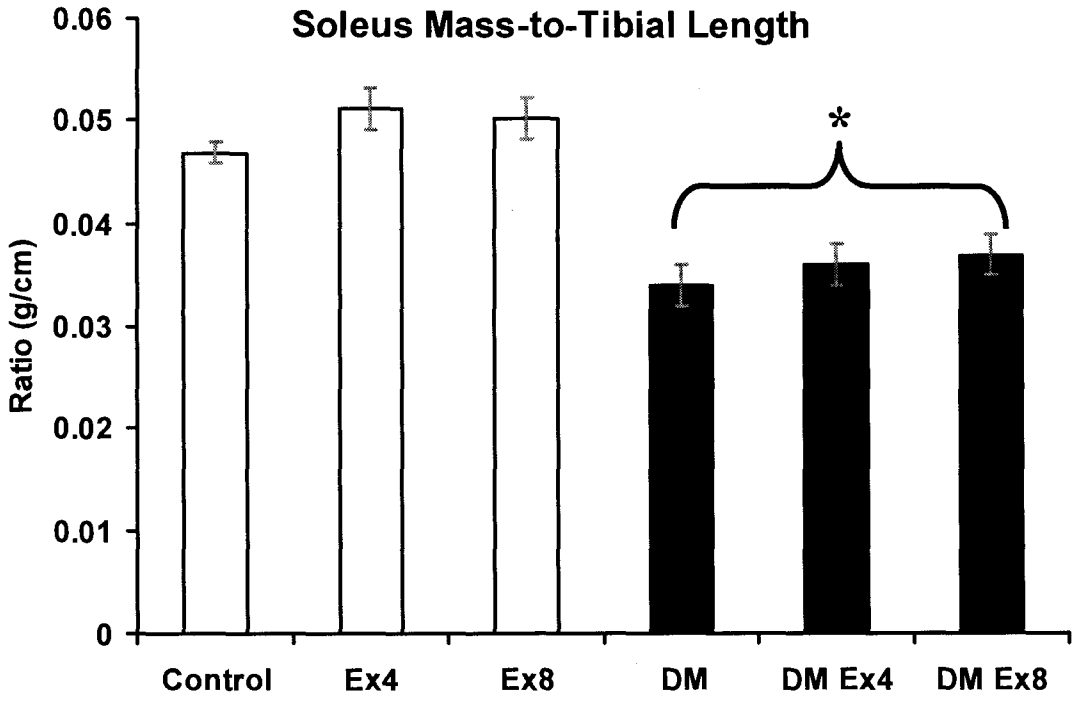

Soleus Mass-to-Tibial Length
Ratio (g/cm)
0.06
0.05
0.04
0.03
0.02
0.01
0
*
Control
Ex4
Ex8
DM
DM Ex4
DM Ex8

Figure 2.8. Extensor Digitorum Longus muscle mass-to-tibial length ratio. The highly glycolytic extensor digitorum longus muscle demonstrated significantly reduced ($P < 0.05$) mass was when compared to Non-DM animals. Exercise initiated at either the onset or 4 weeks after DM induction did not increase muscle mass. Data expressed as mean ± SEM (n = 10). *Significantly reduced ($P < 0.05$) extensor digitorum longus mass-to-tibial length ratio compared to Non-DM exercised animals.

Figure 2.8

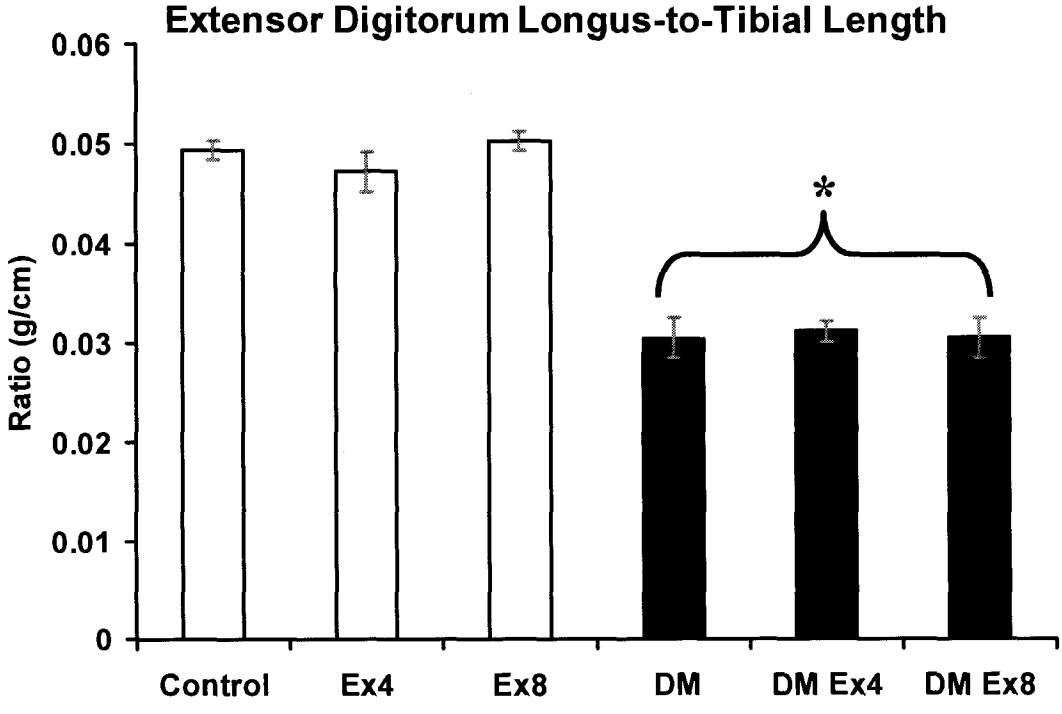

Extensor Digitorum Longus-to-Tibial Length
Ratio (g/cm)
0.06
0.05
0.04
0.03
0.02
0.01
0
*
Control
Ex4
Ex8
DM
DM Ex4
DM Ex8

Figure 2.9. Tibialis Anterior muscle mass-to-tibial length ratio. The tibialis anterior muscle demonstrated significantly reduced ($P < 0.05$) mass was when compared to Non-DM animals. Exercise initiated at either the onset or 4 weeks after DM induction did not increase muscle mass. Data expressed as mean ± SEM (n = 10). *Significantly reduced ($P < 0.05$) tibialis anterior mass-to-tibial legnth ratio compared to Non-DM exercised animals.

Figure 2.9

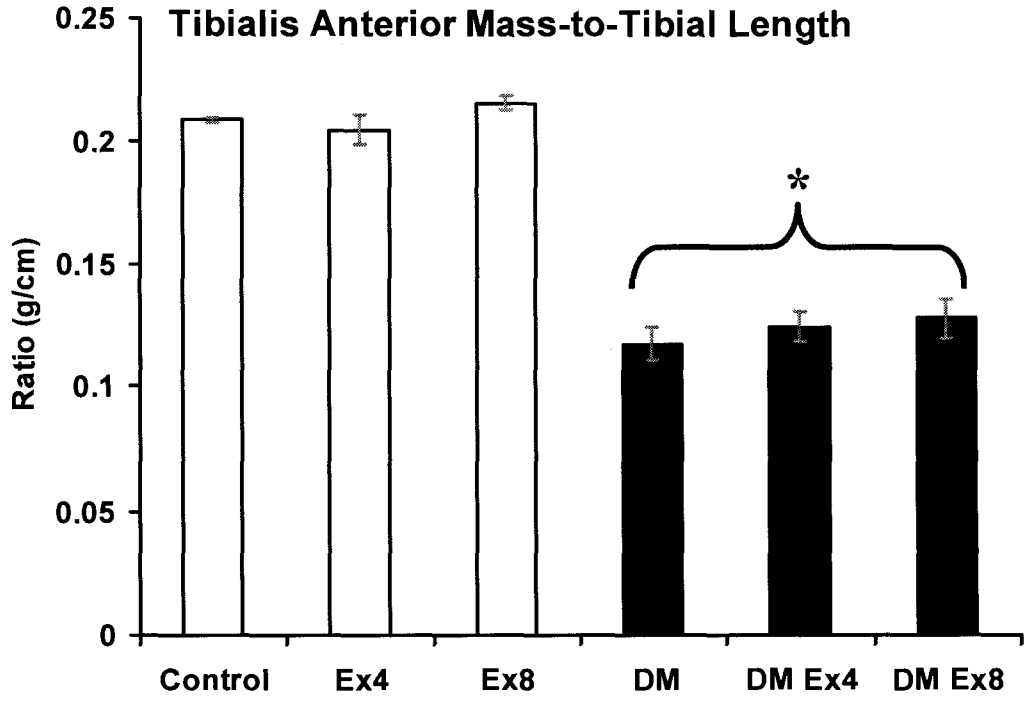

Tibialis Anterior Mass-to-Tibial Length
Ratio (g/cm)
0.25
0.2
0.15
0.1
0.05
0
*
Control
Ex4
Ex8
DM
DM Ex4
DM Ex8

Figure 2.10*A*. A comparison of absolute kidney mass between DM and Non-DM animals. Kidney mass was significantly increased ($P < 0.05$) after 8 weeks of DM. Exercise did not affect kidney mass. Data expressed as mean ± SEM (n = 10). *Significantly elevated ($P < 0.05$) kidney mass compared to Non-DM exercised animals.

Figure 2.10*B*. Kidney mass-to-tibial length ratio following DM induction and/or exercise. Organ-to-tibial length ratio was significantly elevated ($P < 0.05$) after 8 weeks of DM. Exercise did not affect organ mass-to-tibial length ratio. Data expressed as mean ± SEM (n = 10). *Significantly elevated ($P < 0.05$) kidney mass ratio compared to Non-DM exercised animals.

Figure 2.10*A*

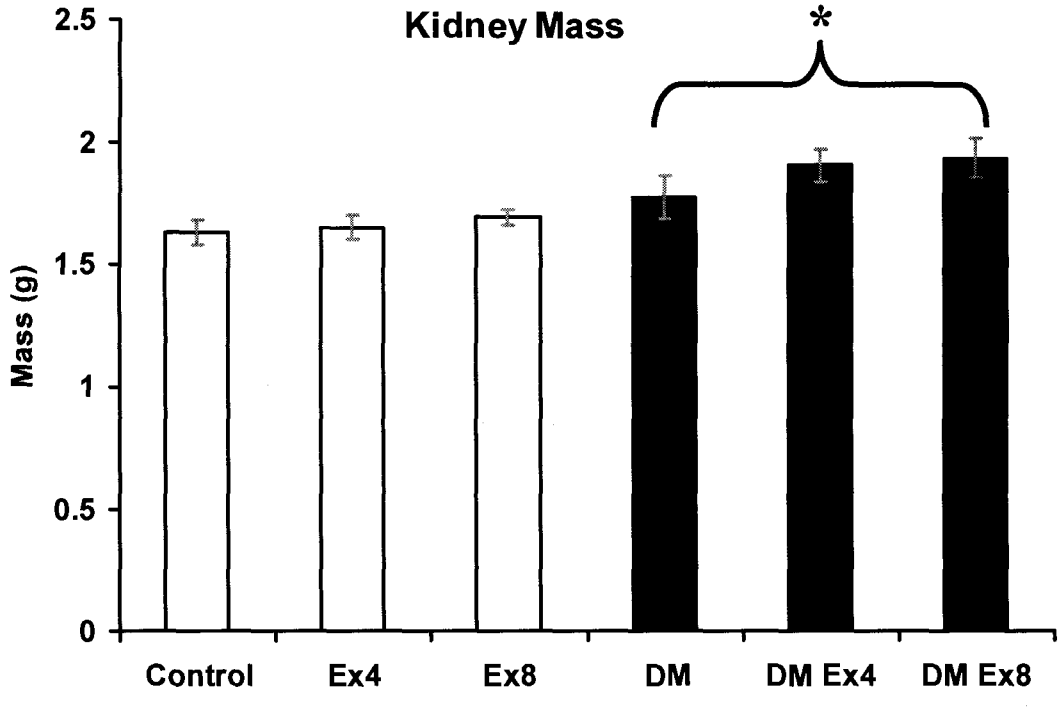


Figure 2.10*B*

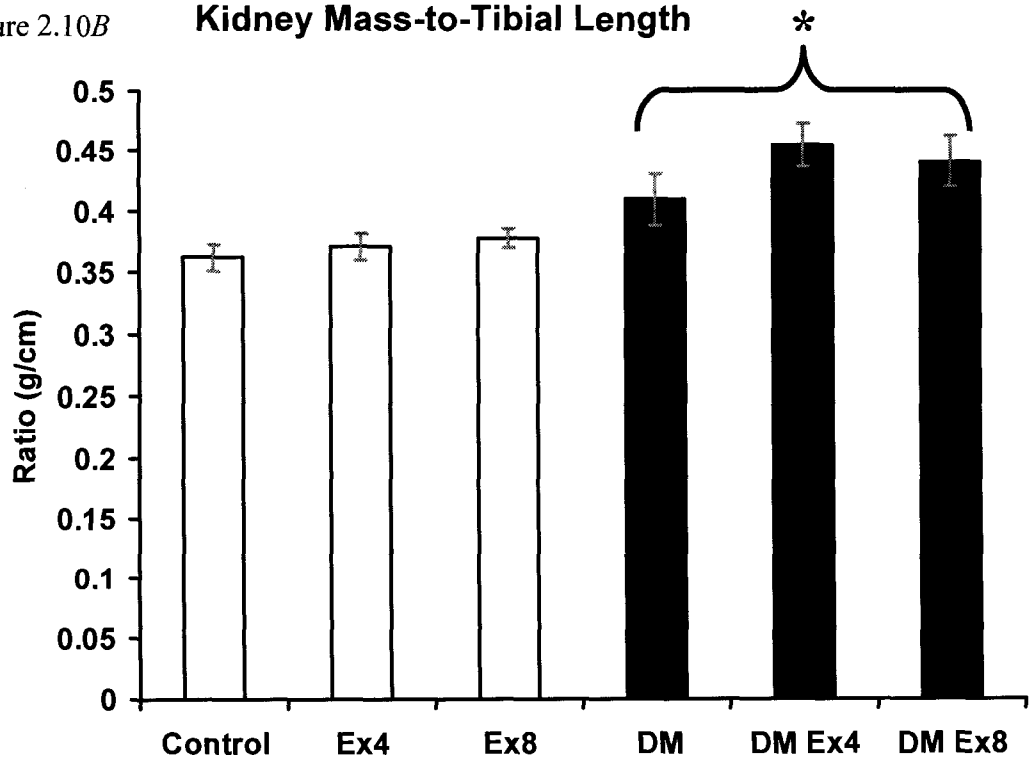


Figure 2.11*A*. A comparison of absolute adrenal gland mass between DM and Non-DM animals. Adrenal gland mass was significantly increased ($P < 0.05$) after 8 weeks of DM. Exercise did not affect gland mass. Data expressed as mean ± SEM (n = 10). *Significantly elevated ($P < 0.05$) adrenal gland mass compared to Non-DM exercised animals.

Figure 2.11*B*. Adrenal gland mass-to-tibial length ratio following DM induction and/or exercise. Gland-to-tibial length ratio was significantly elevated ($P < 0.05$) after 8 weeks of DM. Exercise did not affect gland mass ratio. Data expressed as mean ± SEM (n = 10). *Significantly elevated ($P < 0.05$) adrenal gland mass ratio compared to Non-DM exercised animals.

Figure 2.11*A*

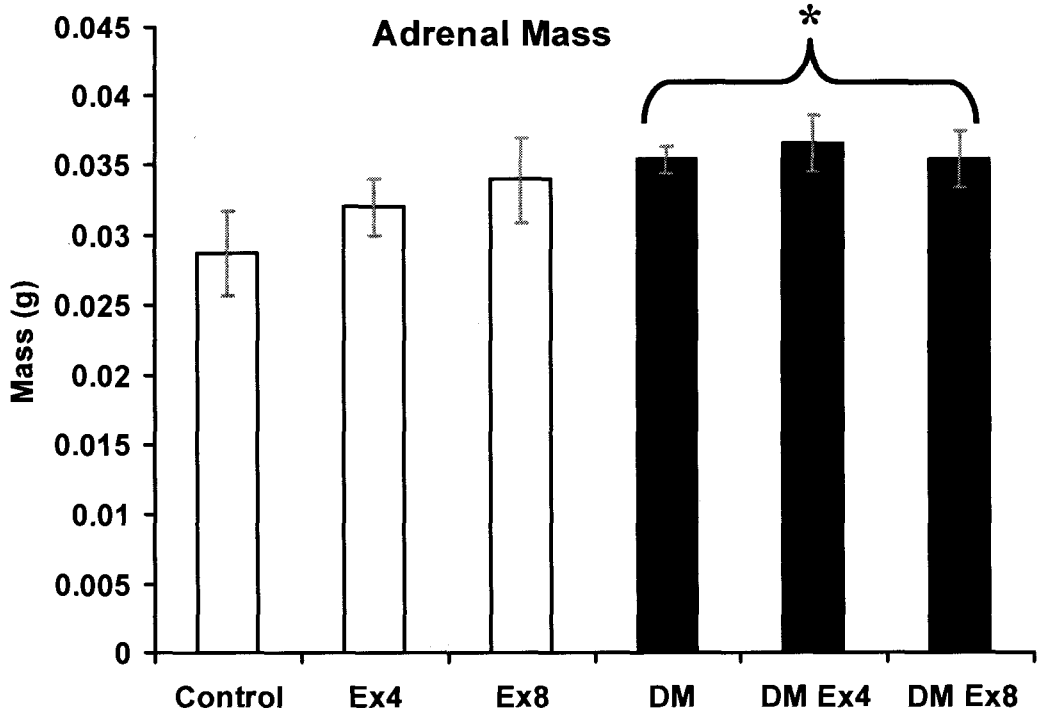


Figure 2.11*B*

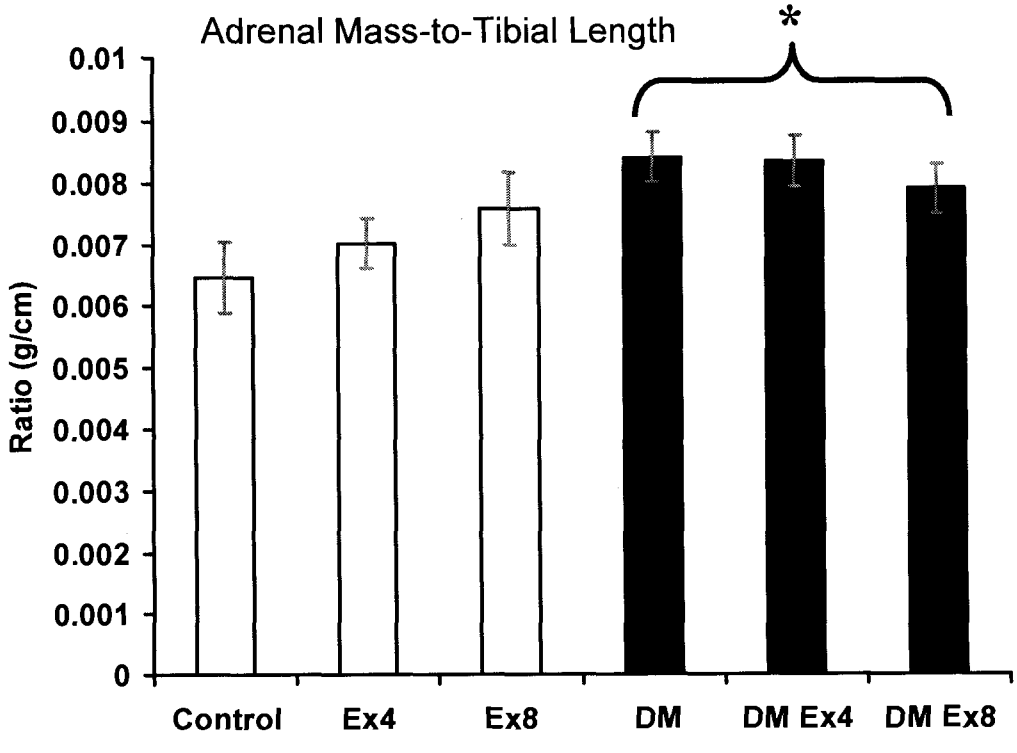


Figure 2.12*A*. Total bone content of inorganic hydroxyapatite. Absolute mineral content was significantly diminished ($P < 0.05$) in DM animals. Values obtained following exercise did not demonstrate improved bone mineral content. Data expressed as mean ± SEM (n = 10). *Significantly reduced ($P < 0.05$) bone content of hydroxyapatite compared to Non-DM animals.

Figure 2.12*B*. Whole body bone mineral content expressed relative to tibial length. Bone mineral content was significantly reduced ($P < 0.05$) in DM animals. Exercise initiated at the onset of, or 4 weeks after, DM did not demonstrate an improved bone mineral content-to-tibial length ratio. Data expressed as mean ± SEM (n = 10). *Significantly reduced ($P < 0.05$) bone content of hydroxyapatite indexed to tibial length when compared to Non-DM exercised animals.

Figure 2.12*A*

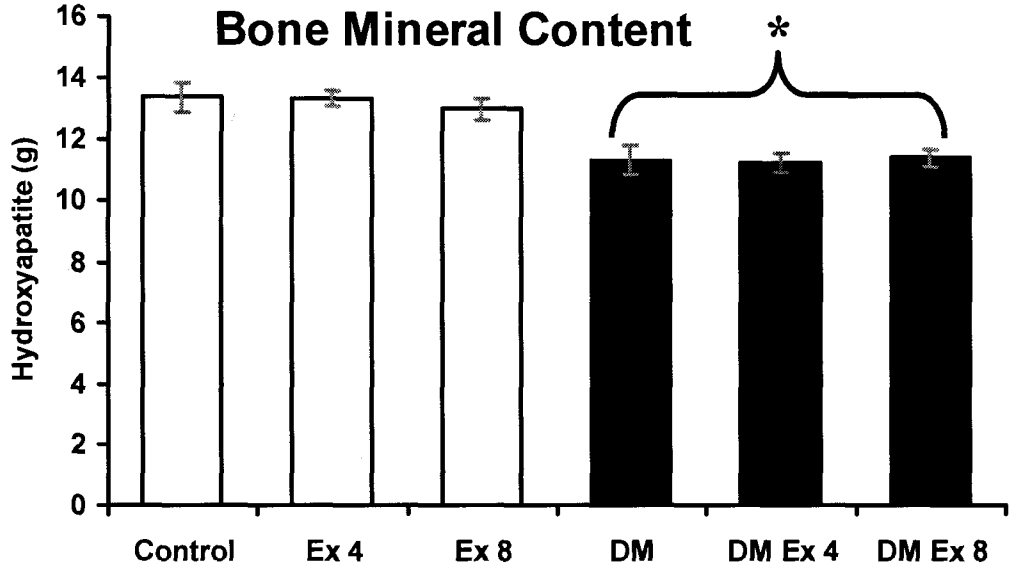
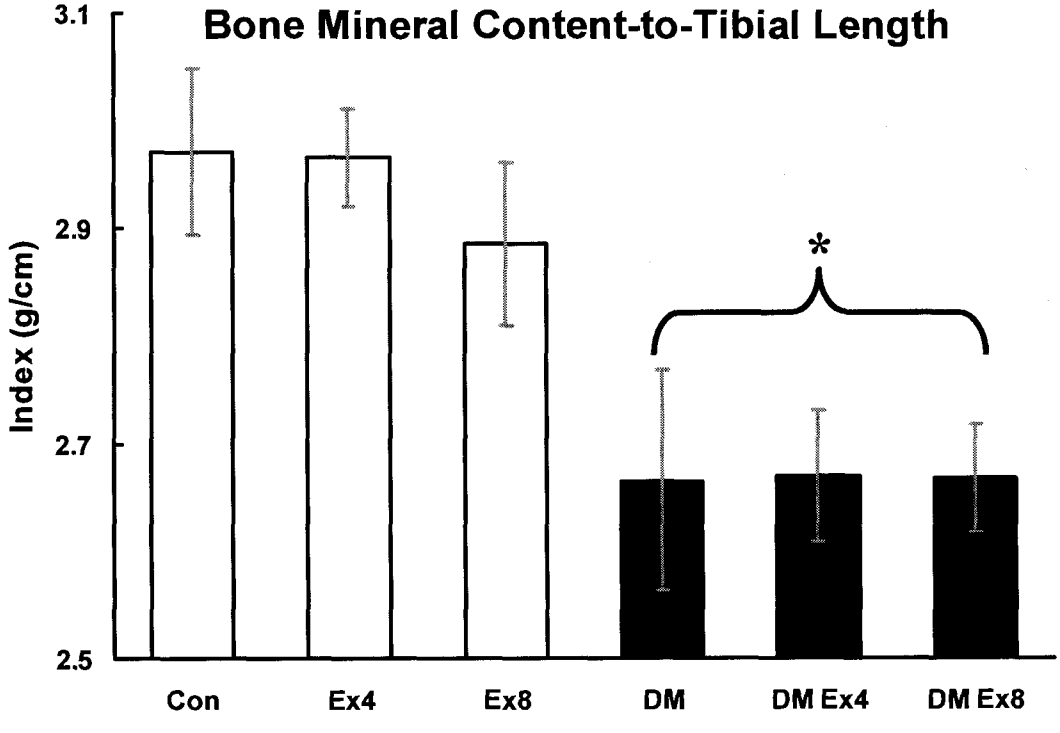


Figure 2.12*B*



2.4 DISCUSSION

It is well established that severe complications develop as a natural progression of diabetes, which involve multiple systems, structures and organs. However, exercise is consistently associated with improved function and protein expression in most tissues, even amidst a formidable diabetic pathology (Hsieh and Wang, 2005; Hersey et al., 1994). The present study was predicated on the assumption that the severity of diabetic complications may be ameliorated by exercise. We determined whether DM-induced anthropomorphic changes and elevated risk factors may be prevented by introducing exercise at the onset of DM and also determined whether complications in established DM may be reversed with exercise. There were four main findings in the present study. First, unfavorable blood lipids and risk factors (cholesterol, triglycerides and glycated proteins) were elevated, whereas HDL and cortisol were both unchanged by DM. This pattern was unaltered by exercise. Second, growth of all muscles (cardiac and skeletal) was reduced by DM and exercise did not normalize the growth of DM muscle. Third, kidney and adrenal hypertrophy were observed in DM animals and exercise did not attenuate this growth. Fourth, bone mineral content was reduced in DM animals and did not improve with exercise.

Blood measures of risk factors. The model employed in this study is well established (Wei et al, 2003; Like and Rossini, 1976; Junod et al., 1969) and consistently results in the selective destruction of pancreatic beta cells. Based on previous reports (Geethan and Prince, 2008; Amatyakul et al., 2006; ADA, 2003; Halle, 1999), and a globally elevated reliance on fat metabolism, it was assumed that DM would elevate total cholesterol,

triglycerides, cortisol and glycated proteins and decrease HDL levels. However, it was uncertain whether exercise would reduce elevated risk factors, as previous reports demonstrated both positive (Halle, 1999) and negative (Ribeiro et al., 2008) outcomes following exercise in humans and positive effects in the STZ-DM model (Amatyakul et al., 2006; Koivisto et al, 1974). For instance, Amatyakul et al. (2006) demonstrated reduced triglycerides employing a low-intensity (15m/min) exercise program with a similar STZ model, whereas the present study showed a trend towards reduced triglycerides, but did not demonstrate a significant difference in triglycerides or any other measured risk factors. It may be that the triglyceride-lowering effect of exercise is dependent on exercise-intensity. Indeed, serum triglyceride levels are dependent on lipoprotein lipase levels and activity, up-regulated by low-intensity exercise (20m/min), and unchanged by high-intensity exercise (30m/min; Hildebrandt et al., 2003) in rats. Nonetheless, it is likely that an improved lipid profile coincides with improved glucose control, as previously shown in the STZ model (Howarth et al., 2007; Amatyakul et al., 2006) and in humans (Halle, 1999), whereas blood glucose remained unchanged in the present study. Moreover, diabetic complications are highly correlated with the percentage of glycated proteins, which spontaneously accumulate in the blood in parallel with glucose concentrations. Since glucose was not decreased following exercise in our study it was reasonable that fructosamines remained unchanged.

Serum levels of HDL cholesterol were unchanged by any treatment in the present study. Geethan and Prince (2008) reported reduced quantities of HDL in the STZ rat, as did Eliza et al. (2008) and Bagri et al. (2008). However, these studies employed Wistar rats, whereas the present study employed Sprague-Dawley rats, which may suggest a

strain-specific lipoprotein profile and exercise response. In support, Morikawa et al. (2007) reported that STZ-induced DM resulted in increased HDL levels in Sprague-Dawley rats. However, both Morikawa et al. (2007) and Geethan and Prince (2008) obtained much lower control levels of HDL compared to the present study; therefore, values obtained in the present study may reflect an increased sensitivity and reduced specificity to HDL cholesterol. However, triglycerides and total cholesterol were increased by DM in the present study, consistent with other reports, and were well within the accepted range for accurately detecting HDL cholesterol when using the Hitachi 911 analyzer (Cobbaert et al., 1998).

Cortisol is secreted via sympathetic activation and was previously shown to be elevated in the STZ model (Radahmadi et al., 2006). Since sympathetic activation has been shown to be reduced following exercise in rats (Bertagnolli et al., 2008) it was expected that cortisol would be elevated with DM and reduced with exercise. The present study showed no difference in cortisol levels, regardless of DM or exercise. However, the study by Radahmadi et al. (2006) revealed much lower cortisol levels in both DM and Non-DM animals (~20 and ~30 nmol/L, respectively) compared with the present study (~45 nmol/L), which may more easily demonstrate significant exercise-induced alterations by requiring less absolute change and may also indicate a strain-specific sympathetic activity and response to exercise (Sprague-Dawley vs. Wistar). Interestingly, binding affinity for 5-HT1A (pituitary-adrenocortical regulator; reviewed by Fuller, 1996) was shown to be different between Sprague-Dawley and Wistar rats (Paré and Tejani-Butt, 1996); hence cortisol-sensitivity to exercise may be partly strain-specific.

Overall, risk factors were either elevated by DM (cholesterol, triglycerides, fructosamines) or remained unchanged (HDL, cortisol). None of the factors were affected by exercise. Taken together, these findings may be a consequence of severe, chronic hyperglycemia/hypoinsulinemia, and factors unaltered by exercise may also reflect an inherently high production and secretion of specific lipid and sympathetic factors in this model of type I DM.

Altered body composition and muscle mass. Type 1 DM in the general population, as well as in the present model of DM, is known to reduce body mass, including lean tissue and especially adipose tissue (Rosenfalck et al., 2002), and also induces skeletal muscle atrophy (Aughsteen et al., 2006; Markuns et al., 1999; Medina-Sanchez, 1991; Armstrong et al., 1975). However, skeletal muscle atrophy may be fibre-type specific in this model of DM, as muscle loss in slow twitch fibres was shown to be minimized, while atrophy of fast twitch fibres was enhanced (Armstrong et al., 1975). In fact, muscle hypertrophy has been demonstrated in type 1 fibres in DM rats (Medina-Sanchez, 1991). Therefore, it was postulated that skeletal muscle mass may be preserved or enhanced following exercise in a muscle-specific manner. However, the results indicated that all DM muscles, including heart muscle, had significantly less mass. Exercise did not preserve or increase mass in any DM muscle. Markuns et al. (1999) showed that DM interrupts several transcriptional regulators, and a physiological level of insulin is necessary to restore transcriptional signaling. In addition, both Armstrong (1975) and Medina-Sanchez (1991) demonstrated preserved and hypertrophied slow fibres, respectively, in muscles composed of a mixed fibre-type. Hence, atrophy of the whole muscle was likely due to atrophy of the much

larger glycolytic fibres, leading to a global reduction in muscle mass, regardless of maintained or enhanced slow fibre mass. Nonetheless, the soleus muscle demonstrated attenuated muscle growth in the present study, despite being composed largely of a slow fibre-type, as previously reported (Frier et al., 2008; Najemnikova et al., 2007).

Copray et al. (2000) reported that the soleus in DM rats is highly susceptible to contraction-mediated damage and the associated atrophy may be related to reduced cations and force production (Chonkar et al., 2006). Therefore, the observation that DM attenuated muscle growth in all muscle types is consistent with previous findings and the pathophysiology of DM in general. Although resistance exercise has been shown to increase soleus mass in DM rats (Farrell et al., 1999), a lack of improvement from endurance exercise in the present study may reflect either an insufficient intensity or enhanced catabolism due to the prolonged nature of the exercise protocol and given a deficiency of glucose availability.

Enhanced organ mass. In contrast to attenuated muscle growth, both the kidney and the adrenal glands demonstrated elevated mass in DM animals. Elevated mass in both kidney and adrenal glands is a commonly reported finding in STZ-induced DM in rats (Pillion et al., 1988; Wehbi et al., 2001; Päivärinne and Kainulainen, 2001; Segev et al., 1997; Rebuffat et al., 1988; Zelena et al., 2006). A distinguishing feature of these 2 organs vs. skeletal muscle is a lack of dependence on insulin for glucose uptake; instead, these organs rely more on a sodium-glucose co-transporter or Glut-1 rather than the insulin-sensitive Glut-4 in skeletal muscle (Duelli and Kuschinsky, 2001; Hediger and Rhoads, 1994; Gould et al., 1994). Increased kidney mass in DM may be related to several

signaling pathways, including reduced angiotensin-2 receptors (Wehbi et al., 2001), accumulated IGF-1 protein and binding protein (Segev et al., 1997), increased TGF-1 (Sharma and Ziyadeh, 1995; Meier et al., 2007) and increased mTOR activity (Lee et al., 2007). In addition to altered cellular signaling, hyperglycemia per se may increase the glucose gradient causing increased glucose uptake, thereby providing excess substrate and anabolic incentive to the kidney and the adrenals.

Hyperglycemia was the primary correlate associated with increased adrenal mass in STZ-induced DM (Zelena et al., 2006). Hypertrophied adrenal glands in DM show increased mitochondrial volume (Rebuffat et al., 1988) and elevated epinephrine content (Schmidt et al., 1992), suggesting that increased mass is a true hypertrophy rather than a simple effect of increased water and glucose content. In addition to DM-augmented adrenal mass, the present study also showed a trend toward increased adrenal mass induced by exercise, independent of DM. In support, other investigators reported a significant exercise-induced adrenal hypertrophy (Schmidt et al., 1992; Watanabe et al., 1991; Stallknecht et al.,1990), including treadmill exercise using the same intensity (27m/min) used in the present study (Buuck and Tharp, 1971). However, these reports generally report hypertrophy on the basis of greater volume (mm^3/kg) as opposed to increased weight. In contrast, Emter et al. (2005) reported no change in adrenal mass following 6 months of treadmill exercise (~15m/min).

Anatomical responses of the kidney and adrenal gland to DM demonstrated a primary sensitivity to hyperglycemia and/or hypoinsulinemia. The fact that both the kidney and adrenals are not dependent on insulin signaling for glucose uptake indicates that restoration of organ mass via insulin treatment is most likely due to the restoration of

blood glucose levels (Olbricht et al.,1992; Rebuffat et al., 1988); however intracellular signaling pathways may still depend on insulin for activation.

Reduced Bone Mineral Content. Absolute and standardized BMC was reduced in DM animals. BMC values standardized to tibial length not only demonstrates that mineral contents are reduced disproportionately to the size of the animal, but indicates that for a given length of bone normal structural integrity provided by a given amount of mineral content is also reduced. The implication for bone health is an increased likelihood of fractures, as evidenced by reduced BMC or mineral density and its correlation with increased fractures in type 1 and type 2 DM (reviewed by Strotmeyer and Cauley, 2007).

STZ-induced DM typically results in diminished BMC (Hamada et al., 2007; Martin and McCabe, 2007; Del Pino-Montes, 2004). Hyperglycemia has been shown to activate several pathways that influence osteoblast activity (Botolin and McCabe, 2006) and may attenuate osteoblast differentiation (Botolin et al., 2005). These researchers also reported increased bone adiposity, which may be exacerbated by elevated blood lipids in DM.

Weight bearing activity is required and is a rate limiting step for progressively increasing bone mineral content. Hence, exercise is an appropriate prescription to removing this limiting step. Indeed, exercise has been shown to increase bone mineral content in rats (Hagihara et al., 2005; Yeh et al., 1993b). However, no increase in BMC was found following exercise in normal animals in the present study. This may be the result of significantly greater exercise intensity compared to the intensity of previous studies showing augmented BMC (15m/min: Hagihara et al., 2005; 20m/min: Yeh et al.,

1993a). In addition, reduced BMC in DM animals was not improved following exercise in the present study, a finding also shown previously by Verhaeghe et al. (2000), when using a variety of exercise intensities (20-60 m/min). Irwin et al. (2006) demonstrated that the loss of insulin in animals with Type 1 diabetes did not prevent normal BMD formation. Hence, the lack of a positive exercise effect on BMC may not reflect an inability to compensate for a lack of insulin, but may reflect the ineffectiveness of exercise to improve glucose and reduce levels of risk factors observed in the present study.

2.5 CONCLUSION

In summary, DM led to a clear elevation of blood-borne risk factors, reduced heart and skeletal muscle mass, increased organ mass and diminished BMC. Although the animals were able to maintain exercise competency there was limited benefit derived from the activity. A possibility exists that the exercise intensity prescribed in the present study was greater than necessary to elicit positive changes and may been an added, detrimental stress rather than a stimulating, beneficial stress. These findings reveal the consequences of blood glucose accumulation and elevated lipids from hypoinsulinemia. On the basis that exercise was unable to reduce blood glucose, cholesterol or triglycerides - and normalized blood glucose and lipids are a *sine qua non* for normalized health - it seems reasonable that exercise was unable to normalize heart and skeletal muscle, or organ and bone mass. It appears that the benefit of exercise may reside in an ability to modify and enhance viable pathways, but in severe, uncompensated DM, exercise may be without direct influence as a flood of glucose dilutes its gesture and weakens its authority.

2.6 REFERENCES

Albright A, Franz M, Hornsby G, Kriska A, Marrero D, Ullrich I, Verity LS. American College of Sports Medicine position stand. Exercise and type 2 diabetes. *Med Sci Sports Exerc*. 2000; 32:1345–1360.

Amatyakul S, Chakraphan D, Chotipaibulpan S, Patumraj S. Role of exercise training on pulpal blood flow in diabetic rats. Clin Hemorheol Microcirc. 2006; 34(1-2):295-301.

American Diabetes Association Diagnosis and classification of diabetes mellitus. *Diabetes Care* 29. 2006; (Suppl 1):S43–S48.

American Diabetes Association: Management of dyslipidemia in adults with diabetes (Position Statement). *Diabetes Care* 26. 2003; (Suppl. 1):S83–S86.

Armstrong RB, Phelps RO. Muscle fiber type composition of the rat hindlimb. *Am J Anat*. 1984; 171(3):259-272.

Armstrong RB, Gollnick PD, Ianuzzo CD. Histochemical properties of skeletal muscle fibers in streptozotocin-diabetic rats. *Cell Tissue Res*. 1975; 162(3):387-394.

Aughsteen AA, Khair AM, Suleiman AA. Quantitative morphometric study of the skeletal muscles of normal and streptozotocin-diabetic rats. *JOP*. 2006; 7(4):382-9.

Bagri P, Ali M, Aeri V, Bhowmik M, Sultana S. Antidiabetic effect of Punica granatum flowers: Effect on hyperlipidemia, pancreatic cells lipid peroxidation and antioxidant enzymes in experimental diabetes. *Food Chem Toxicol*. 2008; Oct 4.

Bertagnolli M, Schenkel PC, Campos C, Mostarda CT, Casarini DE, Belló-Klein A, Irigoyen MC, Rigatto K. Exercise Training Reduces Sympathetic Modulation on Cardiovascular System and Cardiac Oxidative Stress in Spontaneously Hypertensive Rats. *Am J Hypertens*. 2008; Sep 11.

Botolin S, Faugere MC, Malluche H, Orth M, Meyer R, McCabe LR. Increased bone adiposity and peroxisomal proliferato ractivated receptor-gamma2 expression in type I diabetic mice. *Endocrinology*. 2005; 146:3622–3631

Botolin S, McCabe LR. Chronic hyperglycemia modulates osteoblast gene expression through osmotic and non-osmotic pathways. *J Cell Biochem*. 2006; 99(2):411-424.

Braith RW, Mills RM, Welsch MA, Keller JW, Pollock ML. Resistance exercise training restores bone mineral density in heart transplant recipients. *J Am Coll Cardiol*. 1996; 28(6):1471-1477.

Buuck RJ, Tharp GD. Effect of chronic exercise on adrenocortical function and structure in the rat. *J Appl Physiol*. 1971; 31(6):880-883.

Chonkar A, Hopkin R, Adeghate E, Singh J. Contraction and cation contents of skeletal soleus and EDL muscles in age-matched control and diabetic rats. *Ann N Y Acad Sci.* 2006; 1084:442-51.

Cobbaert C, Zwang L, Ceriotti F, Modenese A, Cremer P, Herrmann W, Hoss G, Jarausch J, Türk R, März W, Nauck M. Reference standardization and triglyceride interference of a new homogeneous HDL-cholesterol assay compared with a former chemical precipitation assay. *Clin Chem.* 1998; 44(4):779-789.

Copray S, Liem R, Brouwer N, Greenhaff P, Habens F, Fernyhough P. Contraction-induced muscle fiber damage is increased in soleus muscle of streptozotocin-diabetic rats and is associated with elevated expression of brain-derived neurotrophic factor mRNA in muscle fibers and activated satellite cells. *Exp Neurol.* 2000; 161(2):597-608.

Del Pino-Montes J, Benito GE, Fernández-Salazar MP, Coveñas R, Calvo JJ, Bouillon R, Quesada JM. Calcitriol improves streptozotocin-induced diabetes and recovers bone mineral density in diabetic rats. *Calcif Tissue Int.* 2004; 75(6):526-532.

Dennison EM, Syddall HE, Aihie Sayer A, Martin HJ, Cooper C; Hertfordshire Cohort Study Group. Lipid profile, obesity and bone mineral density: the Hertfordshire Cohort Study. *QJM.* 2007; 100(5):297-303.

Duelli R, Kuschinsky W. Brain glucose transporters: relationship to local energy demand. *News Physiol Sci.* 2001; 16:71-76.

Emter CA, McCune SA, Sparagna GC, Radin MJ, Moore RL. Low-intensity exercise training delays onset of decompensated heart failure in spontaneously hypertensive heart failure rats. *Am J Physiol Heart Circ Physiol.* 2005; 289(5):H2030-2038.
Farrell PA, Fedele MJ, Hernandez J, Fluckey JD, Miller JL 3rd, Lang CH, Vary TC, Kimball SR, Jefferson LS. Hypertrophy of skeletal muscle in diabetic rats in response to chronic resistance exercise *J Appl Physiol.* 1999; 87(3):1075-82.

Felig P, Wahren J, Sherwin R, Palaiologos G. Amino acid and protein metabolism in diabetes mellitus. *Arch Intern Med.* 1977; 137:507–513.

Frier BC, Noble EG, Locke M. Diabetes-induced atrophy is associated with a muscle-specific alteration in NF-kappaB activation and expression. *Cell Stress Chaperones.* 2008; 13(3):287-96.

Fuller RW. Serotonin receptors involved in regulation of pituitary-adrenocortical function in rats. *Behav Brain Res.* 1996; 73(1-2):215-219.

Geethan PK, Prince PS. Antihyperlipidemic effect of D-pinitol on streptozotocin-induced diabetic Wistar rats. *J Biochem Mol Toxicol.* 2008; 22(4):220-224.

Gould GW, Merrall NW, Martin S, Jess TJ, Campbell IW, Calderhead DM, Gibbs EM, Holman GD, Plevin RJ. Growth factor-induced stimulation of hexose transport in 3T3-L1 adipocytes: evidence that insulin-induced translocation of GLUT4 is independent of activation of MAP kinase. *Cell Signal.* 1994; 6(3):313-320.

Hagihara Y, Fukuda S, Goto S, Iida H, Yamazaki M, Moriya H. How many days per week should rats undergo running exercise to increase BMD? *J Bone Miner Metab.* 2005; 23(4):289-94.

Halle M, Berg A, Garwers U, Baumstark MW, Knisel W, Grathwohl D, König D, Keul J. Influence of 4 weeks' intervention by exercise and diet on low-density lipoprotein subfractions in obese men with type 2 diabetes. *Metabolism.* 1999; 48(5):641-644.

Hamada Y, Kitazawa S, Kitazawa R, Fujii H, Kasuga M, Fukagawa M. Histomorphometric analysis of diabetic osteopenia in streptozotocin-induced diabetic mice: a possible role of oxidative stress. *Bone.* 2007; 40(5):1408-1414.

Hediger MA, Rhoads DB. Molecular physiology of sodium-glucose cotransporters. *Physiol Rev.* 1994; 74(4):993-1026.

Hersey WC III, Graves JE, Pollock ML, Gingerich R, Shireman RB, Heath GW, Spierto F, McCole SD, Hagberg JM. Endurance exercise training improves body composition and plasma insulin responses in 70- to 79-year-old men and women. *Metabolism.* 1994; 43:847–854.

Hildebrandt AL, Pilegaard H, Neufer PD. Differential transcriptional activation of select metabolic genes in response to variations in exercise intensity and duration. *Am J Physiol Endocrinol Metab.* 2003; 285(5):E1021-1027.

Howarth FC, Qureshi A, Shahin A, Lukic ML. Effects of single high-dose and multiple low-dose streptozotocin on contraction and intracellular Ca2+ in ventricular myocytes from diabetes resistant and susceptible rats. *Mol Cell Biochem.* 2005; 269(1-2):103-108.

Hsieh CJ, Wang PW. Effectiveness of weight loss in the elderly with type 2 diabetes mellitus. *J Endocrinol Invest.* 2005; 28:973–977.

Irwin R, Lin HV, Motyl KJ, McCabe LR. Normal bone density obtained in the absence of insulin receptor expression in bone. *Endocrinology.* 2006; 147(12):5760-5767.

Junod A, Lambert AE, Stauffacher W, Renold AE. Diabetogenic action of streptozotocin: relationship of dose to metabolic response. *J Clin Invest.* 1969; 48(11):2129-2139.

Kerr D, Ackland T, Maslen B, Morton A, Prince R. Resistance training over 2 years increases bone mass in calcium-replete postmenopausal women. *J Bone Miner Res.* 2001; 16(1):175-181.

Kesaniemi YK, Danforth E,Jr, Jensen MD, Kopelman PG, Lefebvre P, Reeder BA. Dose–response issues concerning physical activity and health: an evidence-based symposium. *Med Sci Sports Exerc.* 2001; 33:S351–S358.

Kim E, Sohn S, Lee M, Jung J, Kineman RD, Park S. Differential responses of the growth hormone axis in two rat models of streptozotocin-induced insulinopenic diabetes. *J Endocrinol.* 2006; 188(2):263-70.

Koivisto VA, Akerblom HK, Kiviluoto MK. Metabolic and hormonal effects of exercise in the severely streptozotocin-diabetic rat. *Diabetologia.* 1974; 10(4):329-35.

Lee MJ, Feliers D, Mariappan MM, Sataranatarajan K, Mahimainathan L, Musi N, Foretz M, Viollet B, Weinberg JM, Choudhury GG, Kasinath BS. A role for AMP-activated protein kinase in diabetes-induced renal hypertrophy. *Am J Physiol Renal Physiol.* 2007; 292(2):F617-627.

Levy E, Roy CC, Lepage G, Bendayan M. Lipid abnormalities in pancreatic tissue of streptozotocin-induced diabetic rats. *Lipids.* 1988; 23(8):771-778.

Like AA & Rossini AA Streptozotocin-induced pancreatic insulitis: new model of diabetes mellitus. *Science.* 1976; 193: 415–417.

Markuns JF, Napoli R, Hirshman MF, Davalli AM, Cheatham B, Goodyear LJ. Effects of streptozocin-induced diabetes and islet cell transplantation on insulin signaling in rat skeletal muscle. *Endocrinology.* 1999; 140(1):106-111.

Martin LM, McCabe LR. Type I diabetic bone phenotype is location but not gender dependent. *Histochem Cell Biol.* 2007; 128(2):125-33.

Medina-Sanchez M, Rodriguez-Sanchez C, Vega-Alvarez JA, Menedez-Pelaez A,Perez-Casas A. Proximal skeletal muscle alterations in streptozotocin-diabetic rats: a histochemical and morphometric analysis. *Am J Anat.* 1991; 191(1):48-56.

Meier M, Park JK, Overheu D, Kirsch T, Lindschau C, Gueler F, Leitges M, Menne J, Haller H. Deletion of protein kinase C-beta isoform in vivo reduces renal hypertrophy but not albuminuria in the streptozotocin-induced diabetic mouse model. *Diabetes.* 2007; 56(2):346-354.

Morikawa T, Toyama T, Kudo N, Kawashima Y. Reducing effect of matrix metalloproteinase inhibitors on serum triacylglycerol in streptozotocin-induced diabetic rats and Zucker fa/fa rats. *Biol Pharm Bull.* 2007; 30(8):1461-1467.

Najemnikova E, Rodgers CD, Locke M. Altered heat stress response following streptozotocin-induced diabetes. *Cell Stress Chaperones.* 2007; 12(4):342-52.
Olbricht CJ, Geissinger B. Renal hypertrophy in streptozotocin diabetic rats: role of proteolytic lysosomal enzymes. *Kidney Int.* 1992; 41(4):966-972.

Paulson DJ, Kopp SJ, Peace DG, Tow JP. Myocardial adaptation to endurance exercise training in diabetic rats. *Am J Physiol.* 1987; 252(6 Pt 2):R1073-1081.

Päivärinne H, Kainulainen H. DAPIT, a novel protein down-regulated in insulin-sensitive tissues in streptozotocin-induced diabetes. *Acta Diabetol.* 2001; 38(2):83-86.

Paré WP, Tejani-Butt SM. Effect of stress on the behavior and 5-HT system in Sprague-Dawley and Wistar Kyoto rat strains. *Integr Physiol Behav Sci.* 1996; 31(2):112-121.

Pechhold K, Patterson NB, Blum C, Fleischacker CL, Boehm BO, Harlan DM. Low dose streptozotocin-induced diabetes in rat insulin promoter-mCD80-transgenic mice is T cell autoantigen-specific and CD28 dependent. *J Immunol.* 2001; 166(4):2531-2539.

Pillion DJ, Jenkins RL, Atchison JA, Stockard CR, Clements RS Jr, Grizzle WE. Paradoxical organ-specific adaptations to streptozotocin diabetes mellitus in adult rats. *Am J Physiol.* 1988; 254(6 Pt 1):E749-755.

Radahmadi M, Shadan F, Karimian SM, Sadr SS, Nasimi A. Effects of stress on exacerbation of diabetes mellitus, serum glucose and cortisol levels and body weight in rats. *Pathophysiology.* 2006; 13(1):51-55.

Rebuffat P, Belloni AS, Malendowicz LK, Mazzocchi G, Meneghelli V, Nussdorfer GG. Effects of streptozotocin-induced experimental diabetes on the morphology and function of the zona fasciculata of rat adrenal cortex. *Virchows Arch B Cell Pathol Incl Mol Pathol.* 1988; 56(1):13-19.

Ribeiro IC, Iborra RT, Neves MQ, Lottenberg SA, Charf AM, Nunes VS, Negrão CE, Nakandakare ER, Quintão EC, Passarelli M. HDL atheroprotection by aerobic exercise training in type 2 diabetes mellitus. *Med Sci Sports Exerc.* 2008; 40(5):779-86.

Rosenfalck AM, Almdal T, Hilsted J, Madsbad S. Body composition in adults with Type 1 diabetes at onset and during the first year of insulin therapy. *Diabet Med.* 2002; 19(5):417-23.

Schmidt KN, Gosselin LE, Stanley WC. Endurance exercise training causes adrenal medullary hypertrophy in young and old Fischer 344 rats. *Horm Metab Res.* 1992; 24:511-515.

Segev Y, Landau D, Marbach M, Shehadeh N, Flyvbjerg A, Phillip M. Renal hypertrophy in hyperglycemic non-obese diabetic mice is associated with persistent renal accumulation of insulin-like growth factor I. *J Am Soc Nephrol.* 1997; 8(3):436-444.

Sharma K, Ziyadeh FN. Hyperglycemia and diabetic kidney disease. The case for transforming growth factor-beta as a key mediator. *Diabetes.* 1995; 44(10):1139-1146.

Stallknecht B, Kjaer M, Mikines KJ, Maroun L, Ploug T, Ohkuwa T, Vinten J, Galbo H. Diminished epinephrine response to hypoglycemia despite enlarged adrenal medulla in trained rats. *Am J Physiol.* 1990; 259(5 Pt 2):R998-1003.

Strotmeyer ES, Cauley JA. Diabetes mellitus, bone mineral density, and fracture risk. *Curr Opin Endocrinol Diabetes Obes.* 2007; 14(6):429-435.

Verhaeghe J, Thomsen JS, van Bree R, van Herck E, Bouillon R, Mosekilde L. Effects of exercise and disuse on bone remodeling, bone mass, and biomechanical competence in spontaneously diabetic female rats. *Bone.* 2000; 27(2):249-256.

Watanabe T, Morimoto A, Sakata Y, Wada M, Murakami N. The effect of chronic exercise on the pituitary-adrenocortical response in conscious rats. *J Physiol.* 1991; 439:691-699.

Wehbi GJ, Zimpelmann J, Carey RM, Levine DZ, Burns KD. Early streptozotocin-diabetes mellitus downregulates rat kidney AT2 receptors. *Am J Physiol Renal Physiol.* 2001; 280(2):F254-265.

Wei M, Ong L, Smith MT, Ross FB, Schmid K, Hoey AJ, Burstow D, Brown L. The streptozotocin-diabetic rat as a model of the chronic complications of human diabetes. *Heart Lung Circ.* 2003; 12(1):44-50.

Yeh JK, Aloia JF, Tierney JM, Sprintz S. Effect of treadmill exercise on vertebral and tibial bone mineral content and bone mineral density in the aged adult rat: determined by dual energy X-ray absorptiometry. *Calcif Tissue Int.* 1993a; 52(3):234-8.

Yeh JK, Liu CC, Aloia JF. Effects of exercise and immobilization on bone formation and resorption in young rats. *Am J Physiol.* 1993b; 264(2 Pt 1):E182-9.

Yin FC, Spurgeon HA, Rakusan K, Weisfeldt ML, Lakatta EG. Use of tibial length to quantify cardiac hypertrophy: application in the aging rat. *Am J Physiol.* 1982; 243(6):H941-947.

Zambotti-Villela L, Yamasaki SC, Villarroel JS, Alponti RF, Silveira PF. Prospective evaluation of aminopeptidase activities in plasma and peripheral organs of streptozotocin-induced diabetic rats. *J Endocrinol Invest.* 2008; 31(6):492-498.

Zelena D, Mergl Z, Makara GB. The role of vasopressin in diabetes mellitus-induced hypothalamo-pituitary-adrenal axis activation: studies in Brattleboro rats. *Brain Res Bull.* 2006; 69(1):48-56.

CHAPTER 3

Diabetes-induced atrophy is associated with a muscle-specific alteration in NF-kappaB activation and expression.

3.1 INTRODUCTION

Diabetes Mellitus (DM) is a metabolic pathology characterized by diminished insulin secretion and/or effect at the cellular level resulting in elevated blood glucose. Diabetes-related hyperglycemia is associated with elevated levels of pro-inflammatory factors, cytokines, increased advanced glycation end-products, increased free radical production, and is generally coupled with a chronic inflammatory state in diabetic subjects (reviewed by Engstrom et al., 2003). Specifically, hyperglycemia-induced inflammation appears to require the activation of nuclear factor kappa-B (NF-κB), a dimerized transcription factor composed from its 5 member proteins, including p50, p52, p65, Rel-B, and c-Rel. Under normal conditions, NF-κB remains sequestered in the cytosol by an inhibitor (IκB). When IκB is phosphorylated and subsequently degraded, NF-κB is released, allowing for nuclear translocation and subsequent DNA binding (reviewed by Hoffmann et al., 2006). This can lead to the expression of gene products involved in inflammation, immune responses, or apoptosis. The most typically observed, transcriptionally active dimer (the *canonical or classical* pathway) appears to be the p50-p65 heterodimer, whereas the *non-canonical* or *alternative* pathway is composed of the p52-RelB subunits (reviewed by Gilmore 2006), although other subunit-combinations are known to bind DNA (reviewed by Hoffmann et al., 2006). The known complement of genes transcribed by the various NF-κB dimer combinations is continually expanding and may explain mechanisms by which selective pathways are activated in any number of pathologies.

Certain pathological conditions may preferentially signal the activation of atrophic pathways within skeletal muscles (Lecker et al., 2004). Specifically, skeletal muscle atrophy observed in diabetes, disuse, and cancer cachexia has been linked to NF-κB-mediated inflammatory conditions (Wyke 2005). Specifically, pathological conditions leading to atrophy appear to activate the canonical p50-p65 NF-κB pathway (Guttridge et al., 2000). In addition, there is evidence that during disuse atrophy, Bcl-3, normally an inhibitor of NF-κB activation, may serve a contradictory role as a transcriptional activator of the p50 homodimer (Hunter and Kandarian 2004). Hence, although NF-κB appears to be activated in various conditions leading to atrophy, the catabolic consequence common to a number of pathologies appears not to arise from the activation of a single NF-κB dimer, but may derive from specific NF-κB dimer-combinations unique to any one disease.

Diabetes, induced by streptozotocin (STZ), has long been known to result in attenuated muscle growth and a preferential atrophy of fast fibers compared to the slow fibers (Armstrong et al., 1975). Hence, specific muscles appear to be more susceptible to diabetes-induced atrophy. Moreover, the mechanisms, composition and activation of NF-κB in diabetic muscle remain generally uncharacterized. Since the consequence of hyperglycemia-induced inflammatory signaling and hence local fiber atrophy remains unknown, the purpose of the present study was to characterize NF-κB in muscles of varying fiber-type by measuring NF-κB activation, determining subunit composition, and quantifying the content of NF-κB protein family members within various skeletal muscles from diabetic animals. It was hypothesized that NF-κB would demonstrate a

muscle-specific pattern of activation and expression that would be associated with atrophy in diabetic muscle.

3.2 METHODS AND MATERIALS

The present study used a total of 10 male Sprague-Dawley rats (286-330g; Charles River, Quebec). Animals were maintained on a 12-h light-dark cycle at 20 ± 1°C, with an ambient relative humidity of 50%. Food and water were provided *ad libitum*. All experimental procedures were approved by *The Animal Care Committee of the University of Toronto*. Animals were randomly assigned to 2 groups; a control group (C, n=5), and a diabetic group (D, n=5). Rats from the D group received a tail vein injection with STZ (55mg/kg body weight) and were allowed to develop diabetes for 30 days while age-matched animals (3 months old) from the C group served as controls. Thirty days after the STZ treatment, animals (n=10) from both groups were anesthetized and muscle tissues were harvested following cardiac excision. All tissues were immediately frozen in liquid nitrogen and stored until preparation.

STZ Administration. Animals were placed into a blanket-covered plexiglass constraint. A single dose of streptozotocin (55mg/kg prepared in 0.1M NaCl buffer) injected into the tail vein was administered. The development of diabetes was determined by blood glucose accruement over 30 days. Age-matched animals from the C group not receiving the STZ treatment served as controls. Body weight and morning, non-fasting blood glucose concentration obtained from the tail vein of each animal were measured weekly using the One Touch Basic Blood Glucose Monitoring System (Lifescan, Canada) with One Touch test strips (Lifescan, Canada; range = 0-600mg/dL).

Electrophoretic Mobility Shift Assay (EMSA). Portions of muscle tissue (50 mg) were homogenized in 15 volumes of extraction buffer (25% glycerol, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA (pH 8.0), 20 mM HEPES (pH 7.9), 0.5 mM DTT, 0.5 mM phenylmethylsufonylfluoride) at 4°C. Homogenates were centrifuged at 14,000 rpm at 4°C for 20 minutes in an Eppendorf centrifuge. The supernatant was removed and protein concentration was determined by the method described by Lowry et al., (1951). Analyses of NF-κB-Oligonucleotide binding in extracts were performed using an EMSA according to the procedure described by Locke and Tanguay (1996). Protein extracts (50 ug) from muscles were incubated with a ^{32}P-labeled, self-complementary, ideal NF-κB oligonucleotide (5'- AGT TGA GGG GAC TTT CCC AGG C-3'; E3291, Promega) in binding buffer (10% glycerol, 50 mM NaCl, 1.0 mM EDTA [pH, 8.0], 20 mM Tris [pH, 8.0], 1.0 mM DTT, 0.3 mg/ml BSA) with approximately 0.1 ng (50,000 cpm) of ^{32}P-labeled oligonucleotide and 2.0 µg poly dI dC (Pharmacia Fine Chemicals, Piscataway, NJ, USA) for at least 30 minutes at room temperature. Samples were electrophoresed on 4 % acrylamide gel at 200V for 2-3 hours. Gels were dried using a BioRad Slab dryer (Model 433) for 45 minutes and exposed to radiographic film (Amersham-ECL, Mississauga, Ontario, Canada) for 1 or 2 days at -70°C. Films were scanned using an Agfa Arcus II scanner. Recombinant NF-κB (p50) [CAS# (56-81-5) Promega, Madison, WI, USA] and oligo was electrophoresed on each gel to determine the positive identification and location of the bound NF-κB-Oligonucleotide complex. Correct NF-κB-Oligonucleotide interaction was confirmed by the absence of NF-κB-activation with the addition of an excess of non-labeled NF-κB oligonucleotides. Supershift assays were performed to determine the composition of the activated NF-kB complex. Briefly, 2 µg of

an antibody specific for a single member of the NF-kB family [p50 (NLS; sc-114 X), p52 (K-27; sc-298 X), p65 (C-20; sc-372 X), RelB (C-19; sc-226 X), C-Rel (C; sc-71 X), and Bcl-3 (C-14; sc-185 X); Santa Cruz Biotechnology, California, USA] was added to the labeled oligo/protein extract for 30 minutes prior to electrophoresis. The occurrence of an NF-kB member protein within the dimer complex was determined by upward movement of the protein-DNA complex.

Polyacrylamide Gel Electrophoresis and Immunoblotting. Muscle portions of ~50mg were homogenized in 20x volume of 600 mM NaCl, 15 mM Tris pH 7.5 and protein concentration determined by the method described by Lowry et al., (1951). One dimensional (1-D) sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed according to the method described by Laemmli (1970), except that the separating gel (0.15 x 4.5 x 8 cm) consisted of a 5-15% polyacrylamide gradient. Prestained standards were used as markers (catalogue 161-0324, Bio-Rad. Mississauga, Ontario, Canada). Samples were electrophoresed using a mini protein II (Bio-Rad, Mississauga, Ontario, Canada) at 55 volts for ~30-minutes followed by 90 volts for ~1-hour or until the dye arrived at the bottom of the separating gel.

Following electrophoretic separation, gels and nitrocellulose membranes were equilibrated in Transfer Buffer solution (1X SDS-PAGE running buffer with 20% methanol [pH 8.3]) for 20-minutes. Following equilibration, proteins were transferred to nitrocellulose membranes (0.22 μm pore size, Bio-Rad Laboratories) as described by Towbin et al., (1979) using the Bio-Rad mini-protean II gel transfer system at a voltage of 55 volts for 3-hours. The transfer buffer was cooled by hourly changes of an ice pack. Following protein transfer, nitrocellulose paper was blocked in Blotto (5% non-fat skim

milk powder in Tris Buffer Saline (TBS: 500 mM NaCl and 20 mM Tris, pH 7.5) for one-hour to overnight. Nitrocellulose membranes were rinsed in three washes of TTBS (TBS plus 0.05% Tween-20) followed by incubation with polyclonal antibodies (see EMSA procedure), diluted 1:500 in TTBS with 2% Blotto (Locke et al., 1995). Immunoblots were visualized by reacting an HRP-conjugated, goat anti-rabbit secondary antibody (diluted 1:2000 and incubated for 1 hour) via enhanced chemiluminescence (Amersham Biosciences Corp, Piscataway, NJ). Immunoblots were scanned using an Agfa Arcus II scanner and quantification of bands from the immunoblots was performed using Kodak 1D Image Analysis Software (Kodak Scientific Imaging Systems, New Haven, CT). The content of p50, p52, p65, RelB, and Bcl-3 was determined by analyzing the intensities of bands on Western blots.

Statistical Analysis. Independent T-Tests were performed (SigmaStat, San Jose, USA) using diabetes as an independent variable for each experimental group. Differences between groups were considered statistically significant at a level of $P \leq 0.05$.

3.3 RESULTS

Blood Glucose Levels. Blood glucose values for control and STZ treated animals are shown in Figure 3.1. The initial blood glucose value for the control group was 95.7 ± 2 mg/dl and was not significantly different from the initial value measured in the STZ treated animals (94.2 ± 1 mg/dl). A significant increase ($P < 0.05$) in blood glucose was measured after 30 days in the STZ treated animals (470 ± 36 mg/dl), whereas the control animals did not demonstrate a significant change after 30 days (95 ± 2 mg/dl).

Body Mass and Muscle Mass. Initial body mass values were not significantly different between control and diabetic groups. As expected (from normal growth) a significant increase ($P < 0.05$) in body mass for both control and diabetic animals was observed after 30 days (Figure 3.2*A*). However, diabetic animals showed a significantly lower body mass ($P < 0.05$) after 30 days (initial mass: 286 ± 4 g; final mass: 362 ± 11 g) when compared with the control group (initial mass: 302 ± 7 g; final mass: 464 ± 6 g).

Muscle mass values for the soleus (SOL), plantaris (PL), and gastrocnemius are shown in Figure 3.2*B*. All muscles from diabetic animals demonstrated significantly reduced ($P < 0.05$) mass when compared with the control group. Both body mass and muscle mass values in diabetic animals were approximately 80% of the values measured in the control animals. To determine if the reduced muscle mass in STZ treated animals reflected the overall reduction in body mass, muscle mass was expressed relative to body mass (Figure 3.2*C*). The soleus and plantaris muscles did not have a diminished muscle-to-body mass ratio compared with the controls. In contrast, the gastrocnemius muscle-to-body mass ratio was significantly reduced ($P < 0.05$) in the diabetic animals compared with the control animals, suggesting an atrophic response.

Activation and Composition of NF-kB. Activation of NF-κB as determined by binding to the NF-κB oligo was measured in muscle extracts from control and diabetic animals by EMSA (Figure 3.3). EMSA-derived band quantification indicated NF-κB activation in the SOL, PL, and WG muscle from diabetic animals was not significantly different from controls (Figure 3.4*A-C*). However, NF-κB activation was significantly ($P < 0.05$) reduced in the RG of diabetic animals (Figure 3.4*D*), suggesting a unique cellular response in this tissue to the diabetic condition.

To determine the dimeric composition of the NF-κB transcription factor observed in muscle extracts from control and diabetic animals, EMSA supershifts were performed (Figures 3.5 and 3.6). The addition of antibodies specific for each of the 5 members of the NF-κB family as well as for Bcl-3 revealed a common pattern for all muscles examined. When incubated with a p50-specific antibody, a slower migration (supershift) of the NF-κB-oligo complex was observed. This was consistent for all control and diabetic muscle extracts. Incubation of muscle samples with other antibodies did not appear to result in any supershifts. When all antibodies were combined the same pattern as p50 only was observed and the disappearance of the NF-κB-oligo complex from its original (antibody-free) location also occurred. Since the entire complex was shifted by the addition of the p50 antibody alone, it suggests that the NF-κB-Oligo complex detected in muscles is primarily a p50-p50 homodimer.

NF-κB Protein Content. To determine NF-κB protein subunit content in rat hindlimb muscles from both control and diabetic animals (Figure 3.7), Western blots were performed for the various NF-κB subunits of the canonical and alternative pathway (p50, p52, p65, Rel-B) and Bcl-3. The main constituent of NF-κB dimerization, p50, demonstrated consistently unchanged protein content in all muscles between control and diabetic animals (Figure 3.7*A*). In contrast, p52 showed a significantly different ($P<0.05$) quantity between diabetes and control in the PL, RG, and SOL muscles, whereas the WG remained unchanged (Figure 3.7*B*). However, unlike the PL and SOL muscles, which showed elevated contents of p52 with diabetes, the RG showed reduced p52 content. The PL and SOL muscles also showed significantly elevated ($P<0.05$) contents of p65 in diabetic animals, while the WG and the RG remained unchanged (Figure 3.7*C*). Similar

to p50, Rel-B content also showed no significant alteration in any muscle in the diabetic condition (Figure 3.7*D*). A muscle-specific protein expression in diabetic tissue was found for Bcl-3 (Figure 3.7*E*), in that the PL muscles from diabetic animals showed a significantly decreased ($P<0.05$) Bcl-3 content. In contrast, both the RG and WG muscles showed a significant increase ($P<0.05$) in Bcl-3 content. The SOL Bcl-3 content was unchanged. Taken together, there appear to be muscle-specific changes in expression that distinguishes the gastrocnemius muscle from the PL and SOL muscles.

Figure 3.1 Blood Glucose Values After STZ Treatment. Glucose values (expressed as a percentage of control) are shown for control and diabetic animals before and after 30 days of STZ treatment. Values are mean ± SEM for glucose (mg/dl). *Significantly different ($P<0.05$) from control values.

Figure 3.1

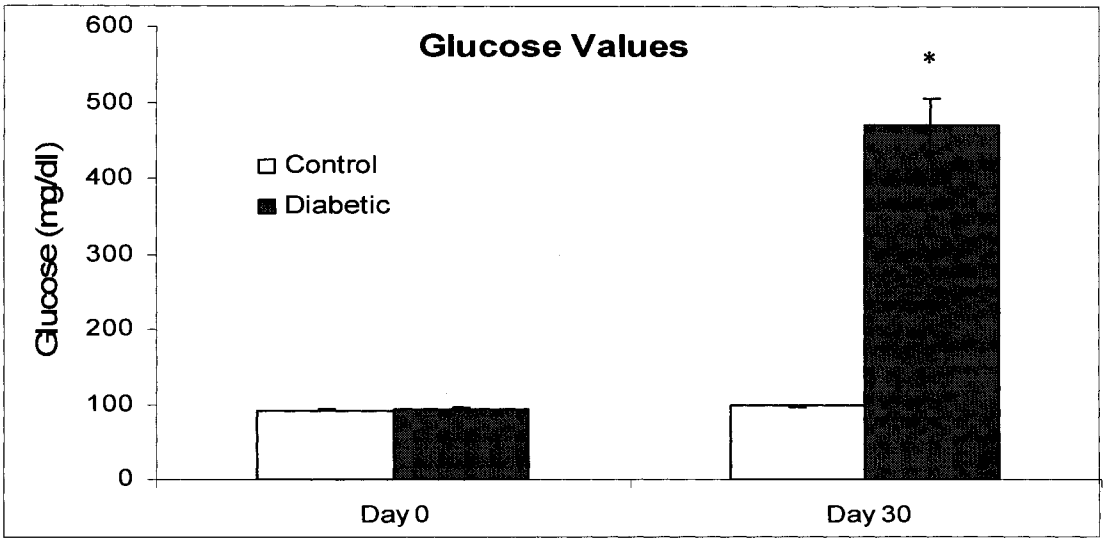

Glucose Values
Glucose (mg/dl)
600
500
400
300
200
100
0
Control
Diabetic
*
Day 0
Day 30

Figure 3.2 Diabetes Alters Body and Muscle Mass.

A: 30 days after STZ injection accumulation of body mass is significantly attenuated ($P<0.05$). Values are mean ± SEM for total body mass (g). *Significantly different ($P<0.05$) from control animals.

B: Muscle mass accumulation is significantly reduced ($P<0.05$) in diabetic animals. Values are percentage of control ± SEM for total muscle mass (g). *Significantly different ($P<0.05$) from control animals.

C: Muscle-to-body mass ratio reflects a muscle specific effect of diabetes. The gastrocnemius demonstrated a significantly attenuated ($P<0.05$) muscle-to-body mass ratio. Values are mean muscle-to-body ratio ± SEM (mg/g). *Significantly different ($P<0.05$) from control animals.

Figure 3.2*A*

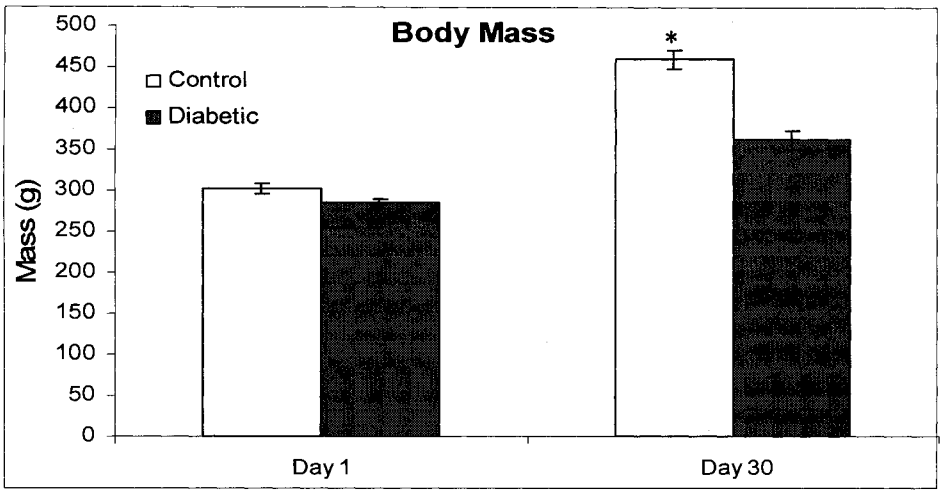


Figure 3.2*B*

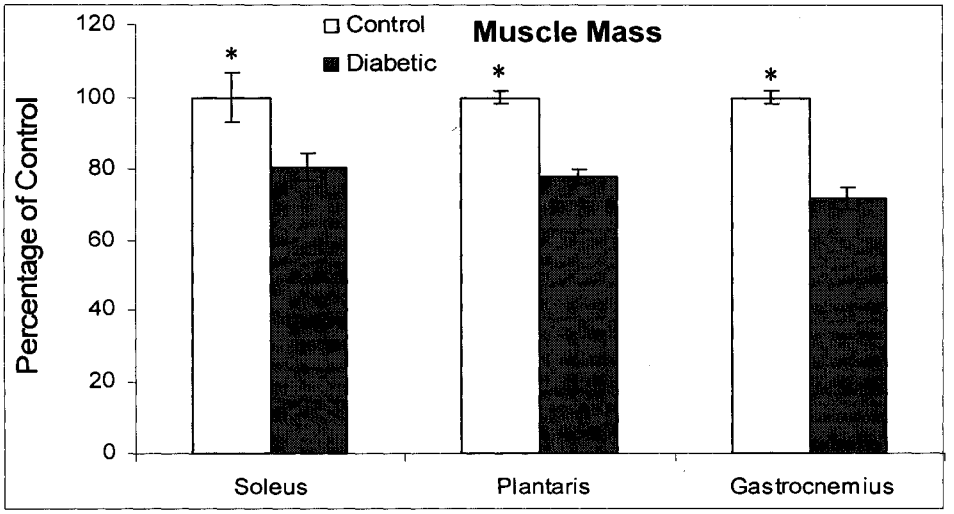


Figure 3.2*C*

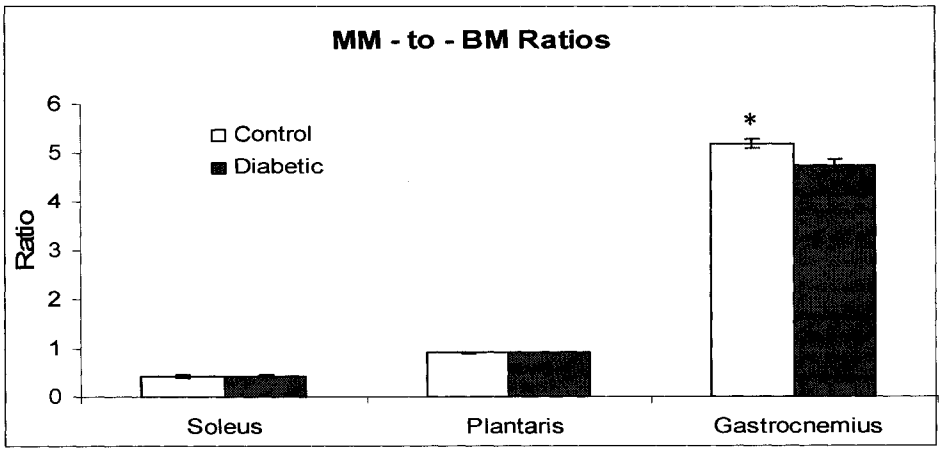


Figure 3.3 Transcriptional Activation of NF-κB. NF-κB activation in WG, RG, SOL, and PL muscle tissues from control and diabetic animals. Protein extracts are as follows: Lane 1: Protein sample from control *animal 1*, lane 2: Protein sample from diabetic *animal 1*, lane 3: Protein sample from control *animal 2*, lane 4: Protein sample from diabetic *animal 2*, lane 5: Protein sample from control *animal 3*, lane 6: Protein sample from diabetic *animal 3*. NF-κB binding is significantly reduced ($P<0.05$) in RG tissues of diabetic animals. *Significantly different ($P<0.05$) from control animals.

Figure 3.3

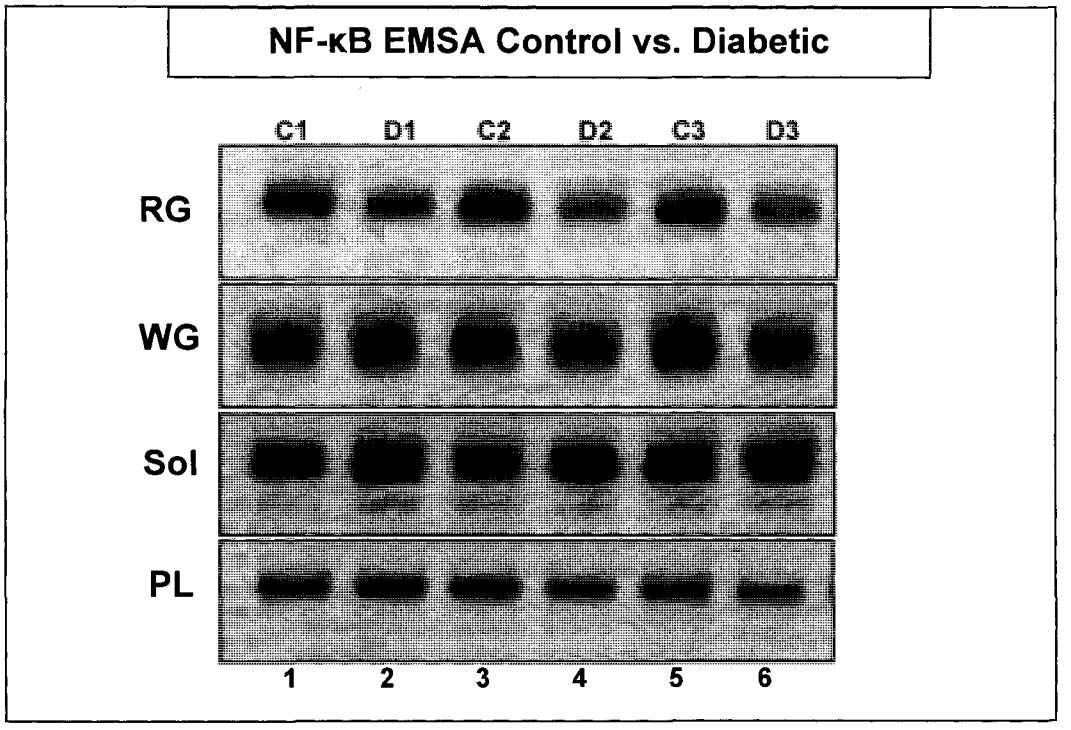

NF-κB EMSA Control vs. Diabetic
C1
D1
C2
D2
C3
D3
RG
WG
Sol
PL
1
2
3
4
5
6

Figure 3.4 Graphical Illustration of NF-κB Activation.

A: Representation of NF-κB binding in SOL muscles expressed as percentage of control ± SEM. No significant difference between control and diabetic animals was observed.

B: Representation of NF-κB binding in PL muscles expressed as percentage of control ± SEM. No significant difference between control and diabetic animals was observed.

C: Representation of NF-κB binding in WG muscles expressed as percentage of control ± SEM. No significant difference between control and diabetic animals was observed.

D: Representation of NF-κB binding in RG muscles expressed as percentage of control ± SEM. NF-κB binding was significant reduced ($P<0.05$) in RG tissues of diabetic animals. *Significantly different ($P<0.05$) from control animals.

Figure 3.4*A*

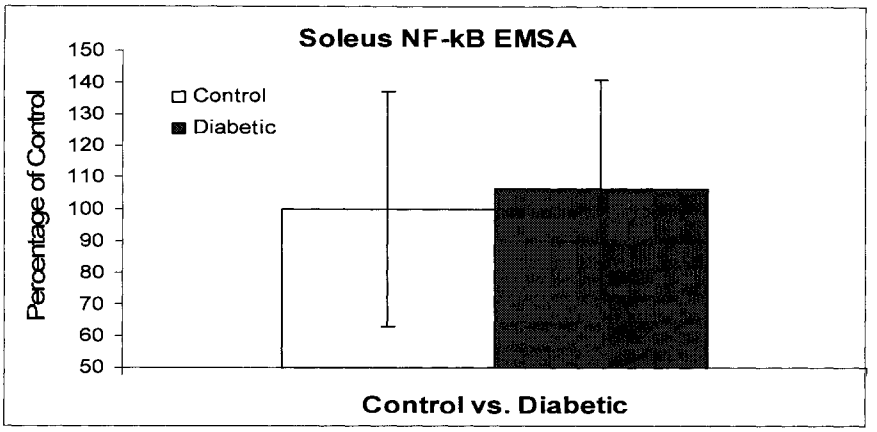


Figure 3.4*B*

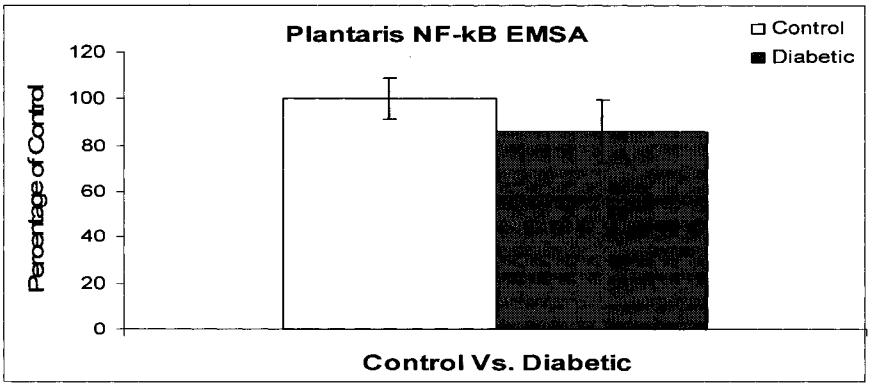


Figure 3.4*C*

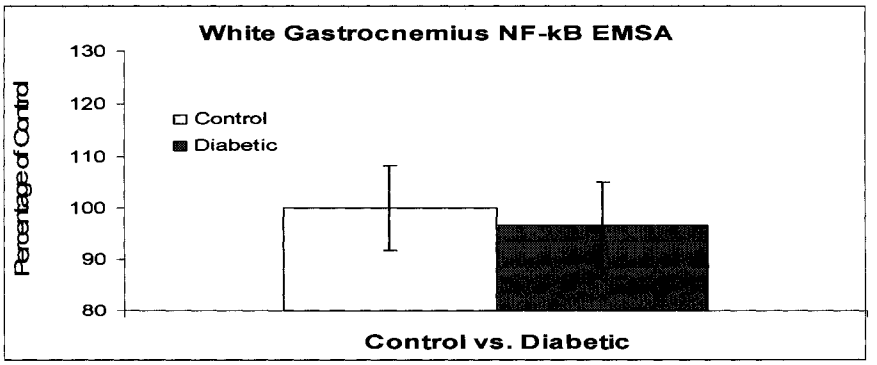


Figure 3.4*D*

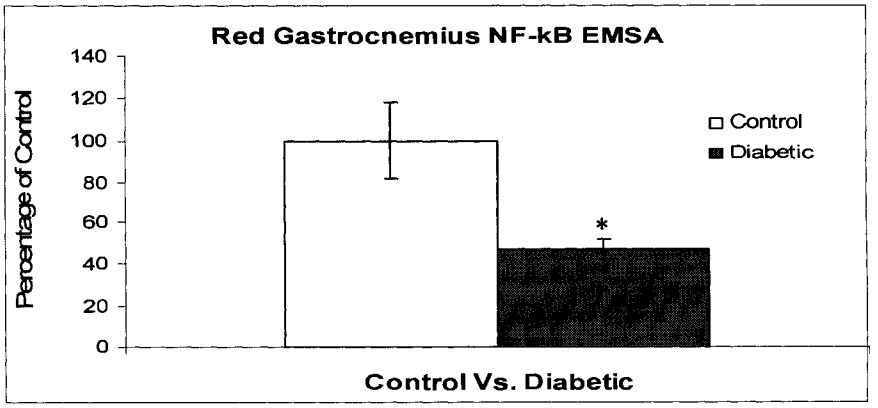


Figure 3.5 Supershifts of NF-κB from Gastrocnemius Muscles from Control and Diabetic Animals. Lane 1: Free Probe (FP), lane 2: Protein sample without antibody, lane 3: Protein sample with the addition of 2μg α-p50, lane 4: Protein sample with the addition of 2μg α-p52, lane 5: Protein sample with the addition of 2μg α-p65, lane 6: Protein sample with the addition of 2μg α-RelB, lane 7: Protein sample with the addition of 2μg α-cRel, lane8: Protein sample with the addition of 2μg α-Bcl-3, lane 9: Protein sample with the addition of 2μg of each antibody, lane 10: Labeled oligo incubated with recombinant p50 (5ng). Disappearance of the band by a p50-specific antibody indicates a homodimer composed predominately of p50.

Figure 3.5

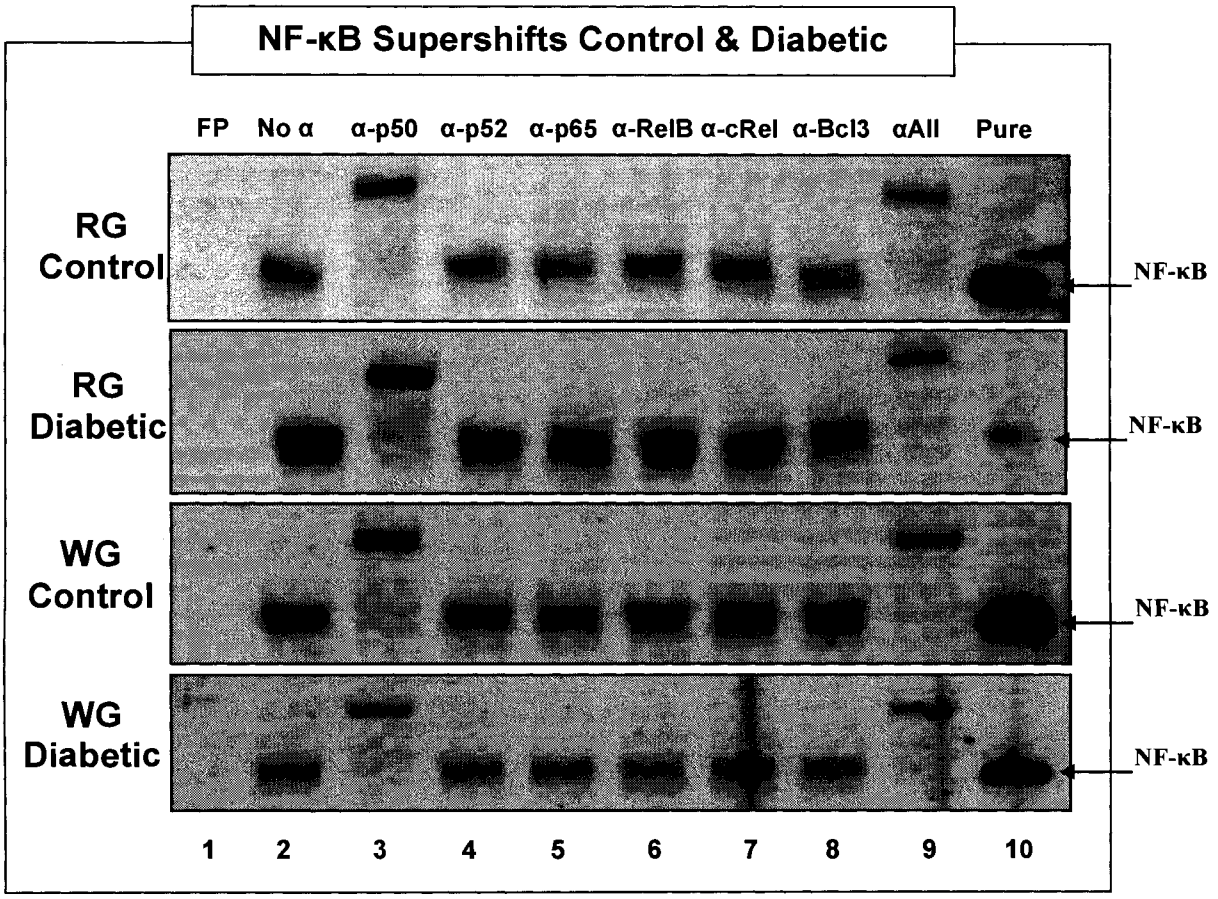

NF-κB Supershifts Control & Diabetic
FP
No α
α-p50
α-p52
α-p65
α-RelB
α-cRel
α-Bcl3
αAll
Pure
RG Control
RG Diabetic
WG Control
WG Diabetic
NF-κB
NF-κB
NF-κB
NF-κB
1
2
3
4
5
6
7
8
9
10

Figure 3.6 Supershifts of NF-κB from Soleus and Plantaris Muscles from Control and Diabetic Animals. Lane 1: Free Probe (FP), lane 2: Protein sample without antibody, lane 3: Protein sample with the addition of 2μg α-p50, lane 4: Protein sample with the addition of 2μg α-p52, lane 5: Protein sample with the addition of 2μg α-p65, lane 6: Protein sample with the addition of 2μg α-RelB, lane 7: Protein sample with the addition of 2μg α-cRel, lane8: Protein sample with the addition of 2μg α-Bcl-3, lane 9: Protein sample with the addition of 2μg of each antibody, lane 10: Labeled oligo incubated with recombinant p50 (5ng).

Figure 3.6

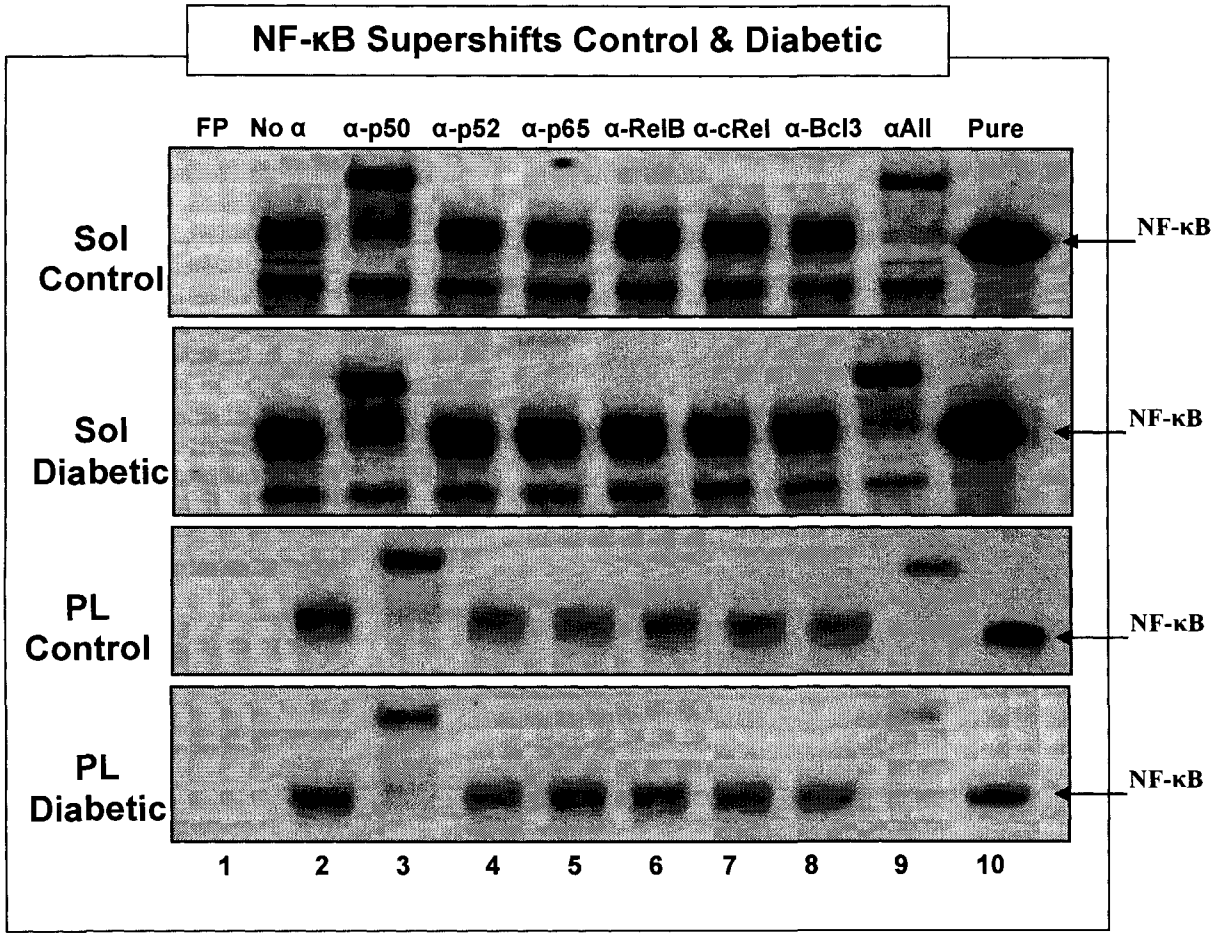

NF-κB Supershifts Control & Diabetic
FP
No α
α-p50
α-p52
α-p65
α-RelB
α-cRel
α-Bcl3
αAll
Pure
Sol Control
Sol Diabetic
PL Control
PL Diabetic
NF-κB
NF-κB
NF-κB
NF-κB
1
2
3
4
5
6
7
8
9
10

Figure 3.7 NF-κB Subunit Content in Control and Diabetic Muscle Tissues. Representative western blots for each protein and muscle are embedded in each bar graph (n=5).

A: Representation of p50 content in control and diabetic muscle tissues expressed as percentage of control. Values are percentage of control ± SEM. No significant difference between control and diabetic animals was observed for any tissues examined.

B: Representation of p52 content in control and diabetic muscle tissues expressed as percentage of control ± SEM. PL and SOL muscles from diabetic animals showed significantly increased ($P<0.05$) p52 content. RG contents of p52 from diabetic animals were significantly decreased ($P<0.05$). *Significantly different ($P<0.05$) from control animals.

C: Representation of p65 content in control and diabetic muscle tissues expressed as percentage of control ± SEM. PL and SOL muscles from diabetic animals showed significantly increased ($P<0.05$) p65 content. No significant difference between control and diabetic animals was observed in either RG or WG tissues.

D: Representation of Rel-B content in control and diabetic muscle tissues expressed as percentage of control. Values are percentage of control ± SEM. No significant difference between control and diabetic animals was observed for any tissues examined.

E: Representation of Bcl-3 content in control and diabetic muscle tissues expressed as percentage of control ± SEM. PL muscle from diabetic animals showed significantly decreased ($P<0.05$) Bcl-3 content. Both RG and WG contents of Bcl-3 from diabetic animals was significantly increased ($P<0.05$). *Significantly different ($P<0.05$) from control animals.

Figure 3.7*A*

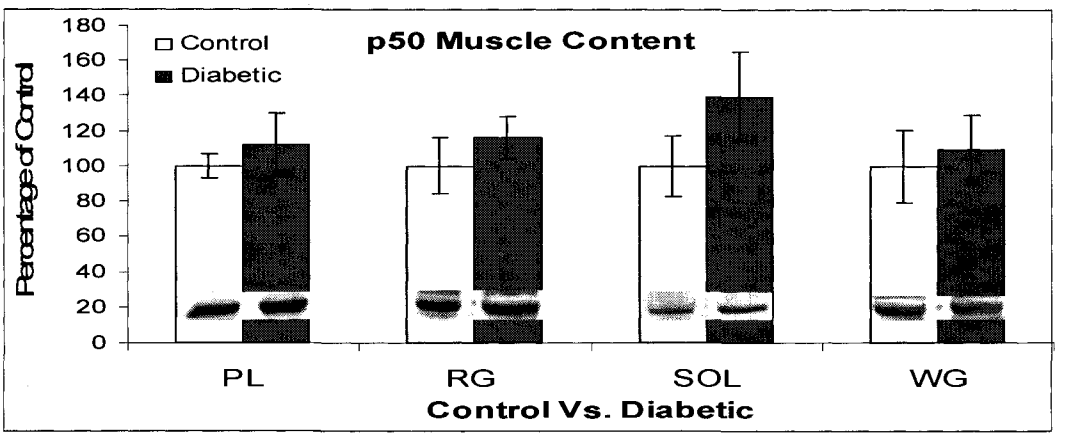


Figure 3.7*B*

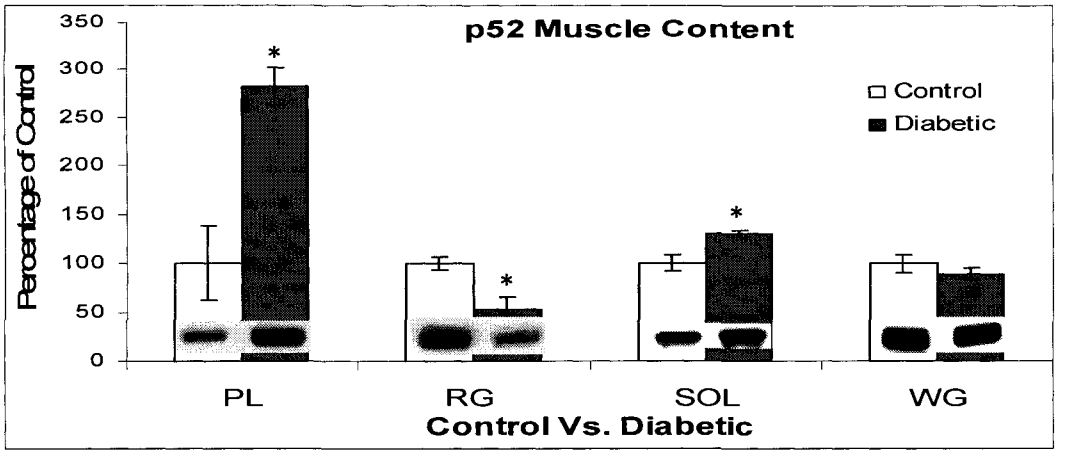


Figure 3.7*C*

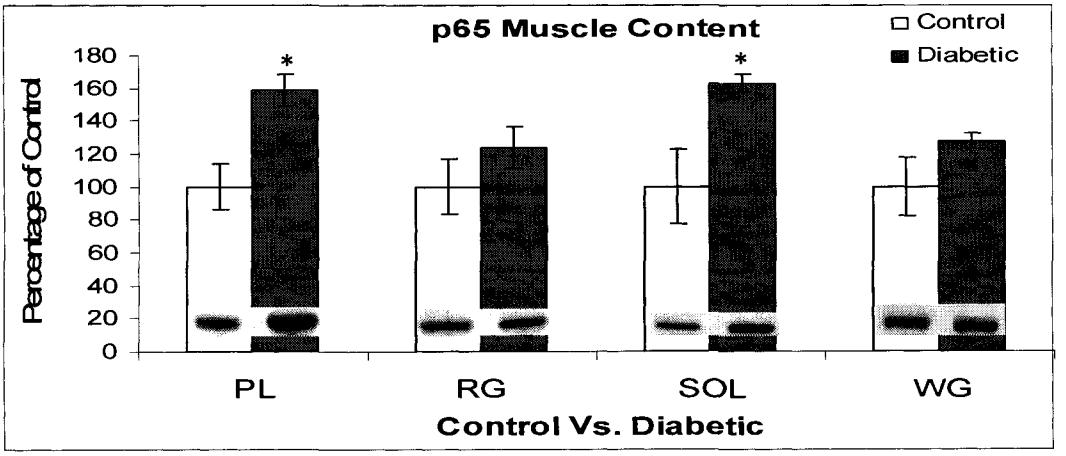


Figure 3.7*D*

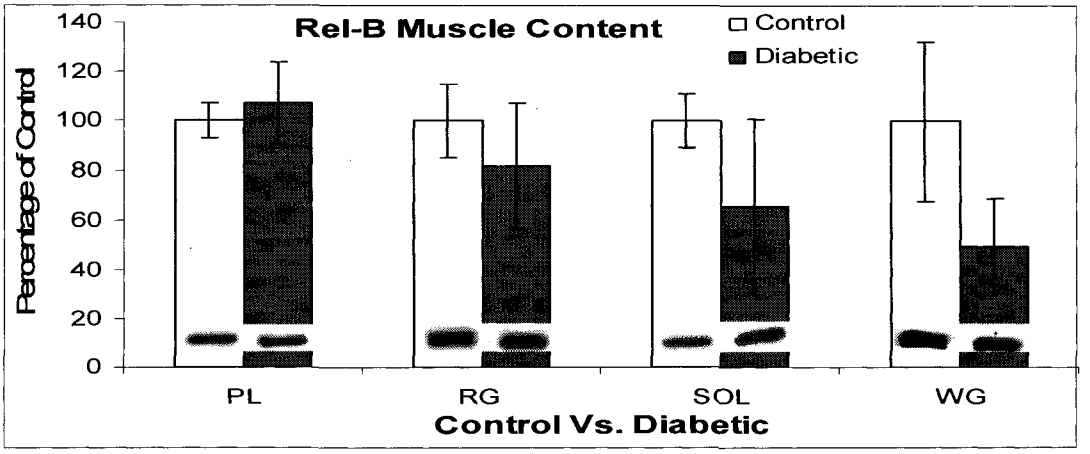
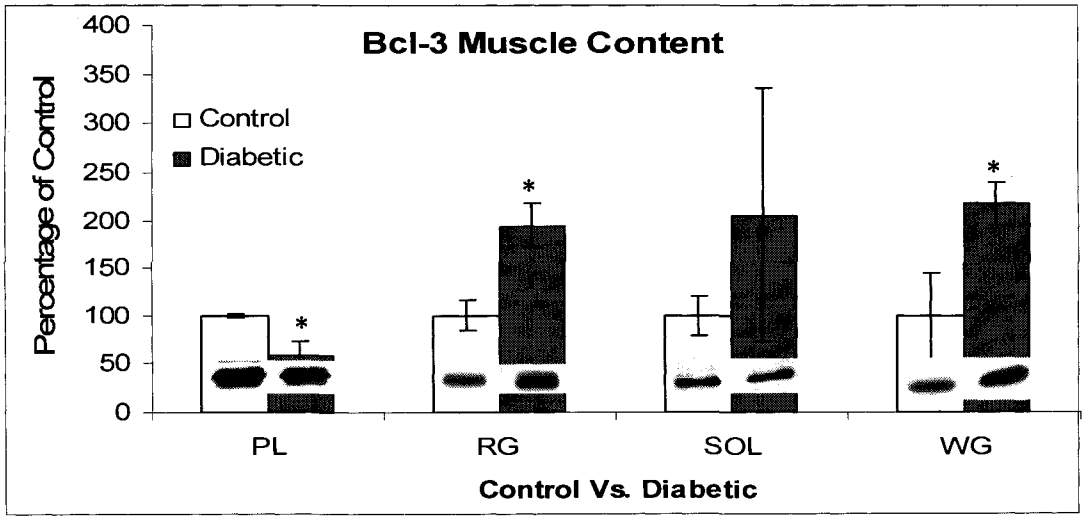


Figure 3.7*E*



3.4 DISCUSSION

A role for NF-κB and its varying composition has been established in atrophy-associated pathologies, including diabetes. In addition, diabetes appears to obtain a fiber-type specific atrophy leading to a greater loss of fast fibers (Armstrong et al., 1975). Therefore, the present study determined the expression and activation of NF-κB in various skeletal muscles in order to assess any muscle-specific relationship between NF-κB and atrophy under diabetic conditions. There were three main findings in the present study. First, NF-κB activation was reduced in the diabetic RG, but was unchanged in other muscles. Second, NF-κB dimer composition was found to be predominantly p50-homodimers in both control and diabetic muscles. Third, NF-κB subunit contents showed a tissue-specific expression and response to diabetes, revealing a unique response by the gastrocnemius, which was the only muscle to show a diminished muscle-to-body mass ratio.

NF-κB Activation is Reduced in Diabetic Muscle. An upregulation of NF-κB appears to be involved in muscle atrophy (Hunter and Kandarian 2004, Cai et al., 2004, Mourkioti et al., 2006). In agreement with this, in the present study only the gastrocnemius muscle demonstrated altered NF-κB activation with diabetes. However, contrary to expectations, the diabetic condition led to unchanged NF-κB activation in WG and a reduced NF-κB activation in RG. NF-κB activation in RG may have been reduced by a generalized reduction in cellular metabolism which may have inhibited NF-κB activation via reduced reactive oxygen species production (Nishikawa et al., 2000). Indeed, treatment with a similar amount of STZ has been shown to reduce oxidative-, and glycolytic enzyme activity selectively in the RG (Chen and Ianuzzo, 1982). However, in both the SOL and

PL, muscle-to-body mass ratio was maintained without a decrease in NF-κB activation; hence RG tissue preservation via reduced NF-κB activation is not supported by the results. Despite the typically reported observation that increased NF-κB activation occurs during stressful conditions it may not be the case that reduced NF-κB activation found in diabetic RG necessarily indicates less stress. In fact, some pathologies, such as incontinentia pigmenti, are associated with reduced NF-κB activation resulting in excessive apoptosis (Smahi et al., 2000). Nonetheless, the diminished NF-κB activity observed in the RG, combined with previous observations that STZ-induced diabetes preferentially affects the fast-glycolytic fibers while preserving slow oxidative fibers (Armstrong et al., 1975), indicates that diminished constitutive NF-κB activation may protect against atrophy in the RG, but not the WG.

The Composition of Activated NF-κB. The subunit composition of NF-κB was determined, using EMSA-supershifts, to assess muscle-specific differences and if diabetes altered the dimeric composition of NF-κB. For both control and diabetic conditions all muscle NF-κB appeared to be composed of p50, suggesting that the constitutive expression of NF-κB may be the p50-p50 homodimer. It should be noted that the predominant p50-homodimers observed in muscles does not necessarily preclude the existence of other dimers, since the p50 subunit protein itself may be transcribed by a dimer composed of p65 or c-Rel (Cogswell et al., 1993). Likeweise, the p52 protein appears to be transcribed by a p52 containing dimer (Liptay et al., 1994), suggesting that the detection of NF-κB proteins in the muscle necessarily indicates that dimers exist within the muscle that are not composed exclusively of p50. Hence, consititutive NF-κB binding by p50 homodimers may only reflect a normal transcriptional repression (Tong et

al., 2004) that is rapidly and transiently displaced by other activated dimers (Bosisio et al., 2006). Moreover, the constitutive binding by p50-homodimers in muscles from both normal and diabetic animals suggests that diabetes per se may not be inherently provocative to these muscles. The only muscle that demonstrated reduced muscle-to-body mass ratio (gastrocnemius) also showed a p50-homodimer in control and diabetic conditions in both WG and RG tissue (with a binding decrease in RG only). Hence, the diabetes-induced NF-κB composition and activity observed in the present study may indicate a muscle-specific, treatment-induced change in NF-κB activation, but did not reveal a clear consequence of altered activation and dimer composition relative to atrophy in this condition.

NF-κB Subunit Quantification. An altered quantity of NF-κB subunits in diabetic muscle supports the concept of a dynamic activation and composition variability. Therefore, altered protein quantities in diabetic tissue may characterize a muscle-specific pattern of NF-κB activity discretely unique to diabetes-related atrophy. Although both p50 and RelB remained unchanged in all tissues, one protein member of the canonical pathway (p65), and one member of the alternate pathway (p52), as well as the p50-homodimer co-activator (Bcl-3), were altered in diabetic muscle. In these cases changes in protein expression distinguished the gastrocnemius muscle from the SOL and PL muscles. Specifically, p52 was elevated in SOL and PL, but was reduced in RG tissue. Elevated p52 processing, which is normally inhibited (Xiao et al., 2001), has been previously reported in lymphomagenesis (Derudder et al., 2003), a pathology often associated with diabetes (Yui and Rothenberg 2004). In the present study, p52 was reduced in RG but was unchanged in WG which may indicate a mechanism by which the gastrocnemius is

protected during diabetes. However, since p52 remained unchanged in the WG, any protection may not be extended to atrophy.

Similarly, quantities of the p65 protein exhibited unchanged levels in WG and RG tissues, but were elevated in SOL and PL muscles. Activated dimers containing p65 have previously been reported to participate in cachexia (Guttridge et al. 2000), oxidative stress (Kefaloyianni et al., 2006), and age-related atrophy (Phillips and Leeuwenburgh, 2005). In addition, elevated contents of p65 have been reported in smooth muscle cells cultured in a high glucose environment, which led to increased p65-mediated transcription (Yerneni et al., 1999), a condition observed in vascular pathologies associated with diabetes. In contrast to these catabolic and pathological affects, elevated p65 protein content has also been reported following insulin-like growth factor II-induced myogenesis, a process requiring NF-κB activation (Kaliman et al., 1999), whereas p65 protein expression may remain unchanged during myostatin induced cachexia (McFarlane et al., 2006). Despite no detection of p65 with EMSA, the possibility remains that p65-associated transcription factors may be transiently activated as evidenced by maintained levels of p50, previously mentioned to be expressed via a p65-associated NF-κB dimer (Cogswell et al., 1993). Therefore, mechanisms of muscle wasting via p65-associated dimer activity may still exist within these muscles.

Bcl-3 was also increased in WG and RG muscles, but diminished in PL, and unchanged in SOL. Normal Bcl-3 expression appears to block cellular arrest and apoptosis (Kashatus et al., 2006), and is up-regulated to protect against reduced growth factors (Rebollo et al., 2000). In contrast, reduced expression may diminish both survival against infection and myogenic differentiation (Schwarz et al., 1997, Shiio et al., 1996).

Although increased Bcl-3 content and binding with p50-homodimers were previously reported to be required for unloading-induced atrophy (Hunter et al., 2002) its altered expression within the present study was not associated with activated p50-homodimers. Nonetheless, it is possible that Bcl-3 was up-regulated within skeletal muscle in response to diabetes, since the gastrocnemius muscle demonstrated both an elevated Bcl-3 protein and a significantly reduced muscle-to-body mass ratio.

Taken together, NF-κB family members demonstrated a tissue-specific expression under diabetic conditions. Although it is not clear what role each protein may play in any diabetes-induced atrophy, there does appear to be a modified pattern of protein expression that distinguishes the gastrocnemius muscle from the SOL and PL muscles.

Body Mass and Muscle Mass Changes. Body mass is typically reduced in the STZ model of diabetes (Armstrong et al., 1975, Price et al., 1996), therefore, attenuated body mass growth was expected. Likewise, it was expected that hindlimb skeletal muscles would demonstrate reduced mass accumulation. Indeed, impaired body mass growth was paralleled by a proportional, attenuated accruement of muscle mass. The exception was the gastrocnemius muscle, which showed a markedly reduced muscle-to-body mass ratio. Armstrong et al., (1975) showed reduced area in fast glycolytic fibers, but no reduction in the slow oxidative- or fast oxidative fiber area-to-body mass ratio in the gastrocnemius of diabetic rats. This suggests that the significantly reduced gastrocnemius muscle-to-body mass ratio in the present study may be mostly due to a susceptibility of fast glycolytic fibers to STZ treatment (Chaudhury et al., 1994) and may be partly attributable to the upregulation of proteolytic mechanisms (Price et al., 1996, Lee et al., 2004) and/or the

loss of anabolic signaling (Chaudhury et al., 1994). However, the atrophic phenomenon observed in the present study was not discernibly fiber-type specific, as the SOL (almost entirely slow fibers) and PL (composed of mostly fast fibers) did not show a diminished muscle-to-body mass ratio. Nonetheless, atrophy was observed in the gastrocnemius. Muscle recruitment patterns indicate that the SOL would be maximally recruited during normal movements while the PL and gastrocnemius would be recruited in parallel with increasing force requirements (Armtrong et al., 1985). However, the gastrocnemius muscle would have a far greater amount of total mass inactive during normal movements compared to both the SOL and PL muscles by virtue of its total mass and quantity of fast fibers. Hence, the possibility exists that the PL and SOL muscles are protected against diabetes-induced atrophy via muscle activity, as a greater percentage of the total mass would be recruited during normal movements whereas the gastrocnemius would remain essentially inactive under the same conditions leading to diabetes-induced, inactivity-mediated atrophy.

3.5 CONCLUSION

DNA binding activity of NF-κB was reduced in diabetic RG muscles only and, in all cases, dimer composition appeared to be composed primarily of p50-homodimers. The finding that p50-homodimers comprised the complement of skeletal muscle NF-κB dimers suggests this to be the constitutive binding dimer, which may act to suppress transcription (Tong et al., 2004), but may not absolutely deny a rapid and transient activation by an assortment of NF-κB transcription factors. While it was proposed that NF-κB would demonstrate muscle-specific activation and would be intimated with

skeletal muscle atrophy, the complexity of a transcription factor responsible for the regulation of copious and largely uncharacterized gene expression did not readily provide an unequivocal role for NF-κB activation, composition, and protein expression in STZ-induced atrophy. The paradox that various forms of the NF-κB dimer appear to be a principle player in both pro- and anti-apoptosis (Fan et al., 2002), and in hypertrophic and atrophy-related pathways testifies to its complexity and importance in gene regulation. Nonetheless, the unique atrophic effect of diabetes on the gastrocnemius muscle observed in the present study revealed a unique profile of NF-κB regulation and protein expression.

3.6 REFERENCES

Armstrong RB, Gollnick PD, Ianuzzo CD. 1975. Histochemical properties of skeletal muscle fibers in streptozotocin-diabetic rats. *Cell Tissue Res* 162: 387-394.

Armstrong RB, Laughlin MH. 1985. Metabolic indicators of fibre recruitment in mammalian muscles during locomotion. *J Exp Biol* 115: 201-213.

Bosisio D, Marazzi I, Agresti A, Shimizu N, Bianchi ME, Natoli G. 2006. A hyper-dynamic equilibrium between promoter-bound and nucleoplasmic dimers controls NF-kappaB-dependent gene activity. *EMBO J* 25: 798-810.

Cai D, Frantz JD, Tawa NE Jr, Melendez PA, et al. 2004. IKKbeta/NF-kappaB activation causes severe muscle wasting in mice. *Cell* 119: 285-298.

Chaudhury SK, Mandal MB, Deshpande SB, Saxena ID. 1994. Effect of streptozotocin-induced diabetes on growth and proteolytic activity of different muscles in rats. *Indian J Exp Biol* 32: 877-880.

Chen V, Ianuzzo CD. 1982. Dosage effect of streptozotocin on rat tissue enzyme activities and glycogen concentration. *Can J Physiol Pharmacol* 60: 1251-1256.

Cogswell PC, Scheinman RI, Baldwin AS Jr. 1993. Promoter of the human NF-kappa B p50/p105 gene. Regulation by NF-kappa B subunits and by c-REL. *J Immunol* 150: 2794-2804.

Derudder E, Laferte A, Ferreira V, Mishal Z, Baud V, Tarantino N, Korner M. 2003. Identification and characterization of p100HB, a new mutant form of p100/NF-kappa B2. *Biochem Biophys Res Commun* 308: 744-749.

Engstrom G, Stavenow L, Hedblad B, Lind P, Eriksson KF, Janzon L, Lindgarde F. 2003. Inflammation-sensitive plasma proteins, diabetes, and mortality and incidence of myocardial infarction and stroke: a population-based study. *Diabetes* 52: 442-447.

Fan C, Yang J, Engelhardt JF. 2002. Temporal pattern of NFkappaB activation influences apoptotic cell fate in a stimuli-dependent fashion. *J Cell Sci* 115: 4843–4853.

Gilmore TD. 2006. Introduction to NF-kB: players, pathways, perspectives. *Oncogene* 25: 6680–6684.

Guttridge DC, Mayo MW, Madrid LV, Wang CY, and Baldwin AS Jr. 2000. NF-kB-induced loss of MyoD messenger RNA: possible role in muscle decay and cachexia. *Science* 289: 2363–2366.

Hoffmann A, Natoli G, Ghosh G. 2006. Transcriptional regulation via the NF-kappaB signaling module. *Oncogene* 25: 6706-6716.

Hunter RB, Kandarian SC. 2004. Disruption of either the Nfkb1 or the Bcl3 gene inhibits skeletal muscle atrophy. *J Clin Invest* 114: 1504-1511.

Hunter RB, Stevenson E, Koncarevic A, Mitchell-Felton H, Essig DA, Kandarian SC. 2002. Activation of an alternative NF-kappaB pathway in skeletal muscle during disuse atrophy. *FASEB J* 16: 529-538.

Kaliman P, Canicio J, Testar X, Palacin M, Zorzano A. 1999. Insulin-like growth factor-II, phosphatidylinositol 3-kinase, nuclear factor-kappaB and inducible nitric-oxide synthase define a common myogenic signaling pathway. *J Biol Chem* 274: 17437-17444.

Kashatus D, Cogswell P, Baldwin AS. 2006. Expression of the Bcl-3 proto-oncogene suppresses p53 activation. Genes Dev. 20: 225-235.

Kefaloyianni E, Gaitanaki C, Beis I. 2006. ERK1/2 and p38-MAPK signalling pathways, through MSK1, are involved in NF-kappaB transactivation during oxidative stress in skeletal myoblasts. *Cell Signal* 18: 2238-2251.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

Lecker SH, Jagoe RT, Gilbert A, et al. 2004. Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. *FASEB J* 18: 39–51.

Lee SW, Dai G, Hu Z, Wang X, Du J, Mitch WE. 2004. Regulation of muscle protein degradation: coordinated control of apoptotic and ubiquitin-proteasome systems by phosphatidylinositol 3 kinase. *J Am Soc Nephrol* 15: 1537-1545.

Liptay S, Schmid RM, Nabel EG, Nabel GJ. 1994. Transcriptional regulation of NF-kappa B2: evidence for kappa B-mediated positive and negative autoregulation. *Mol Cell Biol* 14: 7695-7703.

Locke M, Tanguay RM. 1996. Increased HSF activation in muscles with a high constitutive Hsp70 expression. *Cell Stress Chaperones* 1: 189-196.

Locke M, Tanguay RM, Klabunde RE, Ianuzzo CD. 1995. Enhanced postischemic myocardial recovery following exercise induction of HSP 72. *Am J Physiol* 269: 320-325.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265-275.

McFarlane C, Plummer E, Thomas M, et al. 2006. Myostatin induces cachexia by activating the ubiquitin proteolytic system through an NF-kappaB-independent, FoxO1-dependent mechanism. *J Cell Physiol* 209: 501-514.

Mourkioti F, Kratsios P, Luedde T, et al. 2006. Targeted ablation of IKK2 improves skeletal muscle strength, maintains mass, and promotes regeneration. *J Clin Invest* 116: 2945-2954.

Nishikawa T, Edelstein D, Du XL, et al. 2000. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 404: 787-790.

Phillips T, Leeuwenburgh C. 2005. Muscle fiber specific apoptosis and TNF-alpha signaling in sarcopenia are attenuated by life-long calorie restriction. *FASEB J* 19: 668-670.

Price SR, Bailey JL, Wang X, Jurkovitz C, England BK, Ding X, Phillips LS, Mitch WE. 1996. Muscle wasting in insulinopenic rats results from activation of the ATP-dependent, ubiquitin-proteasome proteolytic pathway by a mechanism including gene transcription. *J Clin Invest* 98: 1703-1708.

Rebollo A, Dumoutier L, Renauld JC, Zaballos A, Ayllon V, Martinez-A C. 2000. Bcl-3 expression promotes cell survival following interleukin-4 deprivation and is controlled by AP1 and AP1-like transcription factors. *Mol Cell Biol* 20: 3407-3416.

Schwarz EM, Krimpenfort P, Berns A, Verma IM. 1997. Immunological defects in mice with a targeted disruption in Bcl-3. *Genes Dev* 11: 187-197.

Shiio Y, Sawada J, Handa H, Yamamoto T, Inoue J. 1996. Activation of the retinoblastoma gene expression by Bcl-3: implication for muscle cell differentiation. *Oncogene* 12: 1837-1845.

Smahi A, Courtois G, Vabres P, et al. 2000. Genomic rearrangement in NEMO impairs NF-kappaB activation and is a cause of incontinentia pigmenti. The International Incontinentia Pigmenti (IP) Consortium. *Nature* 405: 466-472.

Tong X, Yin L, Washington R, Rosenberg DW, Giardina C. 2004. The p50-p50 NF-kappaB complex as a stimulus-specific repressor of gene activation. *Mol Cell Biochem* 265: 171-183.

Towbin H, Staehelin T, Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Biotechnology* 24: 145-149.

Wyke SM, Tisdale MJ. 2005. NF-kappaB mediates proteolysis-inducing factor induced protein degradation and expression of the ubiquitin-proteasome system in skeletal muscle. *Br J Cancer* 92: 711-721.

Xiao G, Harhaj EW, Sun SC. 2001. NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100. *Mol Cell* 7: 401-409.

Yerneni KK, Bai W, Khan BV, Medford RM, Natarajan R. 1999. Hyperglycemia induced activation of nuclear transcription factor kappaB in vascular smooth muscle cells. *Diabetes* 48: 855-864.

Yui MA, Rothenberg EV. 2004. Deranged early T cell development in immunodeficient strains of nonobese diabetic mice. *J Immunol* 173: 5381-5391.

CHAPTER 4

Exercise training and diabetes similarly increase NF-κB-DNA binding in the rat myocardium but may be uniquely regulated by upstream effectors

4.1 INTRODUCTION

There is a well established association between diabetes mellitus (DM) and inflammation (Rekeneire et al., 2006; reviewed by Gleissner et al., 2007, Wellen and Hotamisligil, 2005 and Engstrom et al., 2003). In addition to causing numerous complications, DM typically results in cardiovascular disease which represents the leading cause of death among individuals with DM. Diabetic cardiomyopathy appears to be strongly associated with inflammation in humans (Engström et al., 2003) as well as in animal models of DM (Westermann et al., 2007). There is a possibility that hyperglycemia and/or hyperlipidemia-induced inflammation in DM acts as the primary instigating factor responsible for developing cardiomyopathy, and insulin may act through the NFκB pathway as a key regulating suppressor of inflammation (Dandona et al., 2007; Dandona et al., 2003). Hence, inflammation activated by hyperglycemia remains unabated by the absence of insulin, thereby disrupting normal intracellular signaling in the diabetic myocardium.

As was recently demonstrated (chapter 3), the chief regulator of inflammation, NFκB, was associated with DM-induced skeletal muscle atrophy in a muscle-specific manner. Specifically, portions of the gastrocnemius muscle (those composed of slow myosin) showed reduced NFκB activation, whereas the soleus (also composed of slow myosin) showed normal quantities of NFκB activation. In the diabetic vastus lateralis (mixed fibre-type) Sriwijitkamol et al. (2006) demonstrated that content of the NFκB inhibitor, IκB-α, was reduced. These investigators interpreted reduced inhibitor content to mean that NFκB activation was excessive in this tissue. These findings represent a unique

activation of inflammation in various diabetic muscles, independent of muscle fibre-types.

Owing to a similar fibre-type composition, it has been proposed that because slow myosin fibres resists DM-induced atrophy, heart muscle may be uniquely protected against diabetes and better able to maintain phenotype, metabolic profiles, and peptide chain initiation rates (Armstrong et al., 1975; Armstrong and Ianuzzo, 1976; Jefferson et al., 1974). However, the discovery of altered NF-κB activity within similar fibre-types provides evidence that the diabetic myocardium may have an inflammatory activation pattern very dissimilar to that seen in most skeletal muscle types.

Under normal conditions, constitutive NF-κB binding appears to involve a p50-p50 homodimer, which may act to inhibit transcription by blocking access to the binding site by other NF-κB dimer combinations (reviewed by Hoffmann et al., 2006). Under pathological conditions, there appears to be a rapid and transient transcriptional activation of NF-κB target genes by the p50-p65 protein dimer (reviewed by Gilmore 2006). Interestingly, activated NF-κB from disuse-atrophied muscle has been shown to contain the B-cell lymphoma protein, Bcl-3, within the p50-p50 homodimer (Hunter et al., 2002). Bcl-3 is thought to interact with p50-p50 homodimers and act as a transcriptional activator, while restricting the pathological p50-p65 heterodimer from gaining access to the NF-κB promoter (Carmody et al., 2007).

Hence, NF-κB within the diabetic myocardium may demonstrate a unique protein combination. Moreover, myocardium-specific activation of signaling compounds and the inflammatory pathway may be complicit in manifesting long-term complications seen in the diabetic myocardium, particularly when considering that both inflammation and

cardiomyopathy are inherent to DM and potentially linked in a cause and effect manner. Therefore, determining whether DM alters the quantity of DNA binding or activates the inflammatory pathway through a specific NF-κB protein combination may demonstrate the method and magnitude of its participation. Measuring altered expression and modified behavior of upstream regulators of NF-κB, such as the inhibiting protein (IκB-α) and the inhibitor-degrading kinase (IκK-β), may help explain any revision to NF-κB-DNA binding in the diabetic myocardium. Inhibiting or initiating transcription factor binding often involves proteins complexed with the main factor; therefore, NF-κB may also require addition factors to promote binding (reviewed by Salminen et al., 2008). Specifically, the NF-κB activating kinase has been shown to be complexed with the heat shock protein, HSP90, and its disruption prevents NF-κB binding (Pittet et al., 2005). Hence, determining whether HSP90 is also complexed with the NF-κB-DNA complex or can influence binding activity in exercised or DM myocardia could help delineate additional regulatory mechanisms.

Modified behavior is recommended in cardiac patients by specifically prescribing exercise to improve myocardial function (ADA, 2002). Aerobic exercise was also shown to improve cardiac function in STZ-induced DM in rats (Loganathan et al., 2007). Limited data are available regarding an effect of exercise on the activation of NF-κB. Most research indicates that NF-κB is transiently activated following an acute bout of moderate or intense exercise (Ji et al., 2004; Ho et al. 2005); with little evidence showing reduced activity after acute exercise (Durham et al., 2004; García-López et al., 2007). There is some indication that activation is elevated following aerobic training (Spangenburg et al., 2006), but NF-κB activation was also shown to be reduced following

acute exercise. In addition, aerobic training, due in part to an increase in the IκB inhibitor, reportedly diminished NF-κB activity in skeletal muscle (Sriwijitkamol et al., 2006). The effect of exercise training on NF-κB in the diabetic myocardium is currently unknown. The delineation of this pathway may uncover numerous intermediaries responsible for the progression of the diabetic sequella, potentially identifying therapeutic targets.

Therefore, the purpose of the present study was to characterize the composition and quantify NF-κB activation in the diabetic myocardium and to measure the content and condition of the regulating proteins upstream of its activation. In addition, the current study determined the capacity of exercise to prevent and to mitigate inflammatory stress in the rat myocardium. It was hypothesized that diabetes would alter the NF-κB protein complex and increase its activation by reducing upstream inhibition. It was also hypothesized that exercised cardiac muscle would show normalized NF-κB binding in both diabetic and non-diabetic animals and would obtain a normal NF-κB protein complex.

4.2 METHODS AND MATERIALS

Animal Characteristics and Experimental Groups. All procedures were approved by the Animal Care Subcommittee at The University of Western Ontario. Sixty male Sprague-Dawley rats (~220-250g; Charles River Laboratories, Quebec, Canada) were maintained on a 12-hour dark/light cycle, housed at 20 ±1°C, 50% relative humidity, and provided with standard rat chow and water ad libitum. Animals were divided into DM and non-DM groups. Animals from each group (n=10) were assigned to 1 of 3

treatments: (1) sedentary (SED); (2) 4-weeks sedentary followed by 4-weeks of exercise (EX-4); or (3) 8-weeks of exercise (EX-8).

Diabetes Induction. Animals received a low-dose injection of STZ (20mg/kg; i.p.; Sigma-Aldrich) within 5 minutes of it being dissolved in citrate buffer (0.1 M, pH 4.5) on 5 consecutive days. Non-DM animals were injected with citrate buffer only. DM was confirmed by measuring a blood glucose level >15 mmol. Whole body animal weights and blood glucose values were monitored weekly via blood draw from the saphenous vein using the One Touch Ultra 2 Blood Glucose Monitoring System (Lifescan Canada Ltd, Burnaby, BC,Canada) and One Touch test strips (Lifescan Canada; range = 0–600 mg/dl).

Exercise Training. Rats were familiarized to treadmill running on 2 occasions with brief, 10-minute exposures at 15m/min (5 & 3 days prior to training). At the beginning of the training, rats were run at progressing intensities with unchanging duration and frequency (one hour/day, 5 days/week) starting at 19m/min for 5 days (week 1), followed by 23m/min for 5 days (week 2), 27m/min for 5 days (week 3), and continued thereafter at a constant running intensity of 27m/min (the final intensity corresponding with a work rate of ~75% VO_{2max}). Continuous running during the exercise session was encouraged by compressed air blowing at right angles onto the rat haunches, whenever it moved back beyond a certain point (so as to break a photoelectric beam) on the treadmill. This air jet served to act as a stimulus to encourage running and to act as a warning that the end of the belt was approaching. All animals were able to adjust to increasing exercise intensities and completed the training protocol.

Polyacrylamide Gel Electrophoresis and Immunoblotting. Quantities of the phosphorylated form of IκK-β and total quantities of IκB-α were measured within the present study to determine whether the activity of the upstream NF-κB regulator and quantity of the inhibitor were altered by DM or exercise.

The heart was removed by cardiac excision and the atria and right ventricle were trimmed away. A portion of the left ventricle (50 mg) was homogenized in lysis buffer (137 mM NaCl, 20 mM Tris-HCl, pH 7.5, 10% glycerol, 1% Triton X-100, 0.5% Nonidet P-40, 2 mM EDTA, pH 8.0, 3 μg/ml aprotinin, 3 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 10 mM NaPP, and 2 mM Na3VO4) and protein concentration determined using the Bradford method (1976). One dimensional (1-D) sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed according to the method described by Laemmli (1970), except that the separating gel (0.15 x 4.5 x 8 cm) consisted of a 5-15% polyacrylamide gradient. Following electrophoretic separation, proteins were transferred to nitrocellulose membranes (0.22 μm pore size, Bio-Rad Laboratories) as described by Towbin et al. (1979) using the Bio-Rad mini-protean II gel transfer system. Following protein transfer, blots were reacted with a polyclonal antibody specific for IκB-α (C-21: catalogue # sc-371) and phospho-IκK-α/β (Serine 181: catalogue # sc-23470), Santa Cruz Biotechnology, California, USA., diluted 1:1000 in TTBS with 2% blotto overnight.

A horse radish peroxidase-conjugated secondary antibody was employed (StressGen, Victoria, Canada) and the blot was developed using enhanced chemiluminescence. Briefly, following secondary incubation the blot was immersed in 5 ml of luminal enhancer and 5 ml of stable peroxide solution (ECL, Amersham) for 1

minute and then exposed to x-ray film to visualize the protein bands. To determine nonspecific binding, duplicate gels were run, transferred and reacted, but the primary antibody was omitted. Immunoblots were scanned and quantification of bands from immunoblots was performed by using Scion Image Software (Scion Corporation, Frederick, Maryland, USA).

Electrophoretic Mobility Shift Assay (EMSA). Heart muscle tissue (50 mg) was homogenized in 15 volumes of extraction buffer (25% glycerol, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA (pH 8.0), 20 mM HEPES (pH 7.9), 0.5 mM DTT, 0.5 mM phenylmethylsufonylfluoride) at 4°C. Homogenates were centrifuged at 14,000 rpm at 4°C (16000 rot/min) for 20 minutes in an Eppendorf centrifuge (model #5804R). The supernatant was removed and protein concentration was determined by the method described by Bradford (1976) using BSA as a standard and stored at -70°C. Analyses of NF-kB-oligonucleotide binding in extracts were performed using an EMSA according to the procedure described by Locke and Tanguay (1996). Protein extracts (50 ug) from muscles were incubated with a ^{32}P-labeled, ideal NF-kB oligonucleotide (5'- AGT TGA GGG GAC TTT CCC AGG C-3'; E3291, Promega) in binding buffer (10% glycerol, 50 mM NaCl, 1.0 mM EDTA [pH, 8.0], 20 mM Tris [pH, 8.0], 1.0 mM DTT, 0.3 mg/ml BSA) with approximately 0.1 ng (50,000 cpm) of ^{32}P-labeled oligonucleotide and 2.0 µg poly dI dC (Pharmacia Fine Chemicals, Piscataway, NJ, USA) for at least 30 minutes at room temperature. Samples were electrophoresed on 4 % acrylamide gel (5x gel running buffer (pH 8.5), 30% acrylamide, 2% bis-acrylamide, 50% glycerol, 30% APS, Temed) at 200V for 2-3 hours. Gels were dried using a BioRad Slab dryer (Model 433) for 45 minutes and exposed to radiographic film (Amersham-ECL, Mississauga, Ontario,

Canada) for 1 or 2 days at -70°C. Recombinant NF-kB (p50) [CAS# (56-81-5) Promega, Madison, WI, USA] was run on each gel to determine the positive identification and location of the bound NF-kB-oligonucleotide complex. Correct NF-kB-oligonucleotide interaction was confirmed by the absence of NF-kB-activation with the addition of an excess of non-labeled NF-kB oligonucleotides.

Supershift assays were performed to determine the composition of the NF-kB dimer. Briefly, each lane was loaded with a labeled oligo/protein extract along with 1 μg of an antibody specific for a single member of the NF-kB family [p50 (NLS; sc-114 X), p52 (K-27; sc-298 X), p65 (C-20; sc-372 X), RelB (C-19; sc-226 X), C-Rel (C; sc-71 X), and Bcl-3 (C-14; sc-185 X), Santa Cruz Biotechnology, California, USA]. The occurrence of an NF-kB member protein within the dimer complex was determined by both the reduction of band intensity at the pre-determined molecular weight location in addition to the localization of a band corresponding to a greater molecular weight.

Statistical Analysis. A two-way ANOVA (SPSS) was performed using diabetes and exercise as conditions for each group. A Bonferonni post hoc was used to determine significant differences between groups. Differences between groups were considered statistically significant at a level of $P < 0.05$. Data are expressed as the mean ± SEM.

4.3 RESULTS

Physical characteristics and blood glucose. Measures of blood glucose and body mass were taken once per week for the duration of the experiment. Both blood glucose (shown in Figure 4.1) and body mass (shown in Figure 4.2) demonstrated a clear

difference between DM and Non-DM animals. No differences in blood glucose were found between DM and Non-DM groups prior to STZ injections (6.45 ± .17 mmol/L vs. 6.94 ± .30 mmol/L, respectively). Eight weeks following STZ injections, blood glucose was significantly greater ($P < 0.05$) in STZ-injected animals compared to Non-STZ animals (29.56 ± 0.83mmol/L vs. 6.97 ± 0.34mmol/L, respectively). Prior to STZ injections no difference in body mass was found between DM and Non-DM animals (333 ± 3 g vs. 331 ± 3 g, respectively). Eight weeks following STZ injections, DM animals obtained significantly increased body mass compared to starting values, but Non-DM animals demonstrated greater mass than DM animals (521 ± 7 g vs 362 ± 7 g, respectively) at the end of the experiment.

Quantification of phosphorylated IκK-β and IκB-α. Upstream regulators of NF-κB were determined via western blotting. Quantities of phosphorylated IκK-β are shown in Figure 4.3. There was no significant difference in phosphorylated IκK-β protein quantity between any groups at the end of 8 weeks. As phosphorylated IκK-β is responsible for initiating degradation of the NF-κB inhibitor, these results indicate that in the diabetic myocardium the kinase activity is not different from control tissues, with or without exercise. These data are limited to indicating that the kinase is equally active in DM and Non-DM tissues, but there is no indication regarding its actual phosphorylation of the inhibitor protein.

Once phosphorylated by IκK-β, IκB-α is rapidly degraded, leading to NF-κB release and nuclear translocation. Obtaining a total quantity of IκB-α provided a quantitative indication of how many NF-κB complexes are inhibited within the cell, in addition to quantifying the degradation status of the inhibitor protein. Figure 4.4

illustrates significantly increased ($P < 0.05$) quantities of DM-induced IκB-α protein. Exercise had no effect on IκB-α quantities in either the DM or Non-DM myocardium. Increased IκB-α protein may indicate increased inhibition or an increased number of NF-κB dimers within the DM myocardium with a corresponding increase in inhibitor-transcription factor complexes.

DM and Exercise-induced NF-κB DNA-binding. Electrophoretic mobility shift assays were used to determine the DNA binding status of NF-κB following exercise within the DM myocardium. Figure 4.5 shows NF-κB-DNA binding in all experimental groups. The results indicate that when compared to control tissue, both DM and exercise (independently and in concert) led to significant ($P < 0.05$) NF-κB-DNA binding. Scanned intensities of bound NF-κB were not different between exercised and DM animals. A direct comparison between DM and DM Ex8 groups (shown in Figure 4.6) further provides evidence of similar NF-κB activation in hearts from DM and DM exercised tissue. Hence, the total quantity of NF-κB-DNA binding appears to be elevated similarly by dissimilar conditions, one ultimately harmful and the other beneficial.

Supershift assays for NF-κB protein complex identification. The composition of constitutively bound NF-κB within normal heart tissue is shown in Figure 4.7. The addition of antibodies raised against p50 led to an upward shift in virtually the entire NF-κB-oligomeric complex. The lone addition of all other NF-κB family member proteins, and Bcl-3, did not result in a visible reduction in band intensity or in the localization of any band above the main complex. When antibodies for all of these proteins were combined and loaded into the same well there was an upward shift in virtually the entire NF-κB-Oligo complex, to the same location as when only p50 antibodies were loaded.

This indicates that NF-κB within the normal myocardium is composed primarily of p50-p50 dimers.

The composition of NF-κB within DM tissue is shown in Figure 4.8a. The supershift shows a result similar to that found in normal tissue; again, virtually the entire transcription factor-oligomeric complex is supershifted by the addition of p50, whereas the addition of p65 antibodies does not appear to shift the complex upward. When antibodies against all NF-κB family members were added, the entire complex was shifted. Hence, although there may be some heterodimers within the complex, the constitutive binding of NF-κB appears to be primarily p50-p50 homodimers, even when absolute binding activity was increased by DM. Figure 4.8b shows the results of a supershift assay performed using to determine whether DM is a condition similar to disuse atrophy that results in a transcription factor complexed with Bcl-3. The inclusion of Bcl-3 in 5 different samples demonstrated that Bcl-3 did not appear to be involved in DM-activated NF-κB.

Exercise training-activated NF-κB was also determined via supershift assay and is shown in Figure 4.9. As was observed in DM tissue, NF-κB from exercised tissue showed a strong band shift with the addition of p50 antibodies. The addition of either p65 or Bcl-3 did not result in the localization of a band above the main complex. When antibodies against all NF-κB family members were added, the entire primary complex was shifted. As with DM tissue, these results indicate that although there may be some heterodimers within the complex, the constitutive binding of NF-κB appears to be primarily p50-p50 homodimers, even when absolute binding activity was increased by exercise.

Supershifts from diabetic animals subjected to exercise are shown in Figure 4.10. Just as the composition of activated NF-κB from DM only and exercise only myocardial tissue were shown to be primarily composed of p50-p50 homodimers, exercise training in DM animals did not result in a qualitative shift in dimer composition. Although there may appear to be a subtle reduction in complex intensity when antibodies specific for p65 or Bcl-3 is added to the sample, the predominant band shift appears to be a principally the effect of p50 antibodies. Hence, constitutive binding of NF-κB in tissues from all groups appeared to be predominantly represented by the normally repressive p50-p50 homodimer complex.

HSP90 Regulation of NF-κB binding. As the molecular chaperone HSP90 has been shown to inhibit NF-κB (Pittet et al., 2005), an additional series of supershifts were performed by adding HSP90-specific antibodies to the tissue samples to determine whether HSP90 formed part of the NF-κB complex or whether its blockade would influence binding. Figures 4.11*A-C* illustrates an obvious effect of adding α-HSP90 to samples control, DM and exercised heart tissue. After a brief exposure of the gel to x-ray film, samples without α-HSP90 antibodies showed virtually undetectable NF-κB-oligo binding. In samples with α-HSP90 antibodies added, a large quantity of NF-κB binding was observed. Since NF-κB binding activity is enhanced with the addition of α-HSP90 antibodies, it seems reasonable to conclude that, under normal conditions, HSP90 interacts with the NF-κB complex or with upstream and/or downstream regulators to repress transcriptional activation. Hence, elevated binding following 8 weeks of DM and/or following 8 weeks of exercise may be due in part to diminished HSP90 inhibition.

Figure 4.1. Blood glucose as measured prior to the initial injections of STZ and during the final week of the experiments. Initial blood samples demonstrated no difference between animals divided into Non-Dm and DM groups. Final glucose values showed that animals that received STZ injections obtained significantly greater ($P < 0.05$) blood glucose values compared to animals that did not receive STZ. Data expressed as mean ± SEM (n = 30 per group). *Significantly greater ($P < 0.05$) than Non-DM animals and pre-injection values.

Figure 4.1

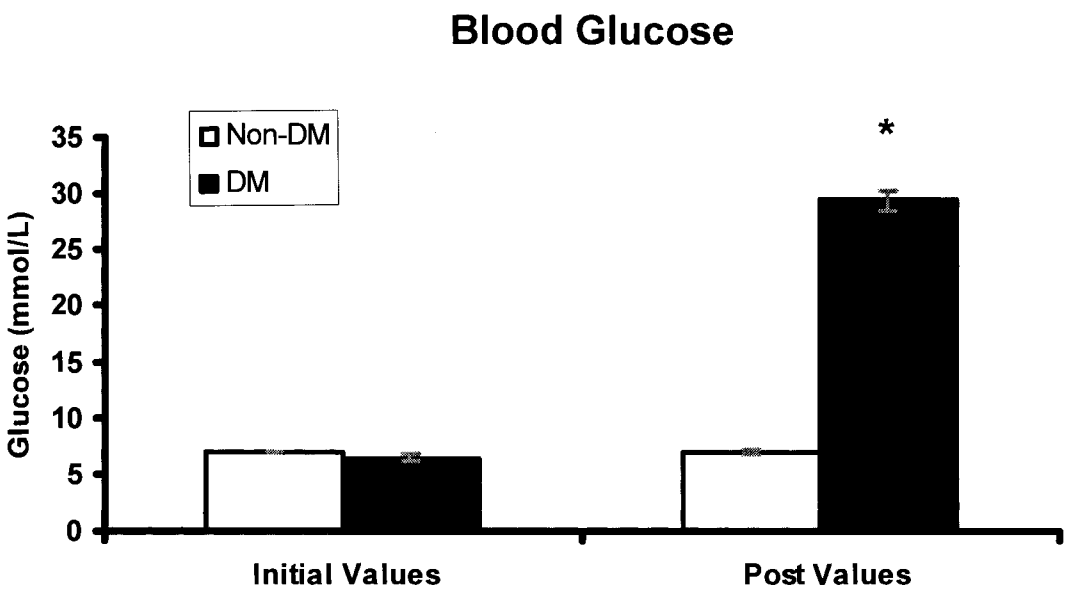

Blood Glucose
Non-DM
DM
Glucose (mmol/L)
35
30
25
20
15
10
5
0
*
Initial Values
Post Values

Figure 4.2. Total animal body mass following 8 weeks of STZ injections and/or exercise. Diabetic animals significantly increased ($P < 0.05$) body mass after 8 weeks. However, Non-DM animals demonstrated significantly greater body mass compared to all other groups after the experimental protocol. Data expressed as mean ± SEM (n = 30 per group). *Significantly greater ($P < 0.05$) than pre-treatment values. †Significantly greater ($P < 0.05$) than pre-treatment values and post treatment values for DM animals.

Figure 4.2

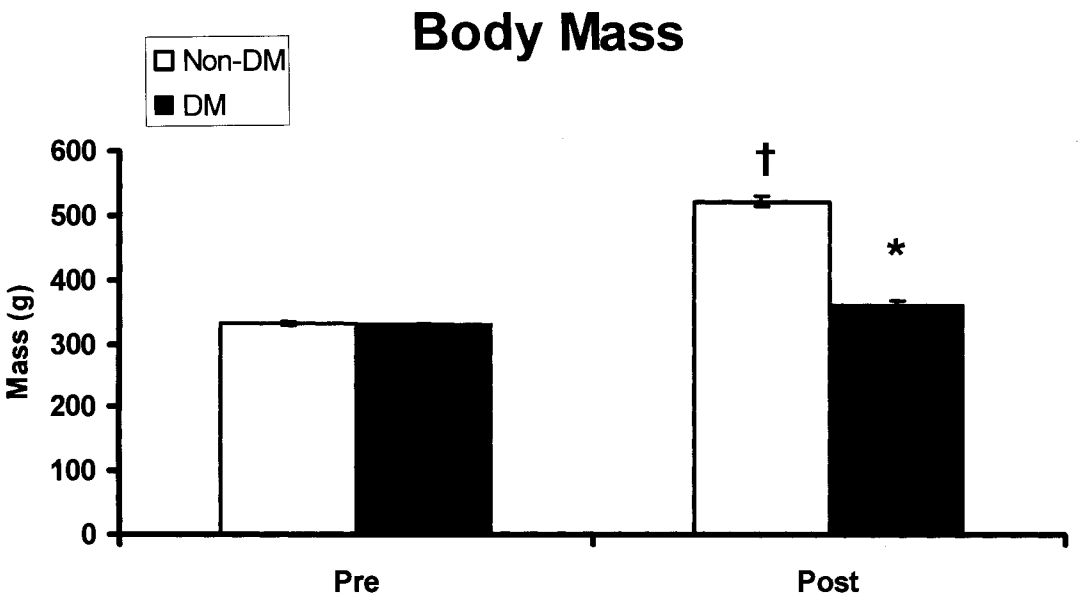


Figure 4.3 The effect of exercise and/or DM on cellular contents of phosphorylated IκK-β. 50 ug of protein from whole cell lysates were loaded into each well and run on a SDS-PAGE and reacted with a phospho-specific antibody to IκK-β.

A: A representative western blot demonstrating total quantities of phosphorylated IκK-β within the myocardium of DM and Non-DM animals.

B: Graphic analysis of phospho-IκK-β in the rat myocardium. Data expressed as mean ± SEM (n = 10 per group). There were no detectable differences in contents of phosphorylated IκK-β between the experimental groups.

Figure 4.3*A*

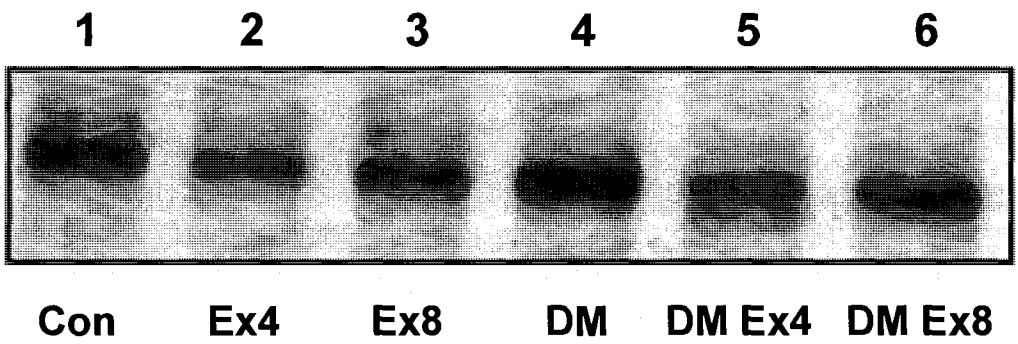


Figure 4.3*B*

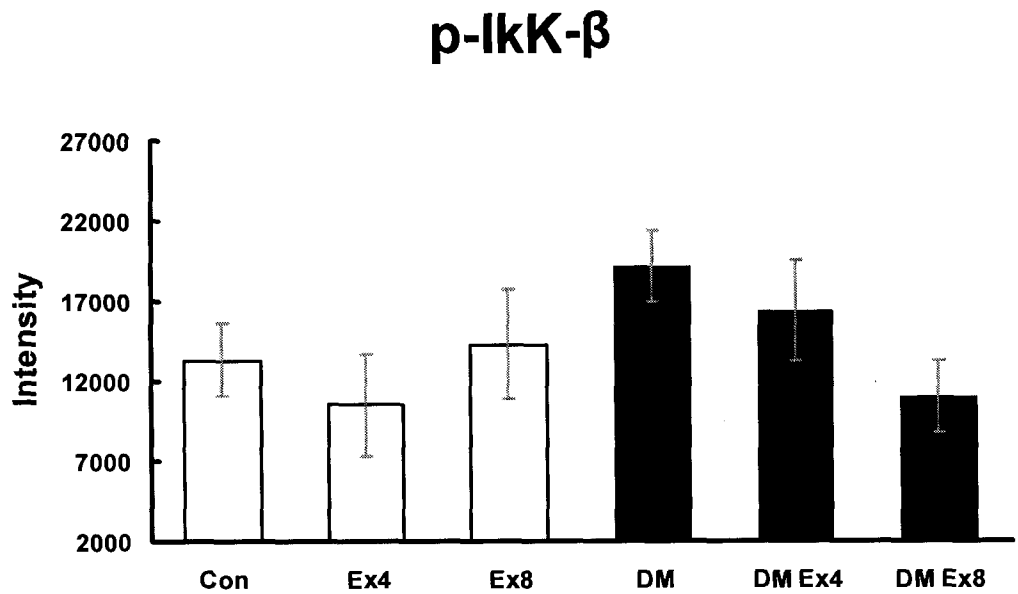


Figure 4.4 The effect of exercise and/or DM on cellular contents of IκB-α. Whole cell protein extracts (50 ug) were on a SDS-PAGE and reacted with an IκB-α-specific antibody.

A: A representative Western blot demonstrating total quantities of phosphorylated IκB-α within the myocardium of DM and Non-DM animals.

B: Graphic analysis of IκB-α in the rat myocardium. Data expressed as mean ± SEM (n = 10 per group). *Significantly greater ($P < 0.05$) than Non-DM animals.

Figure 4.4*A*

Figure 4.4*B*

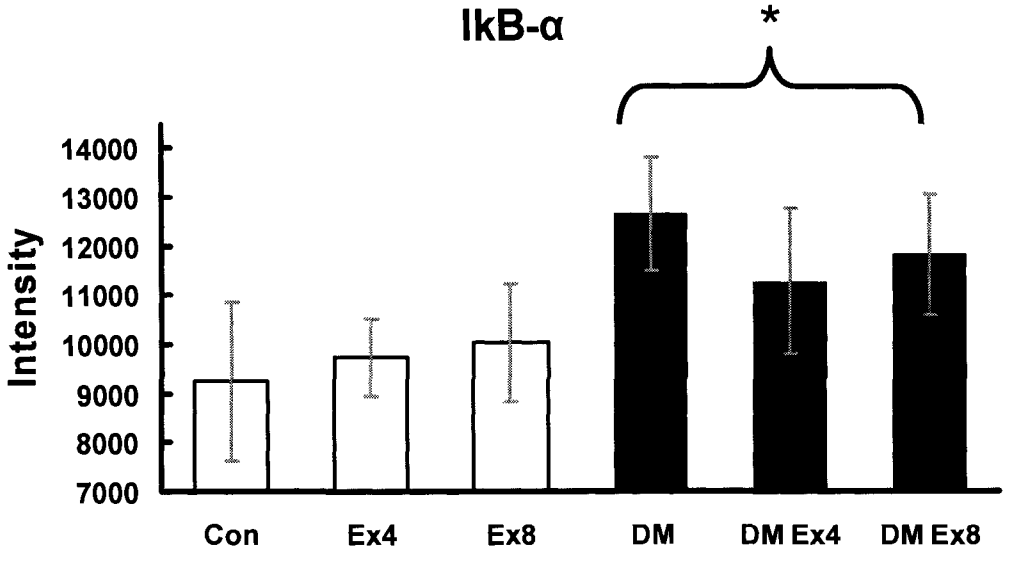


Figure 4.5 Transcriptional activation of NF-κB.

A: Activation of NF-κB in tissue samples from all experimental groups. Protein samples are as follows: Lane 1: Protein sample from control *animal 1*, lane 2: Protein sample from control *animal 2*, lane 3: Protein sample from experimental group animal 1, lane 4: Protein sample from experimental group animal 2, lane 5: Protein sample from experimental group animal 3, lane 6: Protein sample from experimental group animal 4, lane 7: Protein sample from experimental group animal 5, lane 8: Protein sample from experimental group animal 6, lane 9: Protein sample from experimental group animal 7, lane 10: Protein sample from experimental group animal 8.

B: Graphic illustration of NF-κB activation expressed as percentage of control. Data expressed as mean ± SEM (n = 8). *Significantly greater ($P < 0.05$) than control tissues.

Figure 4.5*A*

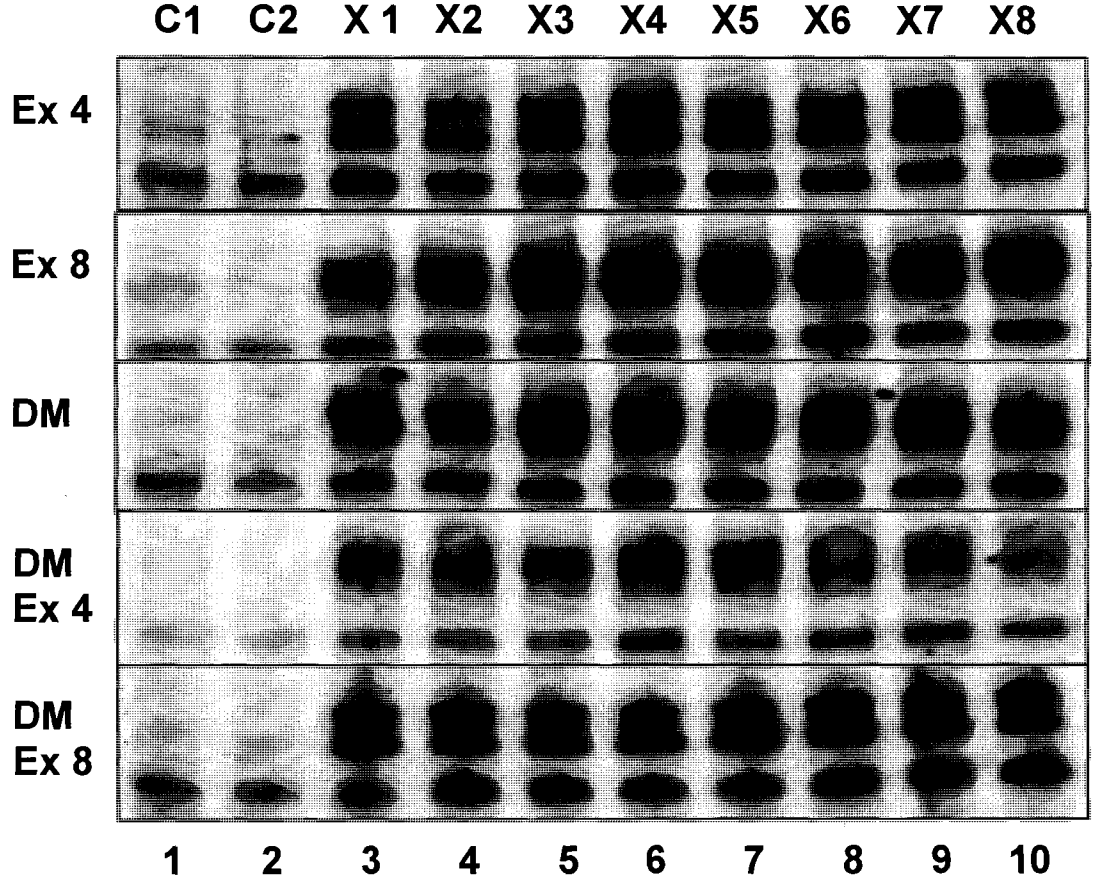


Figure 4.5*B*

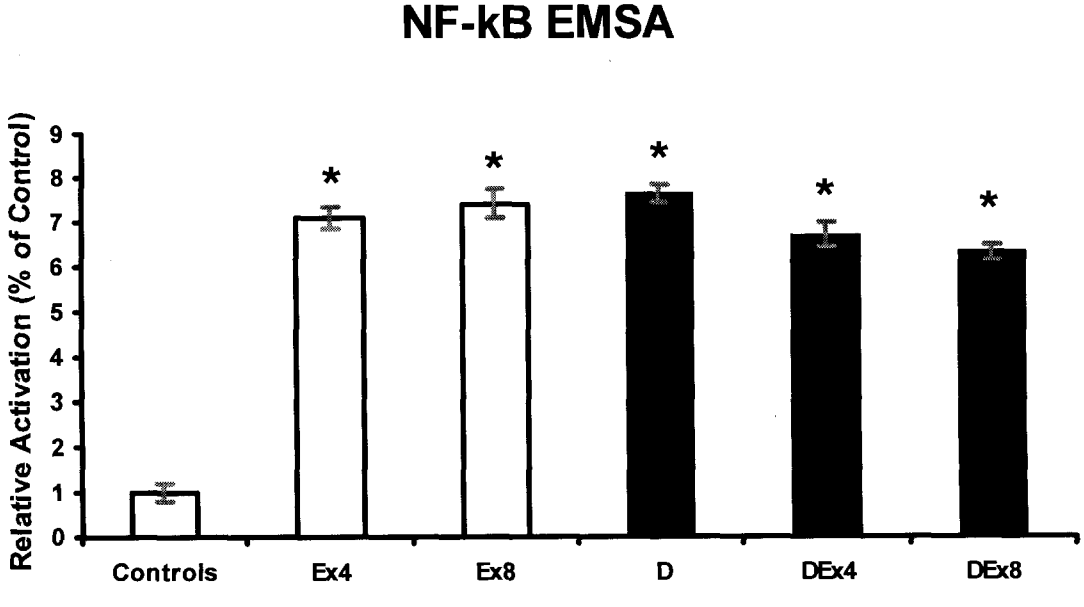


Figure 4.6 The effect of DM on NF-κB transcriptional activation and the effects of exercise on diabetic tissue.

A: A comparative gel-shift assay showing the activation of NF-κB from DM tissues and tissues from DM animals that received 8 weeks of exercise. Protein samples are as follows: Lane 1: Protein sample from group *DM, animal 1*, lane 2: Protein sample from group *DM, animal 2*, lane 3: Protein sample from group *DM*, animal 3, lane 4: Protein sample from group *DM*, animal 4, lane 5: Protein sample from group *DM*, animal 5, lane 6: Protein sample from group *DM Ex8*, animal 1, lane 7: Protein sample from group *DM Ex8*, animal 2, lane 8: Protein sample from group *DM Ex8*, animal 3, lane 9: Protein sample from group *DM Ex8*, animal 4, lane 10: Protein sample from group *DM Ex8*, animal 5.

B: A graphic illustration of a direct comparison between NF-κB binding in tissue samples from animals that received STZ injections or STZ injections and exercise. Data expressed as mean ± SEM (n = 5).

Figure 4.6*A*

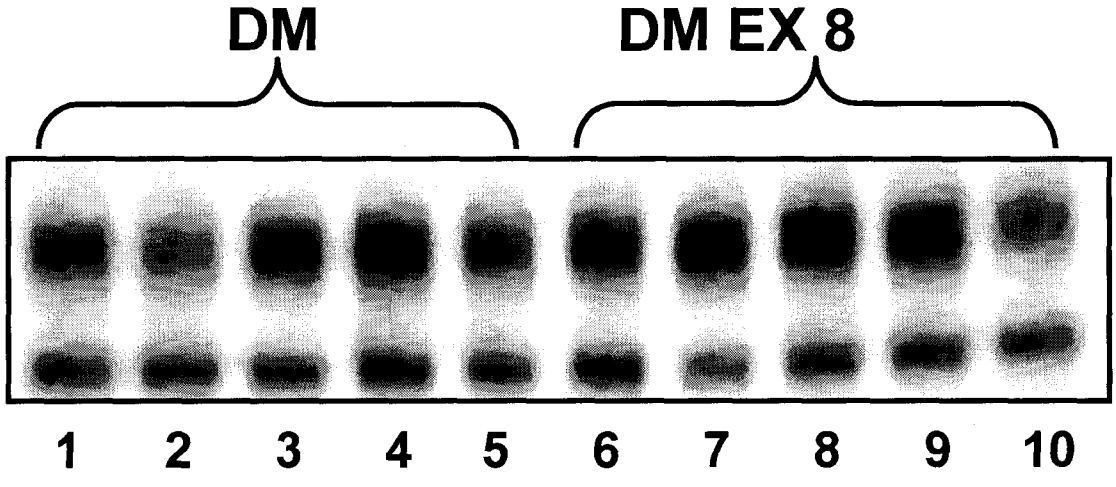


Figure 4.6*B*

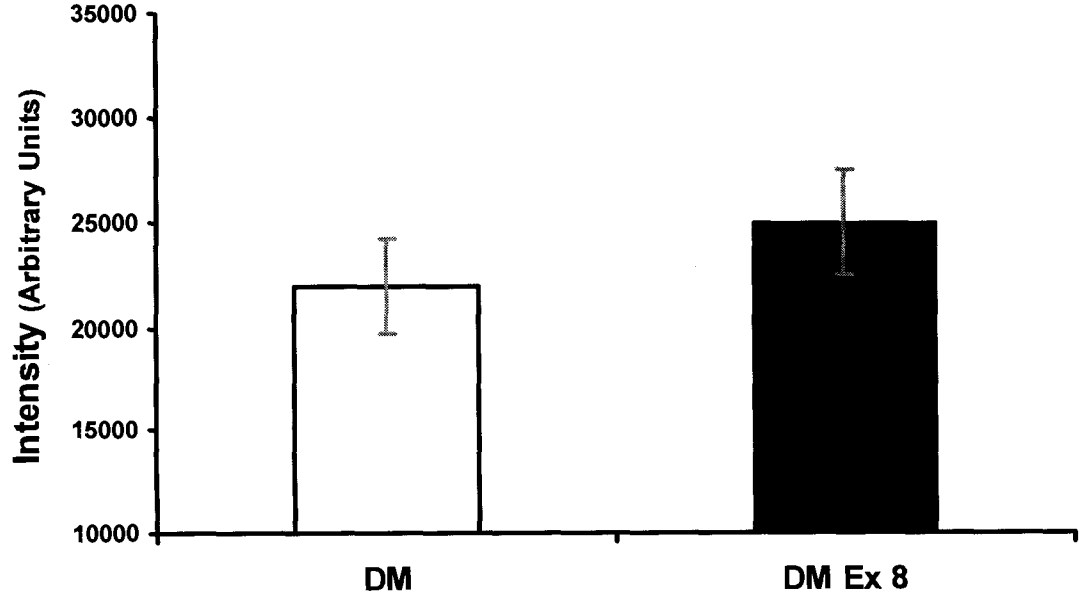


Figure 4.7 Supershift assay from control heart tissue. Lane 1: Free Probe (FP), lane 2: Protein sample without antibody, lane 3: Protein sample with the addition of 1μg α-p50, lane 4: Protein sample with the addition of 1μg α-p52, lane 5: Protein sample with the addition of 1μg α-p65, lane 6: Protein sample with the addition of 1μg α-RelB, lane 7: Protein sample with the addition of 1μg α-cRel, lane8: Protein sample with the addition of 1μg α-Bcl-3, lane 9: Protein sample with the addition of 1μg of each antibody, lane 10: Labeled oligo incubated with recombinant p50 (5ng). An upward shift of the localized NF-κB-oligo complex by a p50-specific antibody indicates a homodimer composed predominately of p50.

Figure 4.7

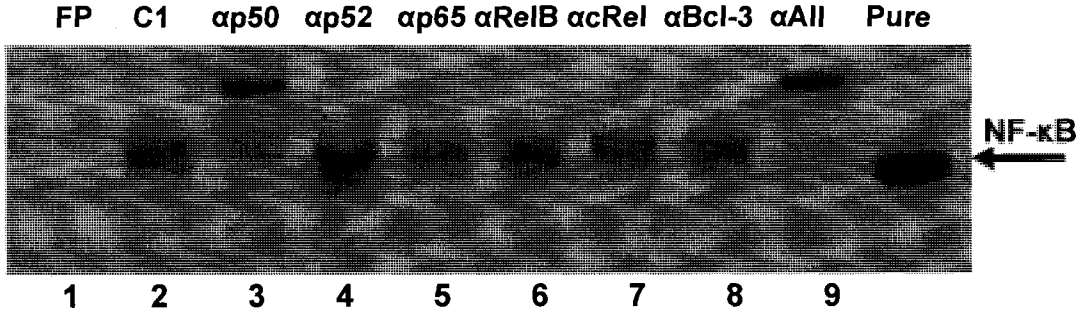

FP
C1
αp50
αp52
αp65
αRelB
αcRel
αBcl-3
αAll
Pure
NF-κB
1
2
3
4
5
6
7
8
9

Figure 4.8 Supershifts of NF-κB within the diabetic myocardium.

A: Lane 1: Diabetic tissue sample without NF-κB antibodies, lane 2: Diabetic tissue sample with the addition of 1μg α-p50, lane 3: Diabetic tissue sample with the addition of 1μg α-p65, lane 4: Diabetic tissue sample with the addition of 1μg all NF-κB antibodies. A supershift is demonstrated with the addition α-p50, but not with α-p65, suggesting DM tissue predominantly contains p50-p50 homodimers.

B: An NF-κB supershift assay in DM tissue to detect Bcl-3 within the NF-κB complex. Every 2 lanes contain a different DM sample; lanes 1, 3, 5, 7, 9 contain no antibodies, whereas every other lane (lanes 2, 4, 6, 8, 10) contains 1μg of α-Bcl-3. + indicates the addition of 1μg of antibodies; - indicates no addition of antibodies. No supershift is detected, hence Bcl-3 is not likely present in activated NF-κB in DM tissue.

Figure 4.8*A*

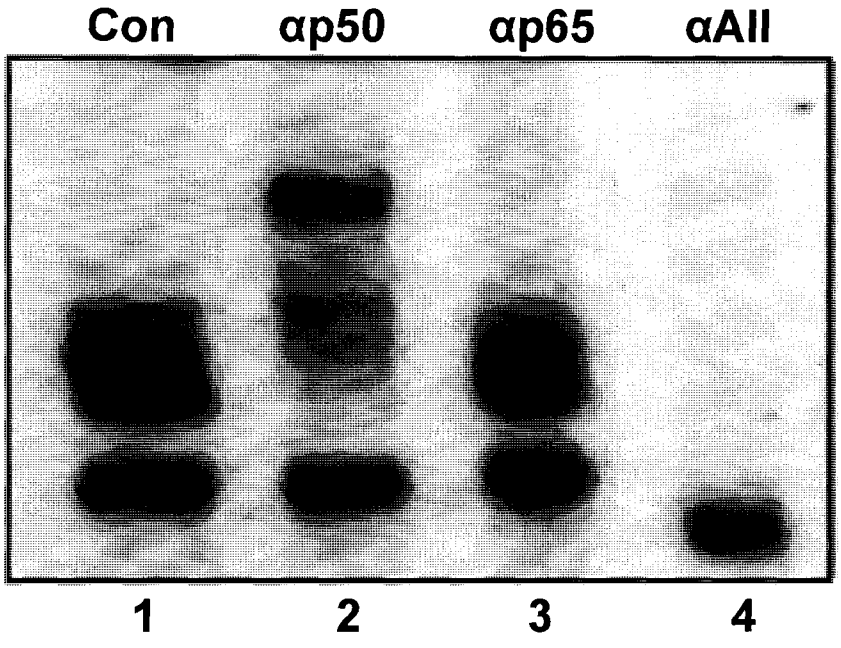


Figure 4.8*B*

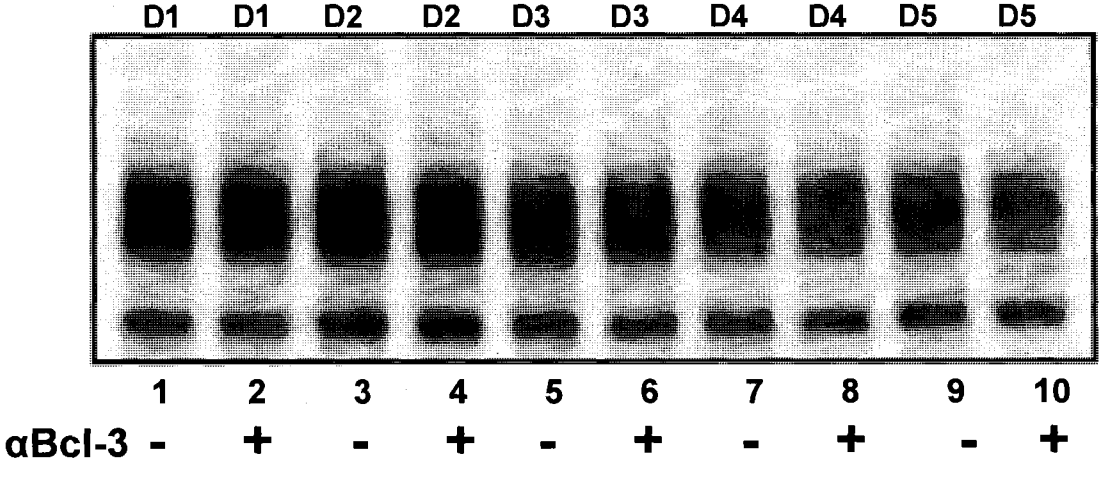


Figure 4.9 NF-κB supershifts in exercised heart tissue.

A: Exercise trained for 8 weeks. Lane 1: Exercised tissue sample without NF-κB antibodies, lane 2: Exercised tissue sample with the addition of 1μg α-p50, lane 3: Exercised tissue sample with the addition of 1μg α-p65, lane 4: Exercised tissue sample with the addition of 1μg all NF-κB antibodies. A supershift is demonstrated with the addition α-p50, but no localized band is detected above the main complex with α-p65, suggesting DM tissue predominantly contains p50-p50 homodimers.

B: Exercise trained for 4 weeks. Lane 1: Exercised-DM tissue sample without NF-κB antibodies, lane 2: Exercised-DM tissue sample with the addition of 1μg α-p50, lane 3: Exercised-DM tissue sample with the addition of 1μg α-p65, lane 4: Exercised-DM tissue sample with the addition of 1μg all NF-κB antibodies. The only supershift detected was with the addition α-p50, suggesting activated NF-κB within exercised-DM tissue predominantly contains p50-p50 homodimers.

Figure 4.9*A*

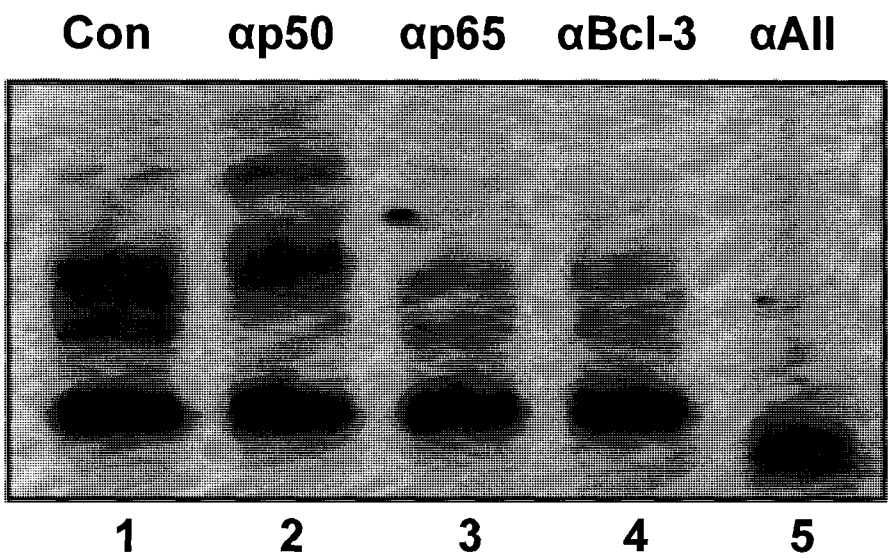


Figure 4.9*B*

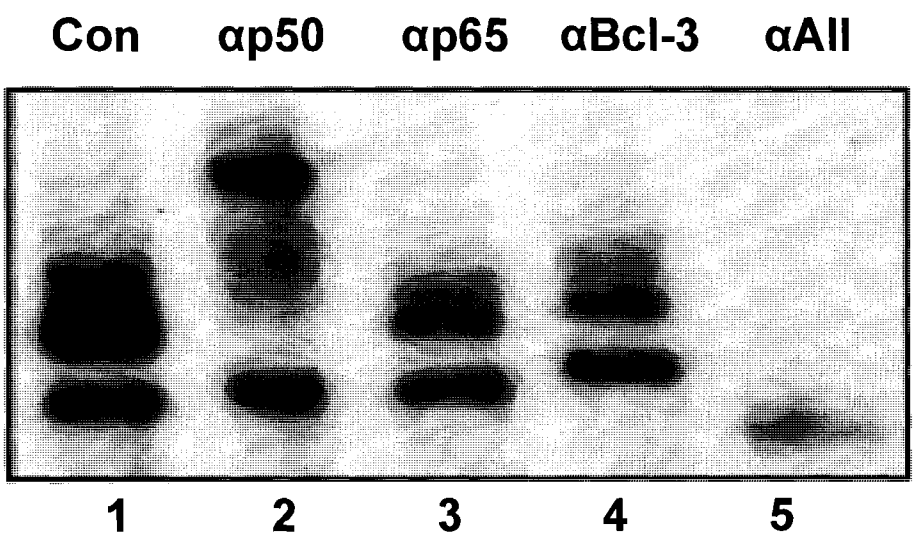


Figure 4.10 NF-κB supershift in diabetic heart tissue exercise trained for 8 weeks. Lane 1: DM Ex8 tissue sample without NF-κB antibodies, lane 2: DM-Ex8 tissue sample with the addition of 1μg α-p50, lane 3: DM Ex8 tissue sample with the addition of 1μg α-p65, lane 4: DM Ex8 tissue sample with the addition of 1μg all NF-κB antibodies. As observed in DM Ex4 tissue, the only supershift detected in DM Ex8 tissue was with the addition α-p50, suggesting activated NF-κB within DM Ex8 tissue predominantly contains p50-p50 homodimers.

Figure 4.10

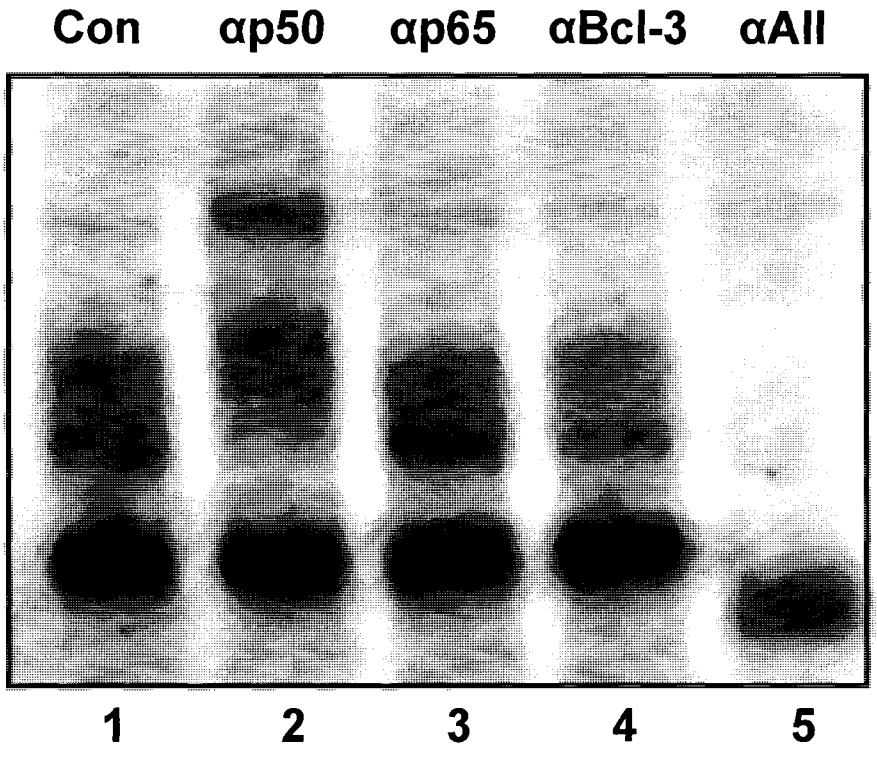


Figure 4.11 Supershift assay investigating NF-κB activation regulation by HSP90. + indicates the addition of 1μg of antibodies; - indicates no addition of antibodies. With or without exercise, interference of HSP90 results in markedly increased NF-κB binding.

A: HSP90 supershifts in Control tissue and 8 weeks of exercise. Lanes 1-5 contain control tissue samples. No antibodies were added to Lane 1 whereas lanes 2-5 contain 1μg α-HSP90. Lanes 6-10 contain tissue samples from animals exercised for 8 weeks. No antibodies were added to Lane 6 whereas lanes 7-10 contain 1μg α-HSP90.

B: HSP90 in DM animals and DM animals exercised for 4 weeks. Lanes 1-5 contains DM tissue samples. No antibodies were added to Lane 1 whereas lanes 2-5 contain 1μg α-HSP90. Lanes 6-10 contain tissue samples from DM animals exercised for 4 weeks. No antibodies were added to Lane 6 whereas lanes 7-10 contain 1μg α-HSP90.

C: HSP90 in DM animals and DM animals exercised for 8 weeks. Lanes 1-5 contains DM tissue samples. No antibodies were added to Lane 1 whereas lanes 2-5 contain 1μg α-HSP90. Lanes 6-10 contain tissue samples from DM animals exercised for 8 weeks. No antibodies were added to Lane 6 whereas lanes 7-10 contain 1μg α-HSP90.

Figure 4.12*A*

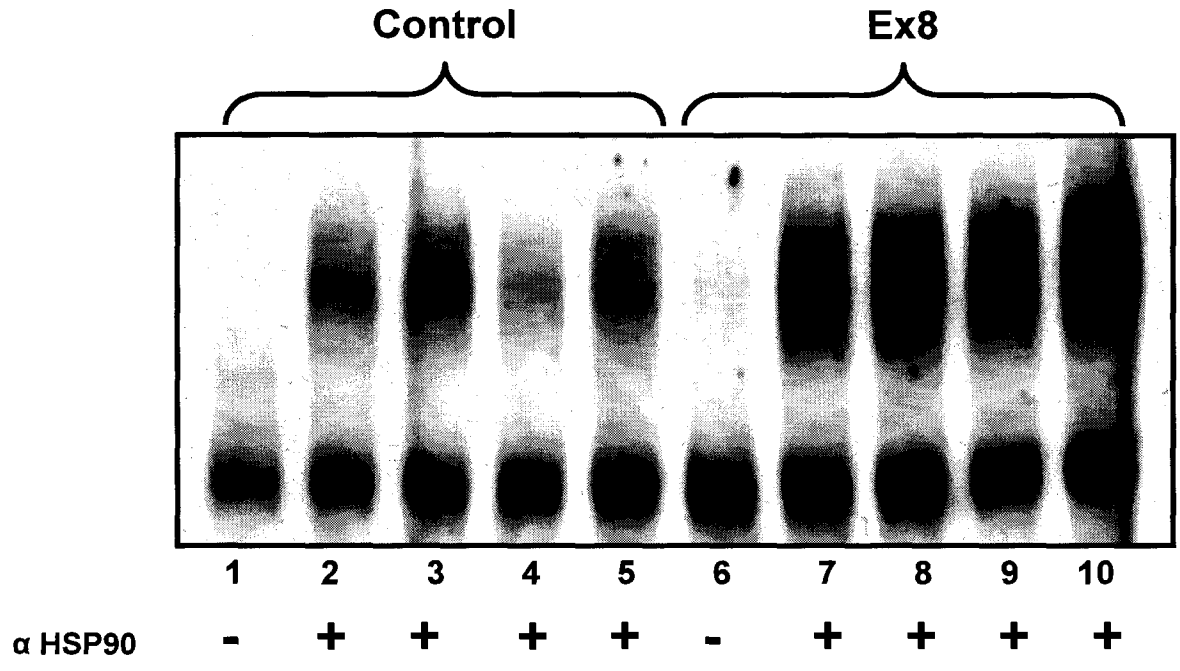


Figure 4.12*B*

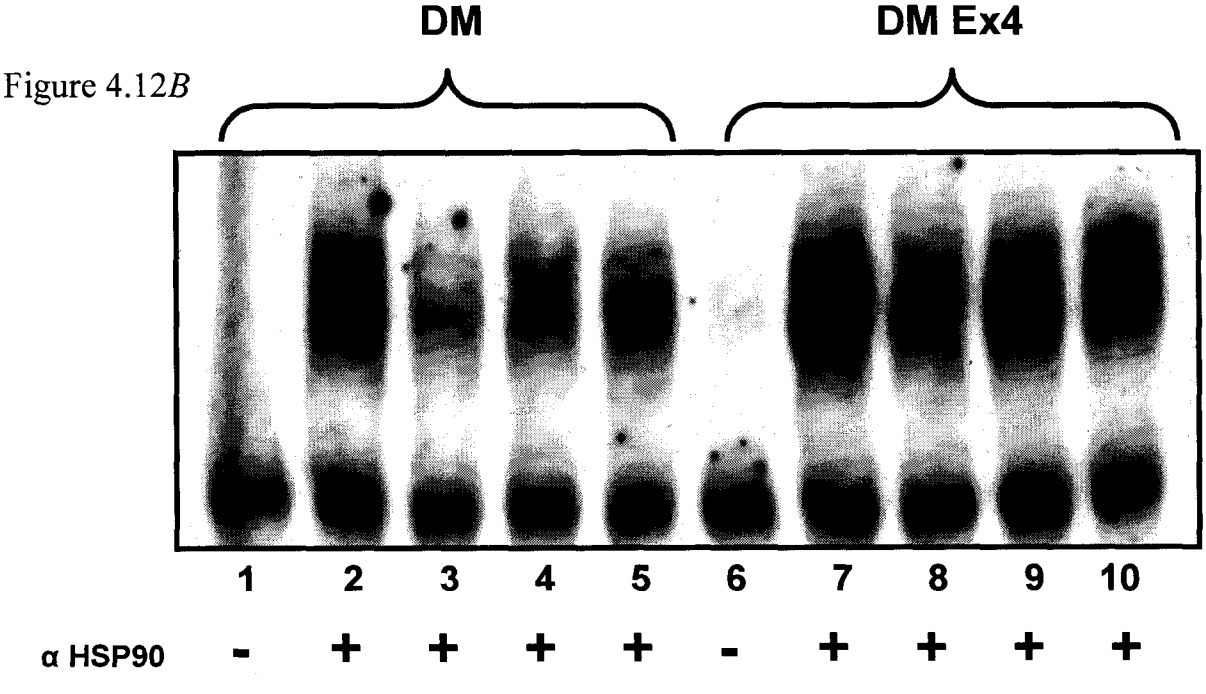
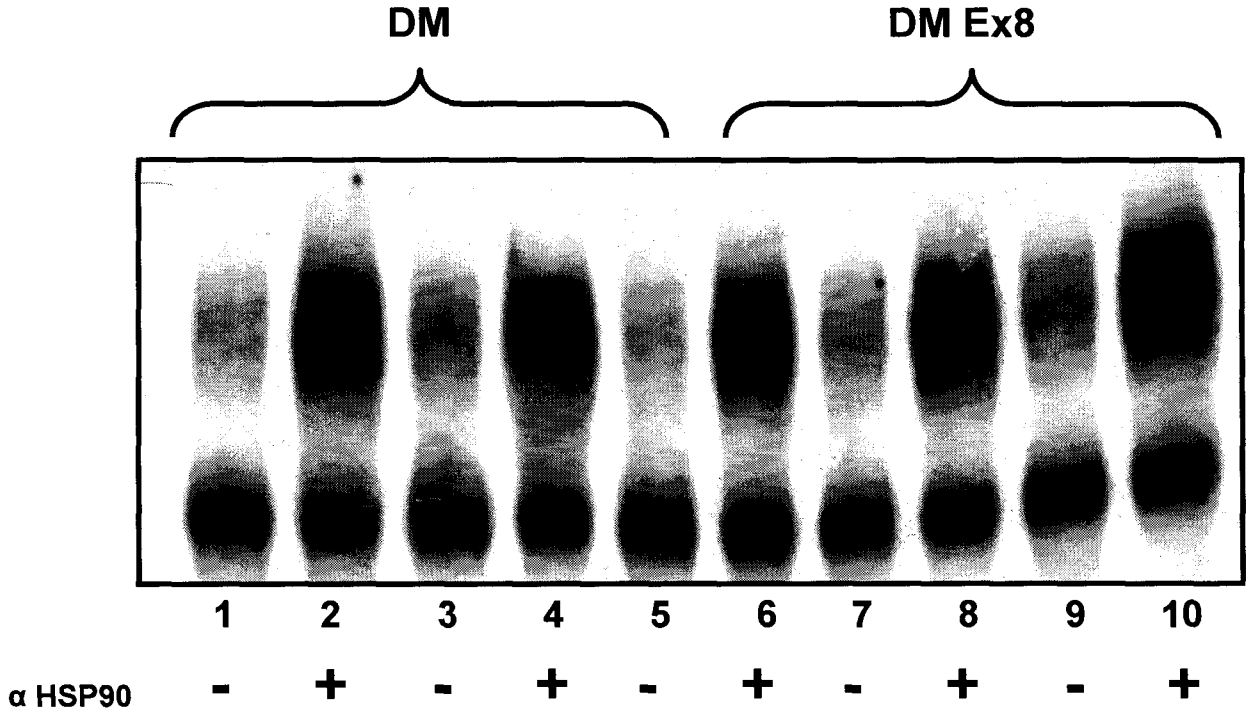


Figure 4.12*C*



4.4 DISCUSSION

Low grade, chronic inflammation is commonly ascribed to the diabetic condition. Epidemiological evidence demonstrates that exercise activity of varying intensities is positively associated with reduced markers of inflammation (reviewed by Ford, 2002). Since the transcription factor, NF-κB, is the primary regulator of inflammation, the present study examined the effect of DM and exercise on NF-κB-DNA binding in the rat myocardium. By convention, NF-κB refers to a specific transcription factor, but by identity, the transcription factor exists as a dimer composed of 5 possible proteins. Hence, the individual members in the constitutively bound state in DM or exercised myocardial tissue may be unique to these conditions and were identified in the present study. The upstream activator and inhibitor of NF-κB binding, IκK-β and IκB-α, respectively, were also assessed for their contribution to inflammatory signaling. Further, possible binding regulation by the molecular chaperone, HSP90, was assessed in control, diabetic and exercised myocardium.

There were four main findings in the present study. First, 8 weeks of DM and exercise, alone or in concert, led to similarly increased NF-κB binding. Second, activation status of the liberating kinase, IκK-β, did not increase with any treatment, whereas the quantity of the inhibiting protein, IκB-α, was significantly increased in DM animals, and exercise did not reduce its elevated quantity. Third, in all groups, the member proteins of DNA-bound NF-κB were identified primarily as p50-p50 dimers. Fourth, HSP90 appeared to influence NF-κB activation, possibly by inhibiting activation, as increased binding was observed when HSP90 was disrupted by exposing tissue samples to HSP90-specific antibodies.

Increased NF-κB binding following DM and exercise. We observed increased myocardial NF-κB binding in both DM and exercise-trained heart muscle. Although no evidence exists that demonstrates NF-κB binding via EMSA in myocardium of DM animals, other structures (such as the aorta) do show sustained binding activity in STZ-induced DM (Kislinger et al., 2001), at least partly mediated by the receptor for advanced glycation end products (RAGE). Indeed, increased glycation products measured in STZ-induced hyperglycemia led to increased RAGE binding and increased expression of the potent NF-κB activator, TNF-α, whereas RAGE blockade inhibited TNF-α expression in STZ-induced hyperglycemia (De Vriese et al., 2003). In support, NF-κB binding was elevated in smooth muscle cells in a hyperglycemic medium (Yerneni et al., 1999). Other reports are limited to inferring increased NF-κB binding by measuring elevated contents of potential activators of NF-κB binding in myocardium of mice with STZ-induced DM (Kaur et al., 2006).

While prior evidence showing increased NF-κB binding in the diabetic myocardium is lacking, the implication of previous findings is supported by elevated NF-κB-DNA binding presented in the present study. If NF-κB is normally transiently activated then the sustained constitutively active NF-κB in DM may reflect not only increased stimulation by upstream activators, but may lend credence to the role of insulin in limiting inflammation through NF-κB (Dandona et al., 2007). Hence, the lack of insulin in DM animals may have allowed the unabated activation of NF-κB. Since the actions of insulin are often proposed to be mimicked by exercise, it was of great interest to examine the effect of exercise on NF-κB activity in normal and DM animals.

Exercise increased NF-κB–DNA binding and did not reduce binding in DM animals. Limited evidence exists to assess NF-κB binding activity following exercise training. Most studies examine transient activation by acute exercise (Ho et al., 2005; Ji et al., 2000), with even fewer examining exercise training effects. Spangenburg et al. (2006) suggested that treadmill exercise training increased NF-κB activity in rat soleus and plantaris muscle, implied by increased contents of IκB-α, IL-6 (an NF-kB target gene) and decreased contents of phosphorylated IκK-β. However, these studies did not demonstrate actual NF-κB binding via EMSA. The present study did measure NF-κB binding in the rat myocardium following 4 and 8 weeks of exercise. After both training durations there was a clear and substantial activation. These results likely reflect long-term training effects rather than an acute, transient exercise effect, since tissues were harvested 48 hours after the last exercise bout. Strictly speaking, NF-κB binding detected via EMSA does not indicate transcriptional activity, hence, it is difficult to distinguish the difference between DM-induced binding and exercise-induced NF-κB binding, as similar binding does not signify similar transcriptional activity. Acute exercise appears to transiently activate inflammation, as measured by elevated cytokines such as C-reactive protein, but training has an anti-inflammatory effect (reviewed by Kasapis and Thompson, 2005). Therefore, increased constitutive NF-κB binding following 4 and 8 weeks of exercise training may not be indicative of elevated inflammation, but, rather, may be the process by which the myocardium responds to frequent intermittent episodes of cellular stress and may reflect a long term adaptation designed to mitigate inflammatory stimuli.

The present study clearly demonstrated increased binding in both DM and exercised animals, but uncovering a qualitative difference between the 2 conditions may be realized by delineating a unique contribution to NF-κB activation by upstream regulators.

Upstream regulators of NF-κB activation in exercised and diabetic myocardia. The NF-κB liberating kinase, IκK-β, was shown to have an equal amount of serine 181 phosphorylation (required to initiate phosphorylation and consequent degradation of the NF-κB inhibitor, IκB-α). This can be interpreted to indicate an equal quantity of inflammatory stimuli between control, DM and exercised animals. However, the lack of NF-κB binding in control tissues suggests otherwise. The commonly recognized phenomenon of receptor down-regulation (reviewed by Shepherd, 1989) may have led to decreased IκK-β proteins, practically providing fewer sites for ligand binding, thus resulting in a net quantity of phosphorylated IκK-β proteins equal to the amount measured in control tissues. By any mechanism no difference was measured in phosphorylated IκK-β, suggesting that the myocardium may have become tolerant to repeated stimulation that normally initiates NF-κB transcriptional activity (Wahlstrom et al., 1999). One other study examined exercise training and found reduced IκK phosphorylation (Spangenburg et al., 2006). However, these investigators measured IκK-α phosphorylated at Serines 176 and 180 (responsible for processing family member proteins), whereas the present study measured IκK-β phosphorylated at Serine 181 (responsible for degrading the inhibitor associated with the pathological p50-p65 herterodimer). Equal quantities of phosphorylated IκK-β may lead to the conclusion that its downstream kinase activity may also be equal among the 3 treatments (DM, exercise

and control). However, the target of its action, IκB-α, showed a measurable difference between DM and Non-DM animals, which reveals that DM may uniquely influence regulation of myocardial NF-κB transcriptional

With or without exercise, DM animals showed significantly increased myocardial contents of the inhibitor protein, IκB-α. Independently, exercise had no influence on IκB-α content. It is reasonable to speculate that the consequence of a greater quantity of inhibitor proteins in DM animals may be that there was greater NF-κB inhibition in DM animals. However, if there are more IκB-α targets available for IκK-β to phosphorylate then there may be a net increase in uninhibited NF-κB. The amount of IκB-α protein able to be phosphorylated by a single active IκK-β is unknown, but its length of activity appears to depend on the activating stimulus (IL-1 = 5 - >60 minutes; TNF-α = 5 - >120 minutes; Zandi et al., 1997), and based on binding data, the stimulus in DM and exercised tissues was relatively sustained. A possibility exists that there was a qualitative shift in NF-κB protein dimer members. It has been demonstrated that IκB-α preferentially binds p50-p65 dimers (Malek et al., 2003). Therefore, the observed difference between exercise- and DM-induced NF-κB binding may be related to the composition of the dimer itself.

Constituent attributes of bound NF-κB in control, DM and exercised heart tissue. The canonical pathway for transcriptional activation via NF-κB is ascribed to the p50-p65 heterodimer, the form predominantly activated by pathological stressors (reviewed by Senftleben and Karin, 2002). It was expected that elevated DM stress would show increased p50-p65 heterodimer binding, whereas normal and exercised tissue would show a p50-p50 binding. It was further expected that exercise would restore the pathological

heterodimer to the inactive p50-p50 transcriptional repressor. Contrary to expectations, all non-control tissues demonstrated increased binding activity by the p50-p50 homodimer. The presence of Bcl-3, reportedly complexed with p50-p50 homodimers in atrophic and cancerous disease states (Hunter and Kandarian, 2004; Thornburg et al., 2003), was not found to be part of the dimer in the present study. Therefore, the nature of exercise and DM stress are independent of other disease states with regard to activating the inflammatory pathway.

Apparently, the constitutive binding state following different chronic stressors exhibited the same bound dimer. This may suggest that DM, exercise and the combination of both are equally stressful, quantitatively and qualitatively. However, the diabetic myocardium, with or without exercise, showed altered upstream effectors, and DM is consistently associated with inflammation whereas exercise is associated with diminished inflammation. Hence, the present study does not clearly delineate the mechanism by which different stressors (one beneficial, the other detrimental) exert unique effects.

The lack of a distinguishing quality between exercise and DM may be explained by the phenomenon of tolerance; that is, the prolonged nature of the agitating stimuli may have initiated a long term adaptation. Since bound p50-p50 cannot initiate transcription by virtue of lacking a trans-activation domain and blocks access by other dimers to binding sites, increased p50-p50 binding may reflect a common protective method motivated by dissimilar needs. In the case of exercise, the inflammatory pathway is acutely and transiently activated, but its positive influence on genetic expression may upregulate proteins known to inhibit NF-κB, such as those produced during the heat

shock response (reviewed by Salminen et al. 2008). Hence, massive binding of NF-κB after training may reflect one method of protection against harmful inflammatory stimuli by exercise. In contrast, inflammatory signals inherent to DM may have led to a desensitization of the myocardium to chronic stimulation, accompanied by an attempt to block p50-p65 heterodimers from accessing DNA binding sites. Indeed, this phenomemon of tolerance was described by Wahlstrom et al. (1999) following chronic NF-κB activation by lipopolysaccharide in murine macrophages. This same tolerance to NF-κB activation was also demonstrated with chronic TNF-α stimulation in the myocardium of mice (Haudek et al., 2001). Not only did p50-p50 homodimers become the predominant form of bound NF-κB, but overstimulation also led to elevated contents of IκB-α, as was demonstrated in the present study. Since IκB-α preferentially binds p50-p65 heterodimers, and does not bind p50-p50 homodimers (Phelps et al., 2000; Li and Nabel, 1997), the effect would be to increase inhibition of the pathological p50-p65 heterodimer while increasing the transcriptionally repressive p50-p50 homodimer.

Since we assessed DNA binding at only a single time-point (8 weeks after initiating physiological and pathological stress) it is possible that at some point along the experimental timeline a more blatant distinction exists between binding quantity and the identity of the constituent members of activated NF-κB. Discovering additional inflammatory regulatory mechanisms within the myocardium may help delineate differences between DM and exercise-induced adaptations. Moreover, the inability of exercise to prevent or reverse NF-κB signaling adaptations may expose the limits of exercise as a treatment modality.

NF-κB binding regulation by HSP90. There was a positive influence on NF-κB-DNA binding when tissue samples from all groups were incubated with HSP90-specific antibodies. The enhanced binding effect was extended to control tissues that demonstrated virtually no binding without HSP90-specific antibodies added. Although some evidence exists indicating that functional HSP90 is required for inflammatory stimuli to activate NF-κB (reviewed by Salminen et al., 2008), this is the first study to demonstrate enhanced binding to the oligomer by disrupting HSP90. Most studies demonstrate reduced transcription factor binding due to disrupted HSP90, either by chemical inhibition (Broemer et al., 2004) or with the addition of antibodies (Ali et al., 1998).

The ability of HSP90 to affect NF-κB-DNA binding may be related to its stabilizing association with the activating kinase, IκK-β (Broemer et al., 2004). These investigators demonstrated that the inhibition of HSP90 led to the dismantling of IκK-β, thus preventing it from phosphorylating the inhibitor and releasing NF-κB via inhibitor degradation. However, this suggests that the more likely finding would have been reduced NF-κB binding with HSP90 disruption, not enhanced binding. There is the possibility that HSP90 has a more direct role in facilitating DNA binding, as it appears to interact directly with the DNA binding domain with an unknown function (Feldman et al., 2007).

Another stress activated transcription factor, the heat shock transcription factor, HSF1, showed reduced heat shock-induced activation when HSP90 was inhibited by HSP90-specific antibodies (Ali et al., 1998). Interestingly, these investigators observed that in Non-stressed cells the addition of HSP90-specific antibodies enhanced HSF1

binding. Hence, there is investigative precedence in support of our findings. However, all of the above mentioned investigations discovered an interactive role in DNA binding by measuring the HSP90-β isoform (Feldman et al., 2007) or employed the use of antibodies specific for HSP90-β (Broemer et al., 2004) or indiscriminant for HSP90-α and HSP90-β (Ali et al., 1998). The antibody used in the present study was specific for HSP90-α, and may demonstrate that HSP90-α protein disruption is required to enhance NF-κB transcriptional activation following inflammatory episodes.

4.5 CONCLUSION

In summary, the current study demonstrated that DM, exercise and the combination of both sufficiently stimulated the inflammatory machinery to induce binding of NF-κB to the DNA promoter in the myocardium. The main effect on upstream effectors was an enhanced quantity of IκB-α in DM tissues, the protein most responsible for inhibiting the pathological form of NF-κB (the p50-p65 dimer). This finding suggests that there may be a larger quantity of the pathological dimer in the diabetic myocardium and that increased inhibition may be a necessary coping strategy. The identity of constitutively bound NF-κB in all tissues was the transcription-repressing p50-p50 dimer. Since this protein combination is uninhibited and free to bind DNA, then increased binding by this dimer may reflect increased cellular quantities of the protein and dimer. This may suggest a strategy by which the myocardium adapts to repeated, intermittent bouts or chronically applied stress of exercise and DM, respectively. The disruption of HSP90 enhanced NF-κB activation, provided a glimpse into the regulatory complexity of inflammatory signaling. The present study recognized a distinction between DM and

exercise stress and proposes that the increase of proteins designed to inhibit the pathological form of NF-κB implies that there is an elevation of the pathological dimer in DM tissues, and exercise is unable to reverse or prevent this change. Moreover, although increased binding by the repressive p50-p50 dimer was common to both DM and exercise, inherently it is easily, although transiently, displaced by p50-p65; hence, this long term adaptation may be insufficiently protective against increased inflammatory activation in DM tissues.

4.6 REFERENCES

Ali A, Bharadwaj S, O'Carroll R, Ovsenek N. HSP90 interacts with and regulates the activity of heat shock factor 1 in Xenopus oocytes. *Mol Cell Biol.* 1998; 18(9):4949-4960.

American Diabetes Association: clinical practice recommendations. Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 2002. *Diabetes Care.* 2002; 25(Suppl 1): S1–S147

Armstrong RB, Gollnick PD, Ianuzzo CD. Histochemical properties of skeletal muscle fibers in streptozotocin-diabetic rats. *Cell Tissue Res.* 1975; 162(3):387-94.

Armstrong RB, Ianuzzo CD. Decay of succinate dehydrogenase activity in rat skeletal muscle following streptozotocin injection. *Horm Metab Res.* 1976;8(5):392-394.

Bradford MM. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72:248-254.

Broemer M, Krappmann D, Scheidereit C. Requirement of Hsp90 activity for IkappaB kinase (IKK) biosynthesis and for constitutive and inducible IKK and NF-kappaB activation. *Oncogene.* 2004; 23(31):5378-5386.

Carmody RJ, Ruan Q, Palmer S, Hilliard B, Chen YH. Negative regulation of toll-like receptor signaling by NF-kappaB p50 ubiquitination blockade. *Science.* 2007; 317(5838):675-678.

Dandona P, Aljada A, Bandyopadhyay A. The potential therapeutic role of insulin in acute myocardial infarction in patients admitted to intensive care and in those with unspecified hyperglycemia. Diabetes Care. 2003 Feb;26(2):516-9.

Dandona P, Chaudhuri A, Ghanim H, Mohanty P. Effect of hyperglycemia and insulin in acute coronary syndromes. *Am J Cardiol.* 2007 4 ;99(11A):12H-18H.

de Rekeneire N, Peila R, Ding J, Colbert LH, Visser M, Shorr RI, Kritchevsky SB, Kuller LH, Strotmeyer ES, Schwartz AV, Vellas B, Harris TB. Diabetes, Hyperglycemia, and Inflammation in Older Individuals. *Diabetes Care.* 2006; 29(8):1902–1908.

De Vriese AS, Flyvbjerg A, Mortier S, Tilton RG, Lameire NH. Inhibition of the interaction of AGE-RAGE prevents hyperglycemia-induced fibrosis of the peritoneal membrane. *J Am Soc Nephrol.* 2003; 14(8):2109-2118.

Delhase M, Hayakawa M, Chen Y, Karin M. Positive and negative regulation of IκB kinase activity through IKKβ subunit phosphorylation. *Science.* 1999; 284:309–313.

Durham WJ, Li YP, Gerken E, Farid M, Arbogast S, Wolfe RR, Reid MB. Fatiguing exercise reduces DNA binding activity of NF-kB in skeletal muscle nuclei. *J Appl Physiol.* 2004;97:1740–1745.

Engström G, Stavenow L, Hedblad B, Lind P, Eriksson KF, Janzon L, Lindgärde F. Inflammation-sensitive plasma proteins, diabetes, and mortality and incidence of myocardial infarction and stroke: a population-based study. *Diabetes.* 2003; 52(2):442-447.

Feldman I, Feldman GM, Mobarak C, Dunkelberg JC, Leslie KK. Identification of proteins within the nuclear factor-kappa B transcriptional complex including estrogen receptor-alpha. *Am J Obstet Gynecol.* 2007; 196(4):394.e1-11.

Ford ES. Does exercise reduce inflammation? Physical activity and C-reactive protein among U.S. adults. Epidemiology. 2002; 13(5):561-568.

García-López D , Cuevas MJ , Almar M , Lima E , De Paz JA , González-Gallego J . Effects of eccentric exercise on NF-kappaB activation in blood mononuclear cells. *Med Sci Sports Exerc.* 2007; 39(4):653-664.

Gilmore TD. Introduction to NF-kappaB: players, pathways, perspectives. *Oncogene.* 2006; 25(51):6680-4.

Gleissner CA, Galkina E, Nadler JL, Ley K. Mechanisms by which diabetes increases cardiovascular disease. *Drug Discov Today Dis Mech.* 2007;4(3):131-140.

Gordin D, Forsblom C, Ronnback M, Parkkonen M, Waden J, Hietala K, Groop PH.Acute hyperglycaemia induces an inflammatory response in young patients with type 1 diabetes. *Ann Med.* 2008; 16:1-7.

Haudek SB, Bryant DD, Giroir BP. Differential regulation of myocardial NF kappa B following acute or chronic TNF-alpha exposure. *J Mol Cell Cardiol.* 2001; 33(6):1263-1271.

Ho RC, Hirshman MF, Li Y, Cai D, Farmer JR, Aschenbach WG, Witczak CA, Shoelson SE, Goodyear LJ. Regulation of IkappaB kinase and NF-kappaB in contracting adult rat skeletal muscle. *Am J Physiol Cell Physiol.* 2005; 289(4):C794-801.

Hoffmann A, Natoli G, Ghosh G. Transcriptional regulation via the NF-κB signaling module. *Oncogene.* 2006; 25:6706–6716.

Hunter RB, Kandarian SC. Disruption of either the Nfkb1 or the Bcl3 gene inhibits skeletal muscle atrophy. *J Clin Invest.* 2004; 114(10):1504-1511.

Hunter RB, Stevenson E, Koncarevic A, Mitchell-Felton H, Essig DA, Kandarian SC. Activation of an alternative NF-kappaB pathway in skeletal muscle during disuse atrophy. *FASEB J.* 2002; 16:529-538.

Jefferson LS, Rannela DE, Munger BL, Morgan HE. Insulin in the regulation of protein turnover in heart and skeletal muscle. *Federation Proc.* 1974;33:1098-1104.

Ji LL, Gomez-Cabrera MC, Steinhafel N, Vina J. Acute exercise activates nuclear factor (NF)-kappaB signaling pathway in rat skeletal muscle. *FASEB J.* 2004;18(13):1499-1506.

Kasapis C, Thompson PD. The effects of physical activity on serum C-reactive protein and inflammatory markers. A systematic review. *J Am Coll Cardiol.* 2005; 45:1563–1569.

Kaur H, Chen S, Xin X, Chiu J, Khan ZA, Chakrabarti S. Diabetes-induced extracellular matrix protein expression is mediated by transcription coactivator p300. *Diabetes.* 2006; 55(11):3104-3111.

Kislinger T, Tanji N, Wendt T, Qu W, Lu Y, Ferran LJ Jr, Taguchi A, Olson K, Bucciarelli L, Goova M, Hofmann MA, Cataldegirmen G, D'Agati V, Pischetsrieder M, Stern DM, Schmidt AM. Receptor for advanced glycation end products mediates inflammation and enhanced expression of tissue factor in vasculature of diabetic apolipoprotein E-null mice. *Arterioscler Thromb Vasc Biol.* 2001; 21(6):905-910.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970; 227:680-685.

Li Z, Nabel GJ. A new member of the I kappaB protein family, I kappaB epsilon, inhibits RelA (p65)-mediated NF-kappaB transcription. *Mol Cell Biol.* 1997; 17:6184–6190.

Loganathan R, Bilgen M, Al-Hafez B, Zhero SV, Alenezy MD, Smirnova IV.Exercise training improves cardiac performance in diabetes: in vivo demonstration with quantitative cine-MRI analyses. *J Appl Physiol.* 2007; 102(2):665-672.

Malek S, Huang DB, Huxford T, Ghosh S, Ghosh G. X-ray crystal structure of an IkappaBbeta x NF-kappaB p65 homodimer complex. *J Biol Chem.* 2003; 278(25):23094-23100.

Phelps CB, Sengchanthalangsy LL, Huxford T, Ghosh G. Mechanism of I kappa B alpha binding to NF-kappa B dimers. *J Biol Chem.* 2000; 275(38):29840-29846.

Pittet JF, Lee H, Pespeni M, O'Mahony A, Roux J, Welch WJ. Stress-induced inhibition of the NF-kappaB signaling pathway results from the insolubilization of the IkappaB kinase complex following its dissociation from heat shock protein 90. *J Immunol.* 2005; 174(1):384-94.

Salminen A, Paimela T, Suuronen T, Kaarniranta K. Innate immunity meets with cellular stress at the IKK complex: regulation of the IKK complex by HSP70 and HSP90. *Immunol Lett.* 2008; 117(1):9-15.

Senftleben U, Karin M. The IKK/NF-kappa B pathway. *Crit Care Med.* 2002; 30(1 Suppl):S18-26.

Shepherd VL. Intracellular pathways and mechanisms of sorting in receptor-mediated endocytosis. Trends Pharmacol Sci. 1989 Nov;10(11):458-462.

Spangenburg EE, Brown DA, Johnson MS, Moore RL. Exercise increases SOCS-3 expression in rat skeletal muscle: potential relationship to IL-6 expression. *J Physiol.* 2006; 572(Pt 3):839-848.

Sriwijitkamol A, Christ-Roberts C, Berria R, Eagan P, Pratipanawatr T, DeFronzo RA, Mandarino LJ, Musi N. Reduced skeletal muscle inhibitor of kappaB beta content is associated with insulin resistance in subjects with type 2 diabetes: reversal by exercise training. *Diabetes.* 2006 ;55:760–767.

Thornburg NJ, Pathmanathan R, Raab-Traub N. Activation of nuclear factor-kappaB p50 homodimer/Bcl-3 complexes in nasopharyngeal carcinoma. *Cancer Res.* 2003; 63(23):8293-8301.

Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Biotechnology.* 1979; 24:145-149.

Wahlstrom K, Bellingham J, Rodriguez JL, West MA. Inhibitory kappaBalpha control of nuclear factor-kappaB is dysregulated in endotoxin tolerant macrophages. *Shock.* 1999; 11(4):242-247.

Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. *J Clin Invest.* 2005; 115(5):1111-1119.

Westermann D, Rutschow S, Jäger S, Linderer A, Anker S, Riad A, Unger T, Schultheiss HP, Pauschinger M, Tschöpe C. Contributions of inflammation and cardiac matrix metalloproteinase activity to cardiac failure in diabetic cardiomyopathy: the role of angiotensin type 1 receptor antagonism. *Diabetes.* 2007; 56(3):641-646.

Yerneni KK, Bai W, Khan BV, Medford RM and Natarajan R. Hyperglycemia-induced activation of nuclear transcription factor kappaB in vascular smooth muscle cells. *Diabetes.* 1999; 48 855-864.

Zandi E, Rothwarf DM, Delhase M, Hayakawa M, Karin M. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. *Cell.* 1997; 91(2):243-252.

CHAPTER 5

IGF-1 receptor signaling in the diabetic myocardium

5.1 INTRODUCTION

Cellular signaling is a sequence of commands between enzymes and substrates designed to influence cellular function and to control genetic expression and protein production. A disturbance in these highly integrated commands provokes a loss of coordination and leads to error in cellular response and function. Diabetes mellitus (DM) not only reduces glucose uptake, but also prevents insulin from acting as a major coordinating and anabolic signaling molecule, leaving the cell dependant on surrogate proteins to perform these roles. Unfortunately, DM progressively deranges intracellular signaling resulting in the production of aberrant proteins. Cardiomyopathy is a primary outcome of lost and altered signaling pathways and is the most common cause of death among diabetics. Ideally, exposing signaling steps which are altered during disease progression may help reveal candidates which are critical to appropriate expression of structural and functional proteins and illuminate the degree to which interventions, such as exercise, may reduce diabetic cardiomyopathy and normalize signaling behavior.

Attempts have been made to distinguish between pathological and physiological myocardial signaling programs activated following varying types of stress (e.g., exercise vs. cardiac disease). Under both normal and pathological conditions, Insulin-like Growth Factor-1 (IGF-1) is the primary regulator of several activating proteins responsible for cardiac hypertrophy (Wilkins et al., 2004), the cellular stress response (He et al., 1998), mitigating negative effects of reactive oxygen species (Kajstura et al., 2001) and regulating activation of the NF-κB inflammatory pathway (Pons S, Torres-Aleman et al., 2000; Eisner et al, 2006; Del Rio and Velez-Pardo, 2006). The IGF-1 signaling pathway operates through a series of phosphorylating intermediaries (in particular,

phosphoinositide 3-kinases (PI-3K) → AKT) leading to the activation or deactivation of glycogen synthase kinase-3β (GSK-3β) and is associated with cardiac growth and function (McMullen et al., 2004; Badorff et al., 2002), hyperglycemia-induced apoptosis (Singleton et al., 1996) and diabetic cardiomyopathy (Gurusamya et al., 2006; Palfi et al., 2006; Konhilas et al. (2006). The remarkably nuanced activity of IGF-1 and expression of its receptor is severely compromised in the rat myocardium of streptozotocin (STZ)-induced DM (Han and Park, 2006). However, the time-line associated with these DM-induced changes is unknown. For example, STZ-induced DM led to increased GSK-3β activity after 3 days, but was decreased after 4 weeks (Gurusamya et al., 2006). Hence, cell signaling may change over time, as may the deleterious changes in DM-induced protein production with progressively deteriorating physiological consequences. An additional branch of the IGF-1 pathway regulates the expression of the muscle-specific uncoupling protein, UCP-3, implicated in reducing metabolites from fatty acid oxidation (Gerber et al., 2006) and reducing production of reactive oxygen species (ROS) (reviewed by Bezaire et al., 2007). As ROS can activate the inflammatory protein, NF-κB (Schreck et al., 1991), also directly linked to DM, a role may exist for UCP-3 in diabetic cardiomyopathy.

Under stressful conditions, the expression and actions of a set of myocardial protective stress proteins, known as heat shock proteins (HSPs), may help protect the heart against DM. In particular, the heat shock transcription factor, HSF-1, and its primary cardio-protective gene product, HSP70, are essential to preserving the structure and function of several other proteins under a variety of stressful conditions (for review see Morimoto et al., 1994). HSP70 overexpression has been shown to prevent insulin

resistance and inhibit NF-κB-activated inflammation in high fat fed mice (Chung et al., 2008). HSP70 induction via HSF-1 activation also preserved myocardial function following ischemia reperfusion (Locke et al., 1995). Interestingly, activated GSK-3β protected against post-infarction remodeling (Palfi, 2006) and reduced HSF-1 transcriptional activity and HSP70 production (He et al., 1998), whereas GSK-3β-specific inhibition increased HSF-1 content (Chung et al., 2008). Therefore, disruption of IGF-1→GSK-3β co-ordination by DM may affect the expression and function of numerous proteins specifically designed to maintain normal function and protect against cellular stress. Disruption of IGF-1 signaling in STZ-induced DM was shown to be the result of increased degradation of the IGF-1 receptor (IGF-1R), facilitated by decreased cellular contents of another stress protein, HSP60 (Chen et al., 2005; Shan et al, 2003). Conversely, HSP60 over-expression at the onset of STZ-induced DM increased IGF-1R content and activation (Lai et al., 2007).

Exercise has long been recognized as a treatment for DM, but mechanistic details regarding its molecular and intracellular reactions are lacking. This is particularly true regarding the well established link between exercise-induced HSPs and cardio-protection. Atalay et al. (2004) reported that STZ-induced DM reduced constitutive and exercise-induced myocardial HSF-1 binding and HSF-1 and HSP70 protein content. In contrast, Najemnikova et al. (2007) showed that constitutive myocardial contents of HSF-1 and HSP70 protein were unchanged in STZ-diabetic rats, but did not determine an exercise-effect. In the normal myocardium, contents of HSP60 are known to increase following endurance training (Samelman, 2000), but HSP60 contents in the diabetic myocardium following exercise is unknown. Therefore, the possibility of exercise to induce HSP60 in

the diabetic myocardium may offer a method to preserve IGF-1 signaling, protein activity and expression, including HSF-1, HSP70, UCP-3, and the regulation of structural and functional proteins, such as myosin and collagen.

Although STZ-induced DM leads to increased myocardial collagen type III contents (Candido et al., 2003), scarce data exists examining exercise-induced changes to collagen in the diseased myocardium. The few available studies indicate that exercise reduced elevated collagen cross-linking in congestive heart failure (Todaka et al., 1997), increased collagen turnover in the aged rat myocardium (Thomas et al., 2000), prevented fibrosis in cardiac hypertrophy (Konhilas et al., 2006) and improved intracellular signaling within the diabetic myocardium in Zucker rats (Lajoie et al., 2004). However, there are conflicting reports regarding the activation status of GSK-3β and improved heart function (Konhilas et al., 2006; Lajoie et al., 2004; Thomas et al., 2000).

Exercise improved the phenotype of structural proteins, quantities of functional proteins, in particular β-myosin heavy chain Type I (β-MHC I), long known to increase in the diabetic myocardium (Dillmann, 1980), however, β-MHC I expression was not improved by treadmill exercise in STZ-induced diabetic rats (Paulson et al., 1992). Hence, it remains unclear which protein changes in the diabetic myocardium are sensitive to exercise. The assertion that IGF-1 mediates β-MHC I contents following myocardial infarction (Nahrendorf et al., 2003), and downstream GSK-3β inhibition increases α-MHC in the mouse myocardium (Hirotani et al., 2007), indicates that IGF-1 may be a key regulator of a multitude of vital cellular regulators. The ability of exercise to benefit the diabetic myocardium may be limited to the extent to which it protects and restores intracellular signaling and protein production. Taken together, it appears that IGF-1

signaling is vital to normal myocardial function, as well as exercise-induced remodeling of the myocardium (Kim et al., 2008).

Therefore, the purpose of the present study was to determine the effects of exercise on the cellular contents of proteins associated with growth, mitigating cellular and oxidative stress and on the expression of contractile and structural proteins in the diabetic myocardium. Specifically, IGF-1R and GSK-3β activation and their association with HSP60, HSP70, HSF-1, UCP-3, Collagen Type III and β-MHC I were examined. It was hypothesized that exercise would normalize IGF-1R and GSK-3β activation and normalize levels of contractile and structural proteins. Improvements were expected to be associated with exercise-induced HSP70, HSP60, and reduced oxidative stress.

5.2 METHODS AND MATERIALS

Animal Characteristics and Experimental Groups. All procedures were approved by the Animal Care Subcommittee at The University of Western Ontario. Sixty male Sprague-Dawley rats (~220-250g; Charles River Laboratories, Quebec, Canada) were maintained on a 12-hour dark/light cycle, housed at 20 ±1°C, 50% relative humidity, and provided with standard rat chow and water ad libitum. Animals were divided into DM and non-DM groups. Animals from each group were assigned to 1 of 3 treatments (n=10 per group): (1) sedentary (Con); (2) 4-weeks sedentary followed by 4-weeks of exercise (Ex4); or (3) 8-weeks of exercise (Ex8).

DM Induction. Animals received a low-dose injection of STZ (20mg/kg; i.p.; Sigma-Aldrich) within 5 minutes of dissolving in citrate buffer (0.1 M, pH 4.5) on 5 consecutive days. Non-DM animals were injected with citrate buffer only. DM was confirmed by

measuring a blood glucose level >15 mmol. Whole body animal weights and blood glucose values were monitored weekly via blood draw from the saphenous vein using the One Touch Ultra 2 Blood Glucose Monitoring System (Lifescan Canada Ltd, Burnaby, BC, Canada) and One Touch test strips (Lifescan Canada; range = 0–600 mg/dl).

Exercise Training. Rats were familiarized to treadmill running on 2 occasions with brief, 10-minute exposures at 15m/min (5 & 3 days prior to the first training run). At the beginning of the exercise training, rats were run at progressing intensities with unchanging duration and frequency (one hour/day, 5 days/week) starting at 19m/min for 5 days (week 1), followed by 23m/min for 5 days (week 2), 27m/min for 5 days (week 3), and continued thereafter at 27m/min (the final intensity corresponding with a work rate of ~75% VO_{2max}). Continuous running during the exercise session was encouraged by an electronically activated puff of compressed air blowing at right angles onto the rat haunches. This air jet served to act as a stimulus to encourage running and to act as a warning that the end of the belt was approaching. All animals were able to adjust to increasing intensities and completed the training protocol.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The heart was removed by cardiac excision and the atria and right ventricle were trimmed away. A portion of the left ventricle (50 mg) was homogenized in lysis buffer (137 mM NaCl, 20 mM Tris-HCl, pH 7.5, 10% glycerol, 1% Triton X-100, 2 mM EDTA, pH 8.0, 3 μg/ml aprotinin, 3 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 10 mM NaPP, and 2 mM Na3VO4) and protein concentration determined using the method of Bradford (1976). One dimensional (1-D) SDS-PAGE was performed according to the method described by Laemmli (1970), except that the separating gel (0.15 x 4.5 x 8 cm)

consisted of a 5-15% polyacrylamide gradient. Following electrophoretic separation, proteins were transferred to nitrocellulose membranes (0.22 μm pore size, Bio-Rad Laboratories) as described by Towbin et al. (1979) using the Bio-Rad mini-protean II gel transfer system.

Immunoblotting and Enzyme Immuno Assay. Following protein transfer, blots were reacted overnight in TTBS with 2% blotto and an antibody specific for one of IGF-1R (C-20 polyclonal: catalogue # sc-713; diluted 1:1000), collagen type III (FH-7A polyclonal: catalogue # sc-59817; diluted 1:1000), Santa Cruz Biotechnology, California, USA.; HSP72 (polyclonal: catalogue # SPA-812; diluted 1:3000), HSF-1 (polyclonal: catalogue # SPA-901; diluted 1:1000), phospho-GSK-3β (polyclonal: catalogue # KAP-ST011; diluted 1:1000), HSP60 (monoclonal: catalogue # SPA-806; diluted 1:3000), Stress-Gen, Ann Arbor, MI, USA; UCP-3 (polyclonal: catalogue # ab3477; diluted 1:1000) Abcam Inc., Cambridge, MA, USA; slow β-MHC I (monoclonal: catalogue # M8421; diluted 1:3000) Sigma-Aldrich, St. Louis, MO, USA; phospho-IGF-1R (monoclonal: 905-643; diluted 1:1000) Assay Designs, Ann Arbor, MI, USA. A horse radish peroxidase-conjugated secondary antibody was employed (StressGen, Victoria, Canada) and the blot was developed using enhanced chemiluminescence. Briefly, following secondary incubation the blot was immersed in 5 ml of luminal enhancer and 5 ml of stable peroxide solution (ECL, Amersham) for 1 minute and then exposed to x-ray film to visualize the protein bands. To determine nonspecific binding, duplicate gels were run, transferred and reacted, but the primary antibody was omitted. Immunoblots were scanned and quantification of bands from immunoblots was performed by using Scion Image Software (Scion Corporation, Frederick, Maryland, USA). An ELISA was

performed in duplicate to quantify serum quantities of isoprostanes (Direct 8-iso-Prostaglandin F2α Enzyme Immuno-Assay kit; Assay Designs Ann Arbor, MI, USA).

Statistical Analysis. A two-way ANOVA (SPSS) was performed using DM and exercise as conditions for each group. A Bonferonni post hoc was used to determine significant differences between groups. Differences between groups were considered statistically significant at a level of $P < 0.05$. Data are expressed as the mean ± SEM. When only one condition was different (usually DM status) and in the absence of interaction, groups were collapsed so as to provide an n=30 per group.

5.3 RESULTS

Physical characteristics and blood glucose. Measures of blood glucose and body mass were taken once per week for the duration of the experiment. Both blood glucose (shown in Figure 5.1) and body mass (shown in Figure 5.2) demonstrated a clear difference between Con-, Ex4-, Ex8– diabetic animals and Con-, Ex4-, Ex8- Non-diabetic animals. Eight weeks following DM induction, blood glucose was significantly greater ($P < 0.05$) in DM animals compared to Non-DM animals (29.3 ± 1.5, 27.8 ± 1.1 and 31.6 ± 1.0 mmol/L vs. 7.12 ± 0.45, 7.23 ± 0.78 and 6.55 ± 0.67 mmol/L, respectively). Eight weeks after DM induction, significantly lower ($P < 0.05$) body mass was found in Control, Ex4, Ex8 (DM) animals vs. Control, Ex4, Ex8 (Non-DM) animals (353 ± 14, 359 ± 10 and 374 ± 14 g vs. 537 ± 15, 505 ± 12 and 520 ± 11 g, respectively).

Quantification of IGF-1R signaling. Status of the IGF-1 receptor and its activation following eight weeks of DM and/or exercise was determined by measuring the total quantity of the receptor (Figure 5.3) and determining its activation by measuring the quantity of receptors phosphorylated at tyrosine 1316 (Figure 5.4). DM significantly

increased ($P < 0.05$) quantities of IGF-1R compared to Non-DM myocardia. Exercise did not influence IGF-1R contents. Total activation of the IGF-1R was not different between any groups; therefore DM-increased IGF-1R content with no change in phosphorylation status in DM animals implies reduced proportional receptor phosphorylation.

Quantification of GSK-3β signaling. Total quantities of phosphorylated GSK-3β were significantly different ($P < 0.05$) between DM and Non-DM animals. All DM animals showed less phosphorylated GSK-3β than Non-DM animals, with or without exercise (Figure 5.5).

Heat shock protein and transcription factor quantification. Total content of cellular HSF-1 was measured and is shown in Figure 5.6. HSF-1 contents are significantly different ($P < 0.05$) among DM animals compared to Non-DM animals. Exercise did not have an effect on HSF-1 quantities. Similarly, exercise did not have an effect on HSP60 contents (Figure 5.7). As well, DM did not affect normal HSP60 protein levels in the myocardium of any animals. However, exercise did increase cellular contents of HSP70 in both DM and Non-DM animals. Hence, HSP70 remained stress inducible and remained sensitive to the influence of exercise in the diabetic myocardium to the same degree as that seen in normal animals. Moreover, DM per se was not sufficiently stressful to induce either HSP60 or HSP70 protein production.

Uncoupling protein-3 and isoprostane quantification. The mitochondrial uncoupling protein, UCP-3, was significantly elevated ($P < 0.05$) by DM (Figure 5.9). Exercise did not change cellular contents of UCP-3 in normal animals nor did it alter the elevating effects of DM. The possibility of UCP-3 influencing free radical production is not determinable by the simple quantification of protein contents, but the measure of whole

body oxidative stress via isoprostane quantification was also accomplished. Figure 5.10 shows a clear effect of exercise on whole body oxidative stress in DM animals. Exercise did not alter isoprostanes levels in normal animals; however isoprostane levels were increased ($P < 0.05$) in sedentary DM animals. Four weeks of exercise initiated 4 weeks after the onset of DM led to significantly normalized isoprostane levels when expressed per ml of serum. Moreover, 8 weeks of exercise led to isoprostane levels significantly less than controls, but also significantly less than the reduced levels shown in DM Ex4 animals. Due to changes in body composition that accompanies DM (see Chapter 2) isoprostanes were also expressed relative to total body mass or lean mass. DM control animals showed significantly elevated oxidative stress, whereas 4 weeks of exercise reduced isoprostanes levels to normal and 8 weeks of exercise reduced isoprostanes to lower than normal levels. Hence, regardless of how oxidative stress was expressed, exercise did reduce whole body oxidative stress, but only in DM animals and its ability to reduce levels of isoprostanes was related to exercise volume.

Quantification of structural and functional proteins. Collagen type III was measured in the myocardium as a measure of the structural effects of DM. Figure 5.12 illustrates that DM increased ($P < 0.05$) collagen type III protein with or without exercise. Protein quantification does not give an indication of myocardial fibrosis, but does indicate that the changes to the extracellular matrix associated with myocardial degeneration and fibrosis is elevated by STZ-induced DM. Similarly, the β-MHC I isoform associated with a re-activation of the fetal gene program seen in heart disease is increased ($P < 0.05$) in diabetic animals (Figure 5.13). Exercise did not change the quantity of myocardial β-MHC I in normal animals nor did it reduce elevated levels measured in DM animals.

Figure 5.1. Blood glucose as measured prior DM induction and during the final week of the experiments. Initial blood samples demonstrated no difference between animals divided into Non-Dm and DM groups. Final glucose values showed that DM animals obtained significantly greater ($P < 0.05$) blood glucose values compared to Non-DM animals. Data expressed as mean ± SEM (n = 30 per group). *Significantly greater ($P < 0.05$) than Non-DM animals and pre-DM values.

Figure 5.1

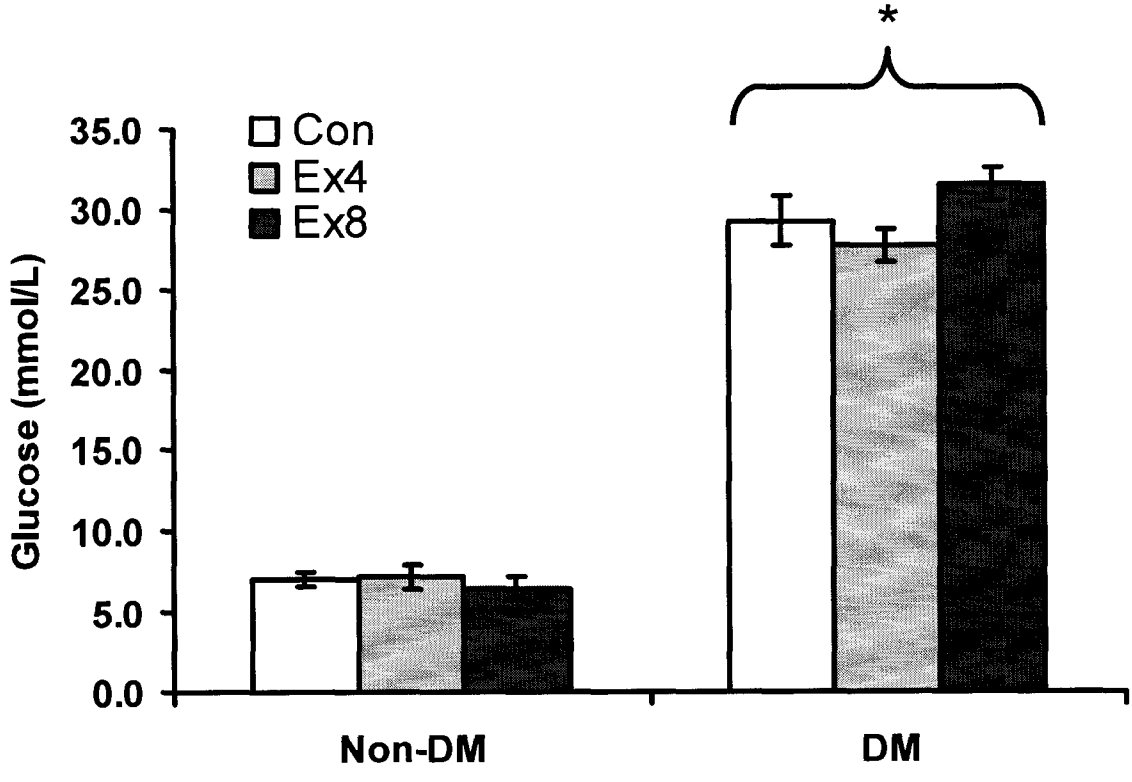


Figure 5.2. Total animal body mass following 8 weeks of DM and/or exercise. Diabetic animals significantly increased ($P < 0.05$) body mass after 8 weeks. However, Non-DM

animals demonstrated significantly greater body mass compared to all other groups after the experimental protocol. Data expressed as mean ± SEM (n = 30 per group). *Significantly greater ($P < 0.05$) than pre-treatment values.

Figure 5.2

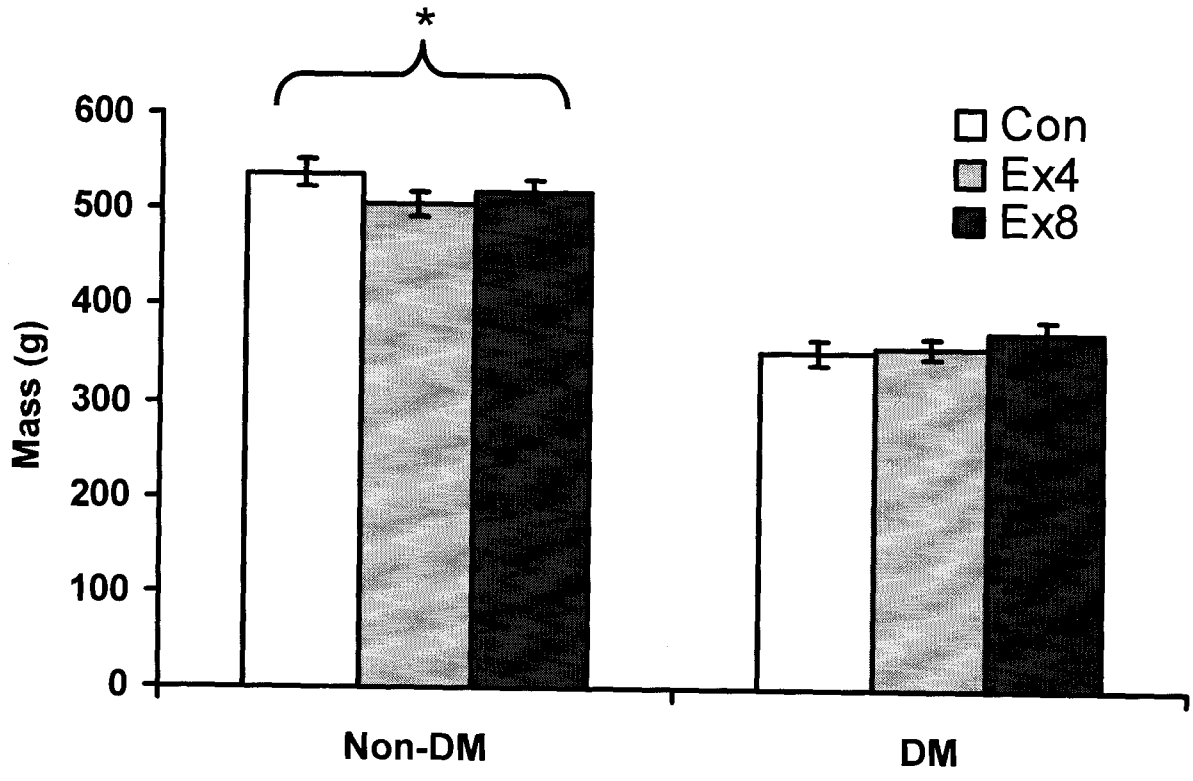


Figure 5.3 Myocardial contents of IGF-1R. 50 ug of protein from whole cell lysates were loaded into each well and run on a SDS-PAGE and reacted with an antibody specific for IGF-1R.

(*A*) A representative western blot demonstrating total quantities of IGF-1R within the myocardium of DM and Non-DM animals.

(*B*) Graphic analysis of IGF-1R in the rat myocardium. Data expressed as mean ± SEM (n = 10 per group). The diabetic myocardium showed significantly greater contents of IGF-1R. Exercise did not influence protein contents. *Significantly greater ($P < 0.05$) than Non-DM animals.

Figure 5.3*A*

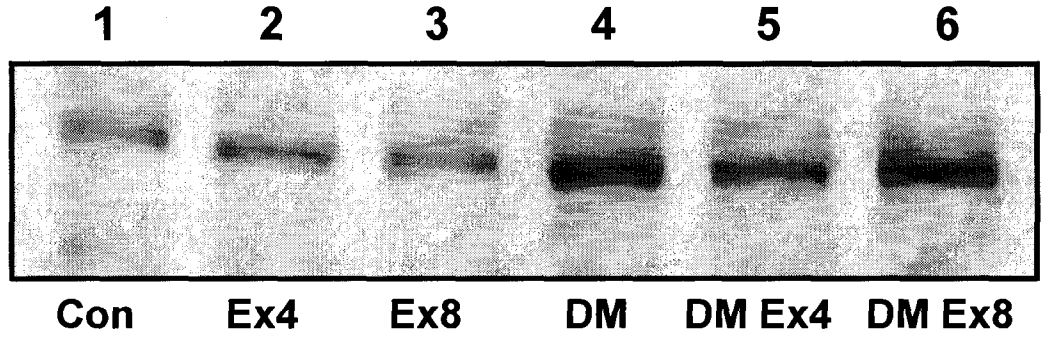


Figure 5.3*B*

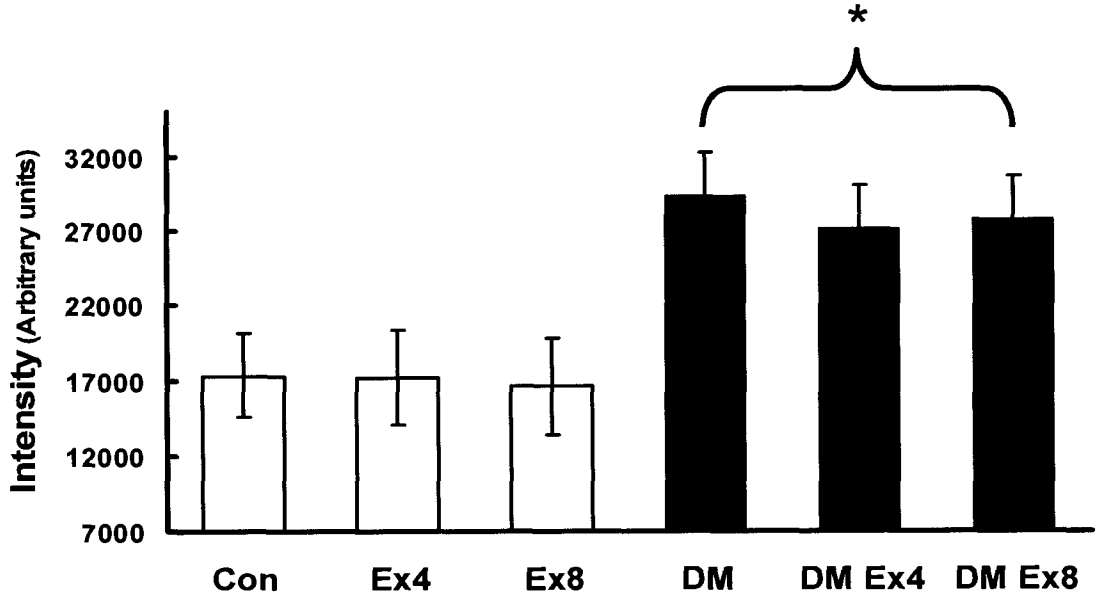


Figure 5.4 The effect of DM and/or exercise on phosphorylated-IGF-1R. 50 ug of protein from whole cell lysates were loaded into each well and run on a SDS-PAGE and reacted with a phospho-specific IGF-1R antibody.

A: A representative western blot demonstrating total quantities of phosphorylated-IGF-1R within the myocardium of DM and Non-DM animals.

B: Graphic analysis of phospho-IGF-1R in the rat myocardium. Data expressed as mean ± SEM (n = 10 per group). DM and/or exercise had no effect on IGF-1R phosphorylation.

Figure 5.4*A*

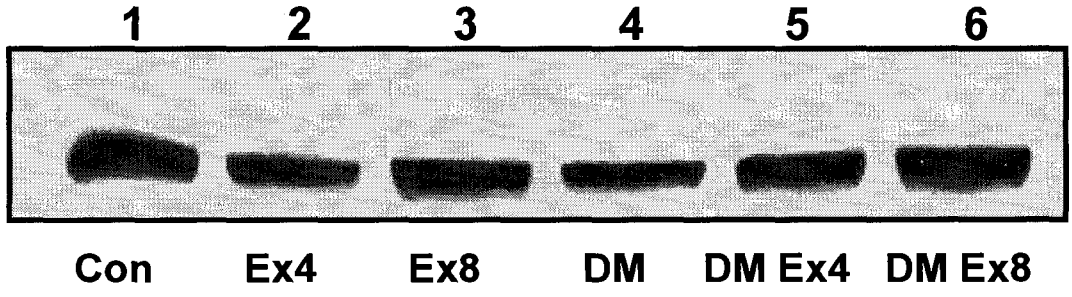
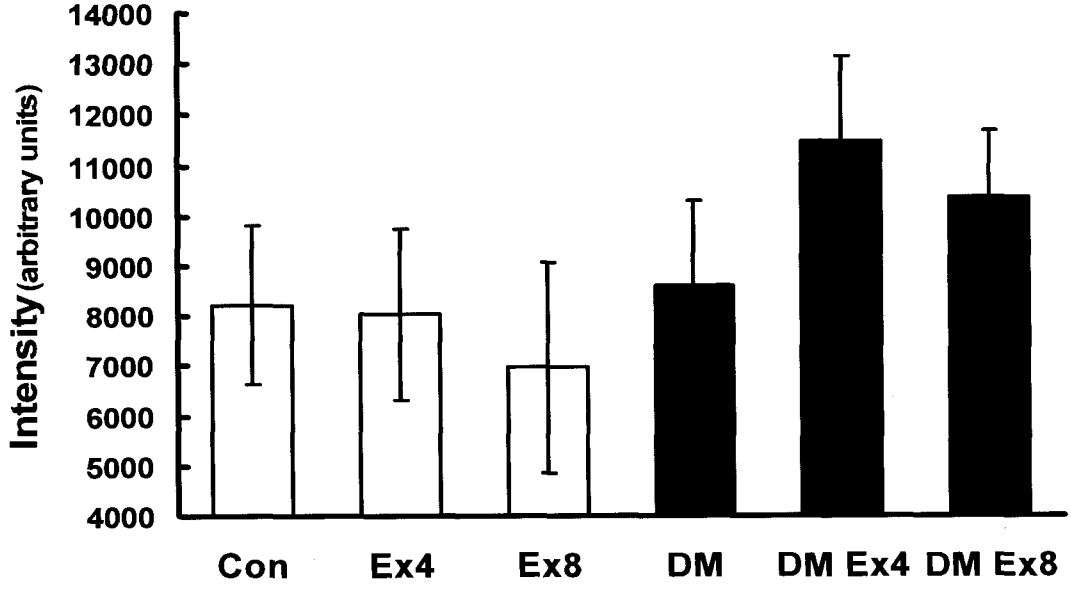


Figure 5.4*B*



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specific (ser9) GSK-3β antibody.

A: A representative western blot demonstrating total quantities of phosphorylated-GSK-3β within the myocardium of DM and Non-DM animals.

B: Graphic analysis of phosphorylated GSK-3β in the rat myocardium. Data expressed as mean ± SEM (n = 10 per group). The diabetic myocardium showed significantly less contents of phosphorylated GSK-3β. Exercise did not influence protein contents. *Significantly less ($P < 0.05$) than Non-DM animals.

Figure 5.5*A*

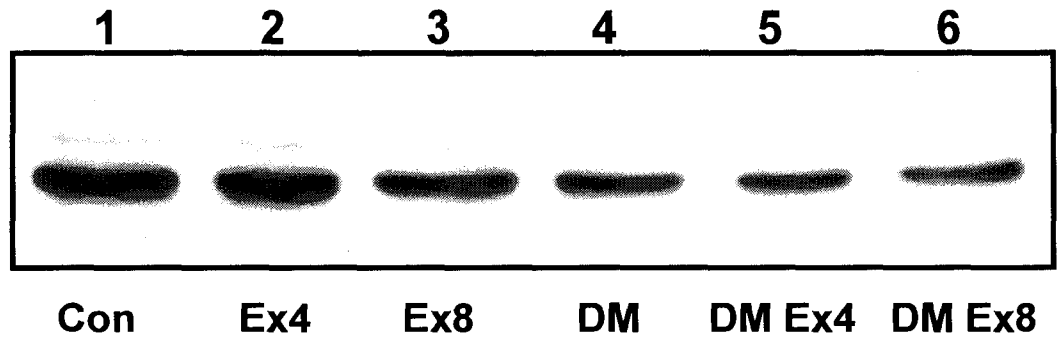


Figure 5.5*B*

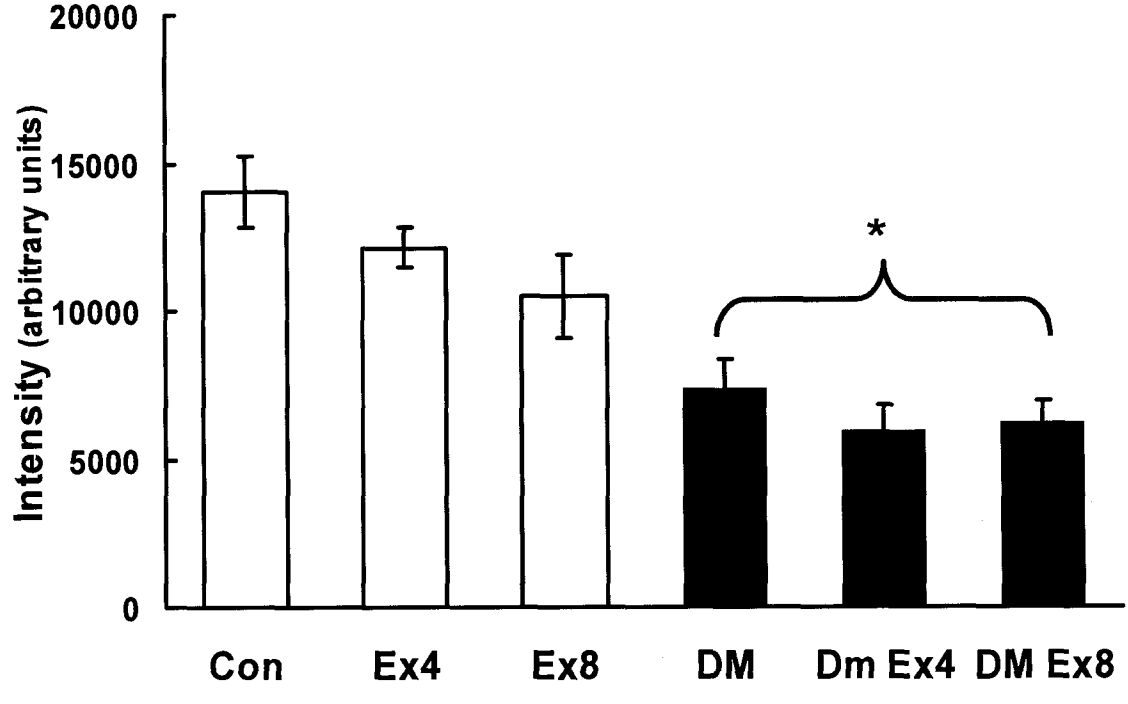


Figure 5.6 Cellular contents of HSF1. An antibody specific for HSF-1 was used against 50 ug of protein per well from whole cell lysates and run on a SDS-PAGE.

A: A representative western blot demonstrating total quantities of HSF-1 within the myocardium of DM and Non-DM animals.

B: Graphic analysis of HSF-1 in the rat myocardium. Data expressed as mean ± SEM (n = 10 per group). The diabetic myocardium showed significantly greater contents of HSF-1. *Significantly greater ($P < 0.05$) than Non-DM animals.

Figure 5.6*A*

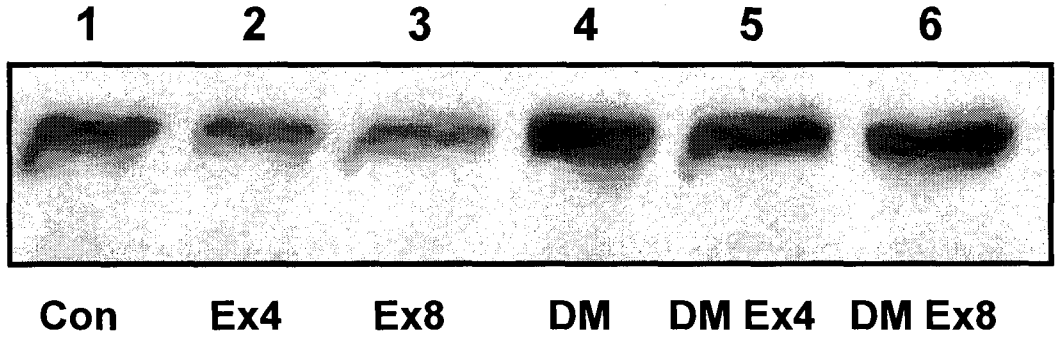


Figure 5.6*B*

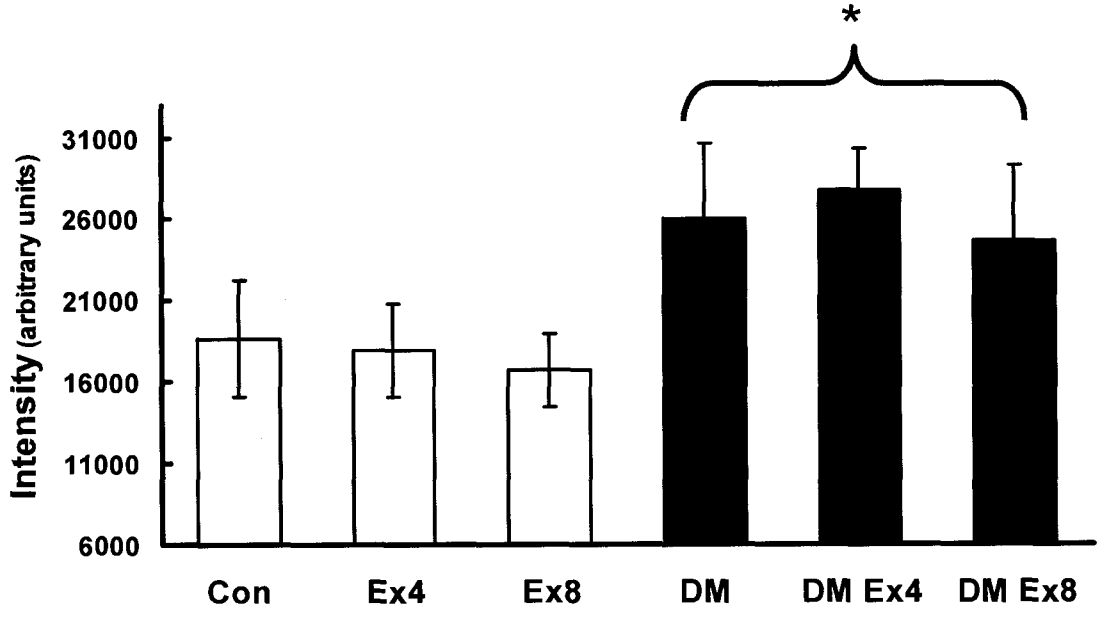


Figure 5.7 Myocardial contents of HSP60. 50 ug of protein from whole cell lysates were loaded into each well and run on a SDS-PAGE and reacted with an HSP60-specific antibody.

A: A representative western blot demonstrating total quantities of HSP60 within the myocardium of DM and Non-DM animals.

B: Graphic analysis of HSP60 in the rat myocardium. Data expressed as mean ± SEM (n = 10 per group). Neither DM nor exercise had an effect on HSP60 contents.

Figure 5.7*A*

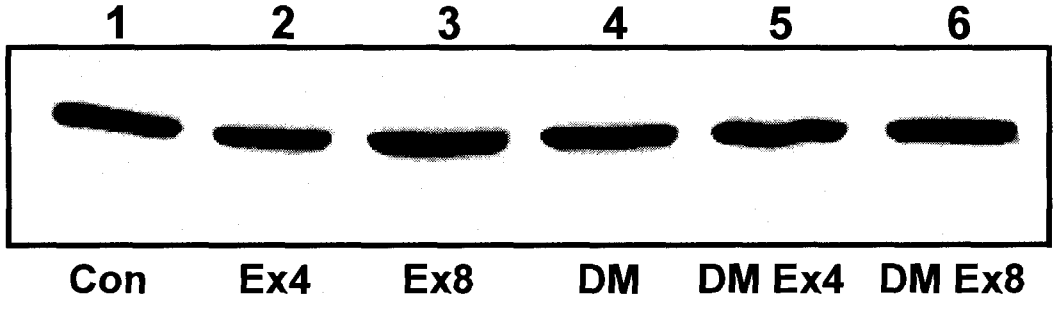


Figure 5.7*B*

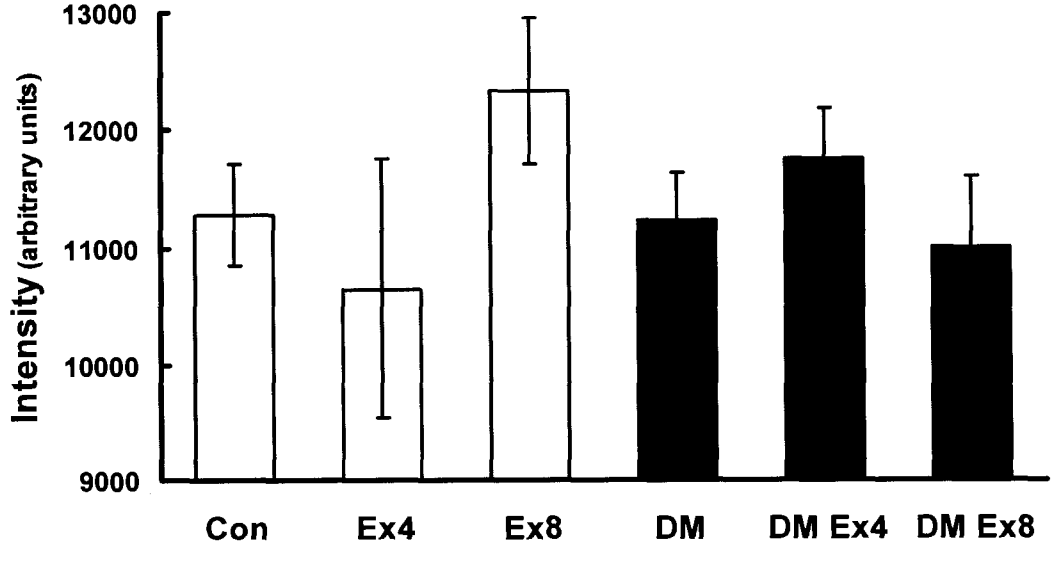


Figure 5.8 Exercise-induced HSP70 in the rat myocardium. 50 ug of protein from whole cell lysates were loaded into each well and run on a SDS-PAGE and reacted with an HSP70-specific antibody.

A: A representative western blot demonstrating total quantities of HSP70 within the myocardium of DM and Non-DM animals.

B: Graphic analysis of HSP70in the rat myocardium. Data expressed as mean ± SEM (n = 10 per group). Exercised myocardium showed significantly greater contents of HSP70. *Significantly greater ($P < 0.05$) than Non-exercised animals. DM had no effect on HSP70 contents.

Figure 5.8*A*

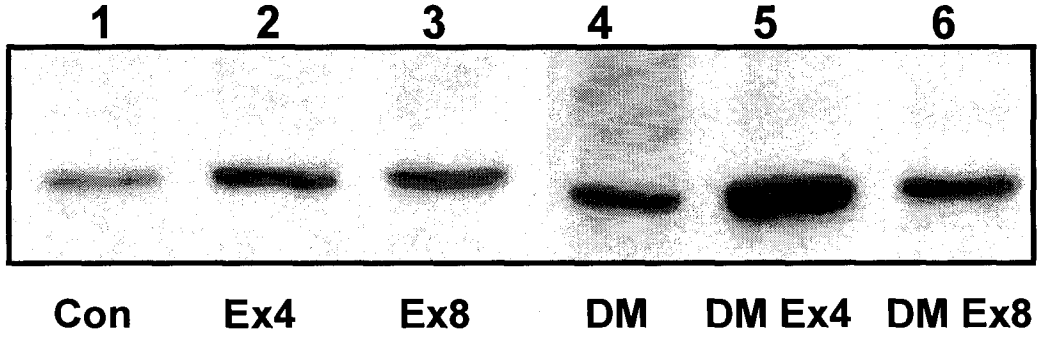


Figure 5.8*B*

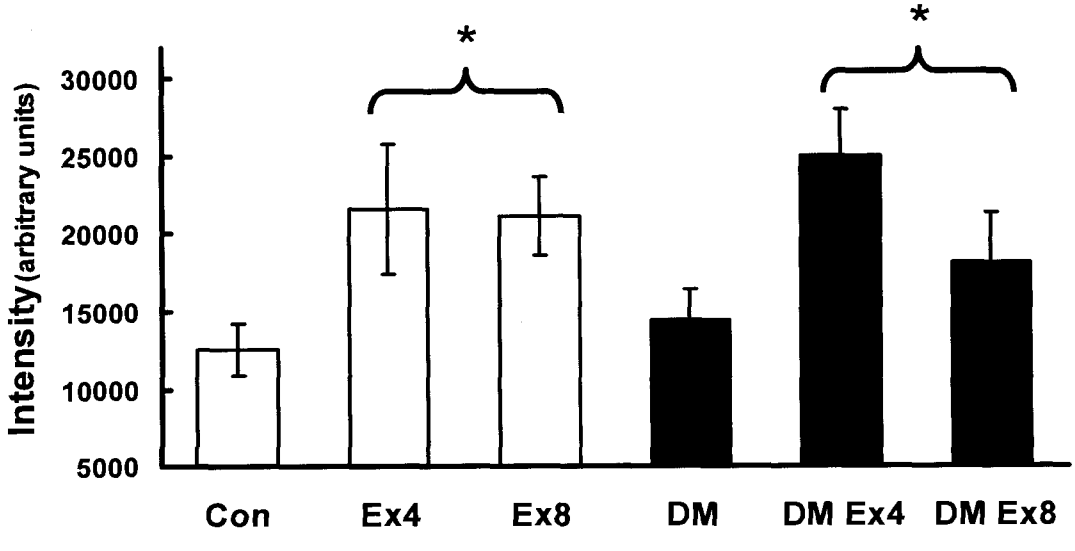


Figure 5.9 Myocardial contents of UCP-3. Whole cell protein exracts (50 ug) were run on a SDS-PAGE and reacted with an antibody specific for UCP-3.

A: A representative western blot demonstrating total quantities of UCP-3 in DM and Non-DM animals.

B: Graphic analysis of UCP-3 in the rat myocardium. Data expressed as mean ± SEM (n = 10 per group). Diabetic myocardium showed significantly greater contents of UCP-3. *Significantly greater ($P < 0.05$) than Non-DM animals. Exercise did not influence UCP-3 contents.

Figure 5.9*A*

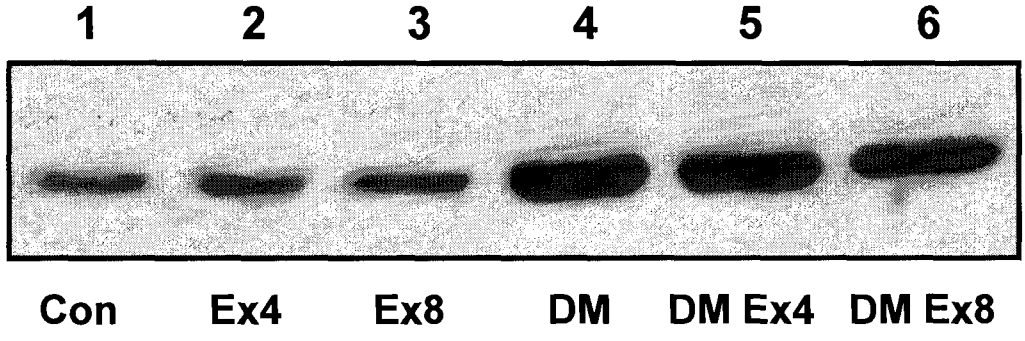


Figure 5.9*B*

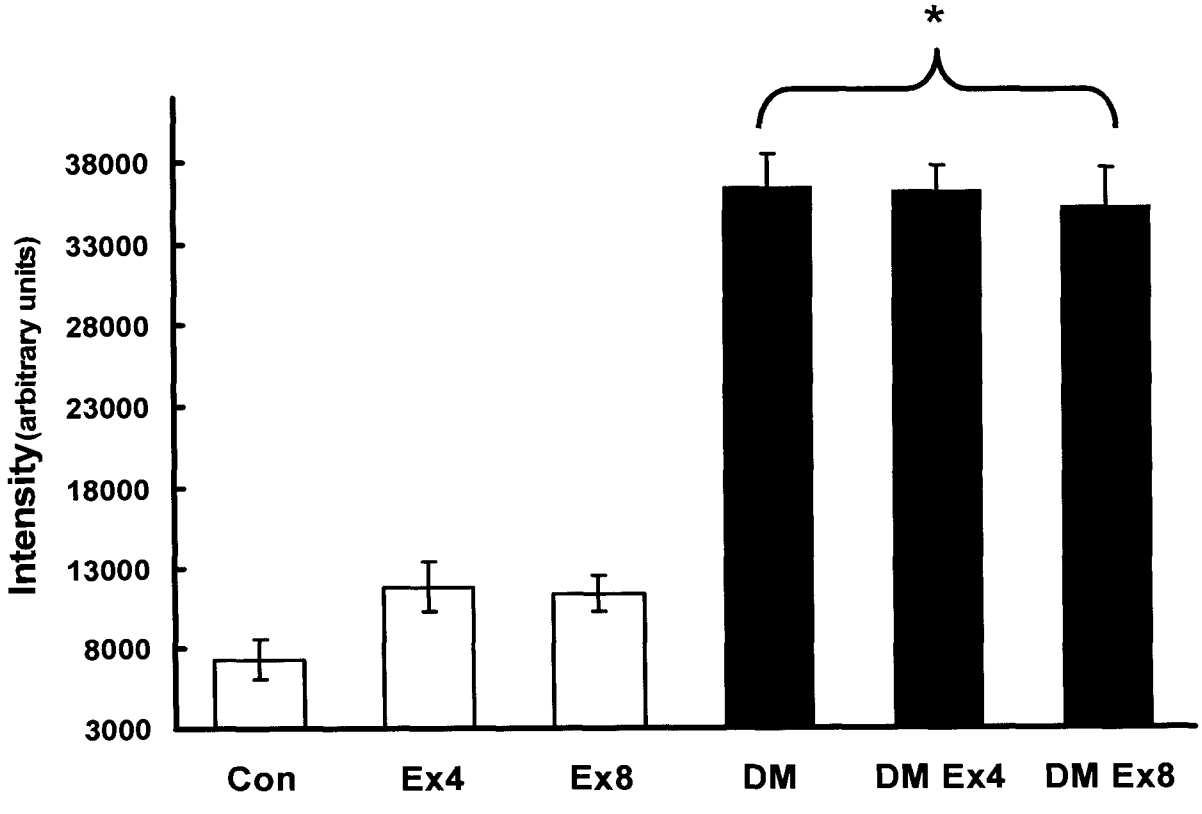


Figure 5.10 Serum levels of isoprostanes are reduced by exercise in the diabetic myocardium. Graphic analysis of 8-isoprostane-$PGF_{2\alpha}$ determined from rat serum by an ELISA. Data expressed as mean ± SEM (n = 10 per group). DM animals showed significantly less isoprostanes when exercised. *Significantly less ($P < 0.05$) 8-isoprostane-$PGF_{2\alpha}$ compared to Non-DM animals and DM controls. Exercise did not reduce isoprostanes in normal animals.

Figure 5.10

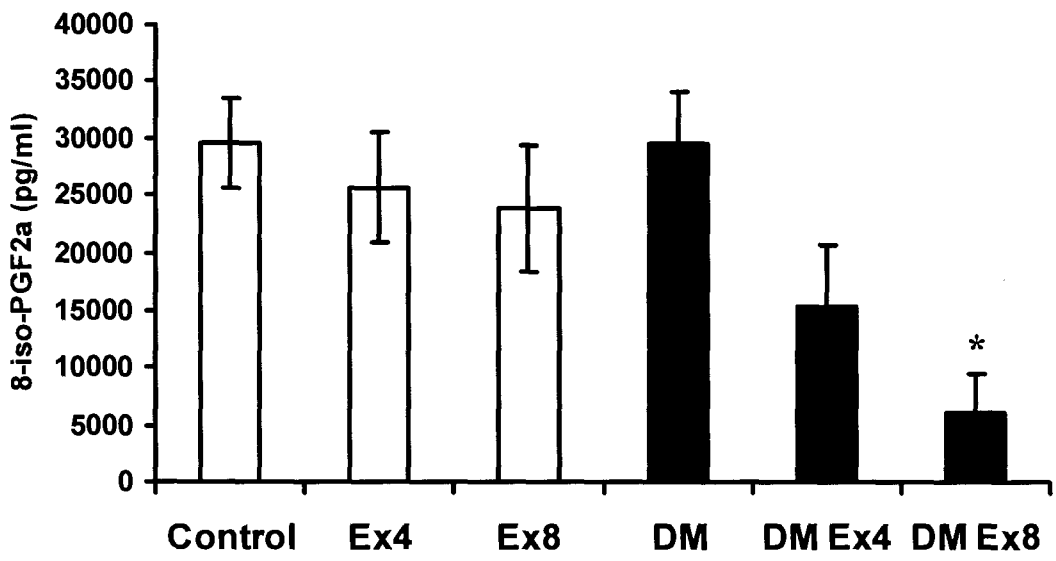


Figure 5.11 Serum levels of isoprostanes are elevated in the diabetic myocardium when expressed relative to lean mass or total body mass. Isoprostanes are returned to

normal levels after 4 weeks of exercise. Isoprostanes are reduced to levels lower than Non-DM levels following 8 weeks of exercise.

A: Graphic analysis of 8-isoprostane-$PGF_{2\alpha}$ expressed relative to total body mass. Data expressed as mean ± SEM (n = 10 per group). *Significantly greater ($P < 0.05$) 8-isoprostane-$PGF_{2\alpha}$ compared to Non-DM animals. †Significantly less ($P < 0.05$) 8-isoprostane-$PGF_{2\alpha}$ than all other groups. Exercise did not reduced isoprostanes in normal animals.

B: Graphic analysis of 8-isoprostane-$PGF_{2\alpha}$ expressed relative to lean mass. *Significantly greater ($P < 0.05$) 8-isoprostane-$PGF_{2\alpha}$ compared to Non-DM animals. †Significantly less ($P < 0.05$) 8-isoprostane-$PGF_{2\alpha}$ than all other groups. Exercise did not reduced isoprostanes in normal animals.

Figure 5.11*A*

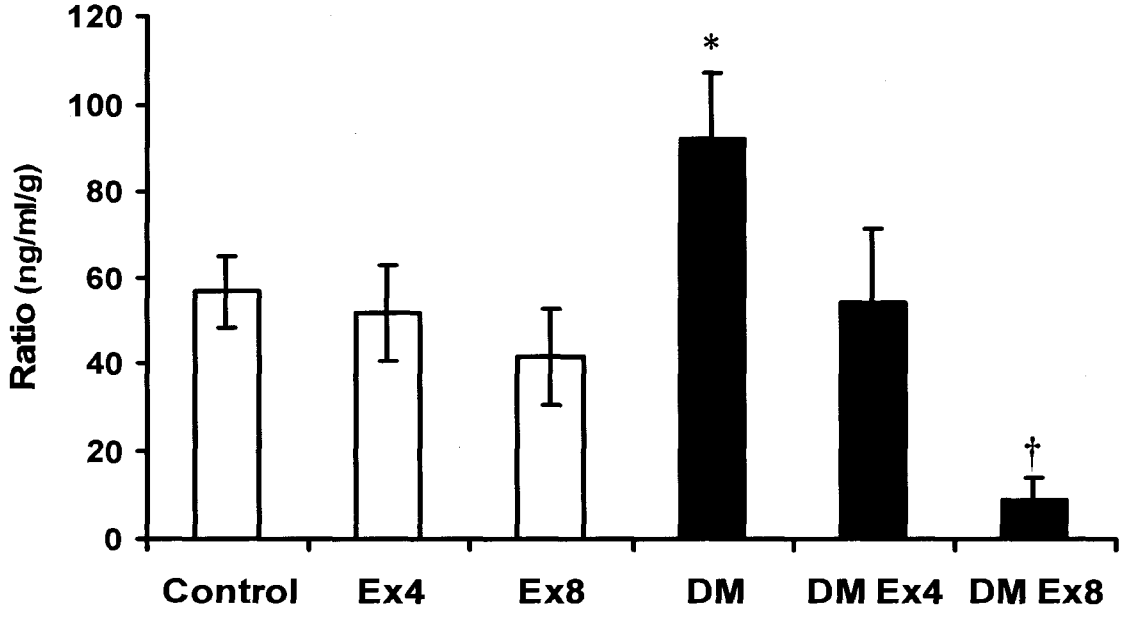


Figure 5.11*B*

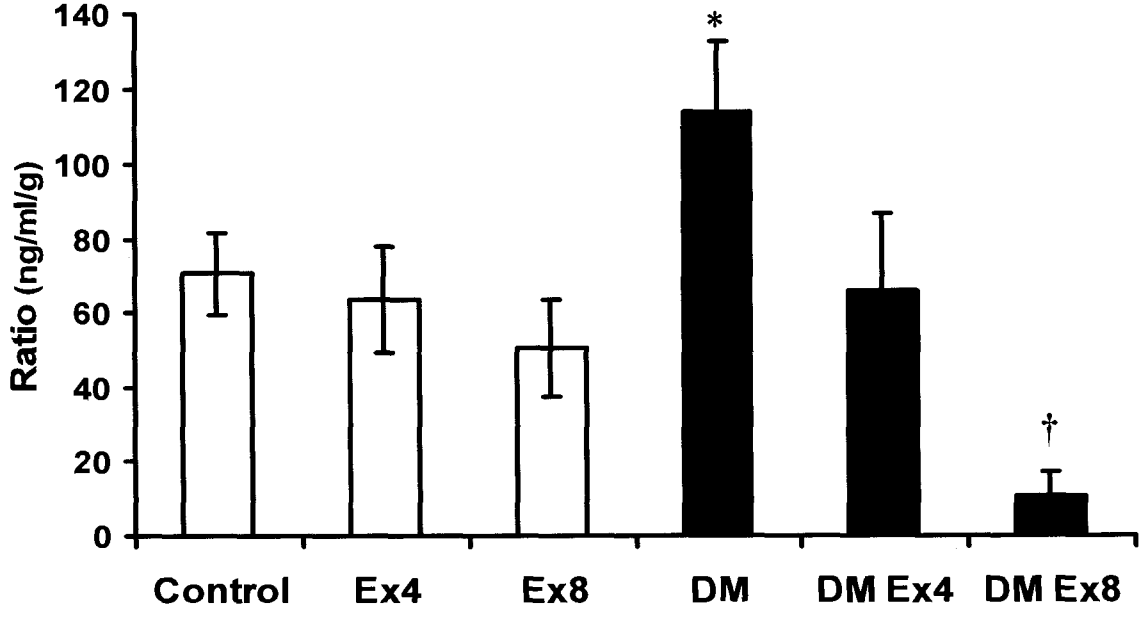


Figure 5.12 Collagen Type III is elevated in the diabetic myocardium. Whole cell

protein extracts (50 ug) were run on a SDS-PAGE and reacted with an antibody specific for Collagen Type III.

A: A representative western blot demonstrating total quantities of Collagen Type III in DM and Non-DM animals.

B: Graphic analysis of Collagen Type III in the rat myocardium. Data expressed as mean ± SEM (n = 10 per group). Data expressed as mean ± SEM (n = 10 per group). DM animals showed significantly greater myocardial contents of Collagen Type III. *Significantly greater ($P < 0.05$) than Non-DM animals. Exercise did not influence Collagen Type III contents.

Figure 5.12*A*

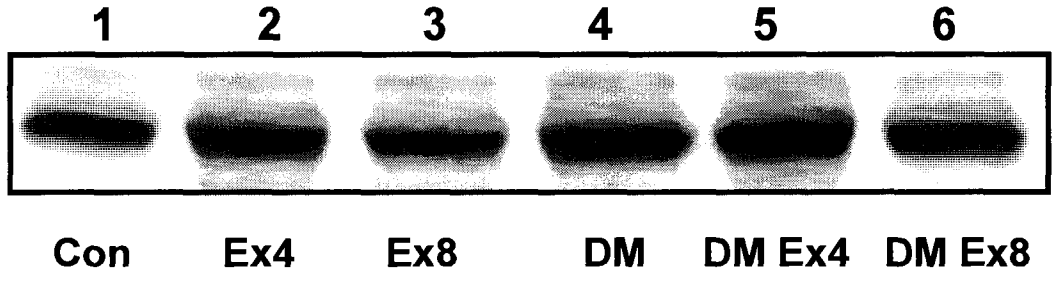

1
2
3
4
5
6
Con
Ex4
Ex8
DM
DM Ex4
DM Ex8

Figure 5.12*B*

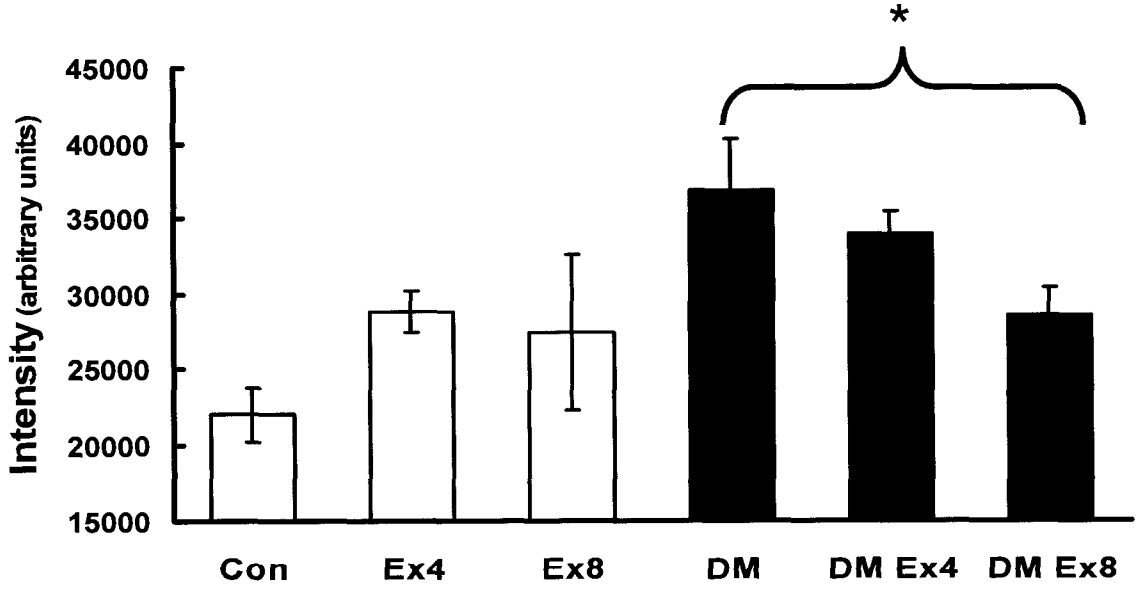

*
45000
40000
35000
30000
25000
20000
15000
Intensity (arbitrary units)
Con
Ex4
Ex8
DM
DM Ex4
DM Ex8

Figure 5.13 β-Myosin Heavy Chain Type I is elevated in the diabetic myocardium. Whole cell protein exracts (20 ug) were run on a SDS-PAGE and reacted with an antibody specific for β-MHC I.

A: A representative western blot demonstrating total quantities of β-MHC I in DM and Non-DM animals.

B: Graphic analysis of β-MHC I in the rat myocardium. Data expressed as mean ± SEM (n = 10 per group). DM animals showed significantly greater myocardial contents of β-MHC I. *Significantly greater ($P < 0.05$) than Non-DM animals. Exercise did not influence β-MHC I contents.

Figure 5.13*A*

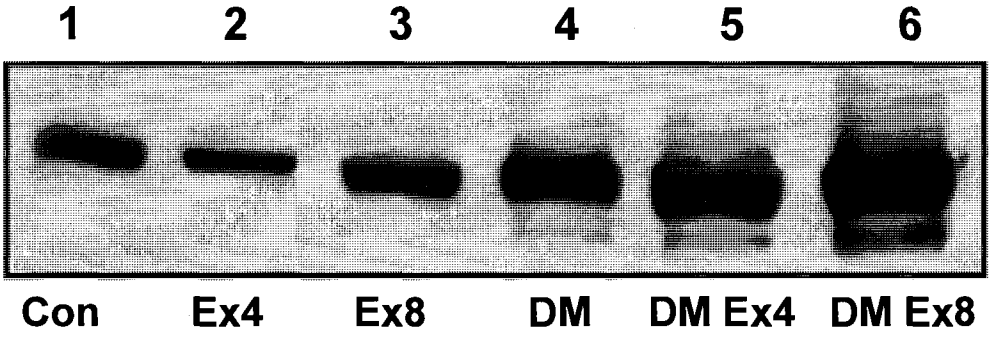
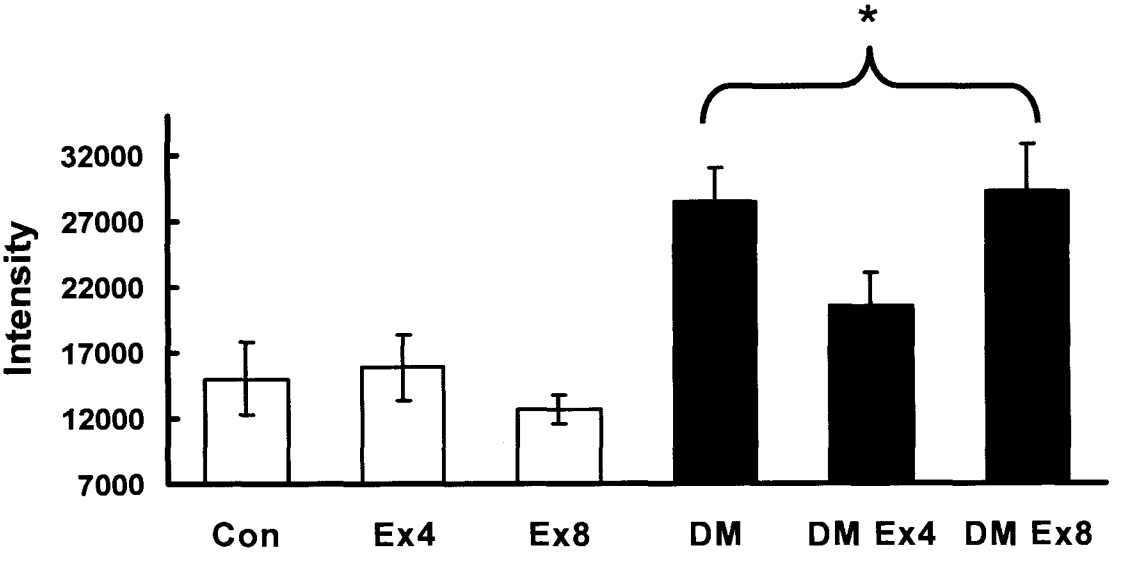


Figure 5.13*B*



5.4 DISCUSSION

DM-induced myocardial remodeling is characterized by abberant protein production and by the re-emergence of the fetal genotype, commonly associated with heart disease (reviewed by Rajabi et al., 2007). Altered collagen content increases stiffness, restricting contractibility and distensability. The accumulation of the slower contracting β-MHC I changes functional design, and provides phenotypic evidence of a fundamental shift in intracellular signaling. Myocardial growth and protein production is regulated through numerous signaling proteins; chief among them is IGF-1R kinase activity, which regulates its downstream targets, and affects proliferation and hypertrophy (Tseng et al., 2006; Antos et al., 2002). Since exercise influences myocardial growth and induces the production of protective proteins in the normal myocardium it was important to determine whether exercise was able to alter the deleterious effects of DM on cell signaling and functional proteins.

There were several findings in the present study. DM increased myocardial contents of IGF-1R, but the total number of activated/phosphorylated receptors was similar between DM and Non-DM animals. Eight weeks of DM reduced the phosphorylation of GSK-3β, suggesting its increased activity. DM also increased HSF-1 and UCP-3 protein, as well as the structural and functional proteins, Collagen type III and β-MHC I, respectively. Contents of HSP60 were similar between DM and Non-DM myocardia. Exercise did not influence myocardial contents of these proteins. However, exercise increased HSP70 regardless of DM, suggesting a preserved cellular stress response amidst severe DM. In addition, exercise reduced oxidative stress, measured by reduced serum contents of isoprostanes, in DM animals only. Diabetic animals that were

exercised for 8 weeks had significantly less serum isoprostanes than DM animals that exercised for 4 weeks. Therefore, in the DM condition, the extent of oxidative stress attenuation was related to total exercise volume over 8 weeks. Figure 5.14 is an integrated composite of the proposed pathway for all of the proteins discussed in the following sections.

IGF-1R and receptor activation. Following 8 weeks of DM, IGF-1R was significantly elevated in the rat myocardium, a finding also observed in rat aorta (Kobayashi et al., 2003). This is in contrast to the findings of Shan et al. (2003) and Chen et al. (2005). However, these contrasting studies measured contents over 10 days and 21 days, respectively, and paralleled HSP60 contents, which were unchanged in the present study. There may have been a temporary reduction in protein expression during the early stages of DM and the much longer duration in the present study (8 weeks) may represent a more stabilized adaptation to severe DM relative to myocardial IGF-1R and HSP60 contents.

The mechanism by which IGF-1R was increased may be related to a general up-regulation of receptors in response to diminished ligand quantity, as seen when agonists are low or receptors are antagonized (Sirohi et al., 2007). The STZ-induced DM model shows reduced quantities of both serum and myocardial of IGF-1 (Han and Park, 2006; Bornfeldt et al., 1992), which then stimulates receptor up-regulation (Eshet et al., 1993), which theoretically increases the probability of ligand-receptor interaction. Also, since the degradation of receptors often requires ligand binding, reduced ligand quantity may have reduced receptor turnover, leading to a net increase in IGF-1R.

The adapted response to DM was increased IGF-1R, but the results also indicate that the receptor was phosphorylated similarly among all groups, suggesting similar

activation. However, it has previously been demonstrated that IGF-1R auto-phosphorylation and constitutive activation occurs when IGF-1R is over-expressed (von Willebrand, 2003; Quong et al., 1994). The concept that receptors can be constitutively activated when unoccupied by an activating ligand is well-established (reviewed by Bond and Ijerman, 2006). However, the present study does not indicate whether the phosphorylated IGF-1R was bound and phosphorylated by IGF-1 or activated by another mechanism, including auto-phosphorylation. Therefore, it was beneficial to measure the activity and expression of divergent targets downstream of IGF-1R to determine whether specific pathways were activated or deactivated.

Myocardial GSK-3β was activated in DM tissues. GSK-3β was activated to a larger extent in DM tissues than in normal tissues (measured by reduced serine 9 phosphorylation). This is in contrast to Gurusamy et al. (2006) who showed reduced activity after 4 weeks in mice. However, these investigators also reported an initial GSK-3β activation measured after 4 days of DM, hence their report that elevated GSK-3β activity decreased after 4 weeks may also be a temporal phenomenon, lending further support that our findings reflect a longer term adaptation. In general, GSK-3β activity is associated with an anti-hypertrophic effect in cardiomyocytes (Tseng et al., 2006), whereas GSK-3β de-activation enables the myocardial capacity to adapt via hypertrophy to increasing functional demands (Hirotani et al., 2007) mediated in large part by its downstream control of the elongation-initiation factor, eIF2B (Pap and Cooper, 2002). However, pathological cardiac hypertrophy following chronic beta-adrenergic stimulation can be reduced by increasing GSK-3β activity (Antos et al., 2002). Hence, a delicate

balance of GSK-3β activation and de-activation is required for discrete cellular proliferation and survival responses to physiological disequilibration.

Since GSK-3β is phosphorylated by the IGF-1→PI-3K→AKT pathway it would be expected that the lack of GSK-3β phosphorylation found in DM animals was the result of reduced upstream activity. Given that IGF-1R was phosphorylated equally among all groups, interrupted kinase activity likely occurs downstream of IGF-1R and upstream of GSK-3β. Specifically, AKT is the kinase responsible for directing the phosphorylation of GSK-3β and deactivating its kinase activity. Previously, Gottlob et al. (2001) reported that AKT requires glucose for its downstream activity. Myocardial AKT phosphorylation was also significantly diminished following 8 weeks of DM in mice (Bilim et al., 2008). Specifically, STZ-DM reduces AKT activity by increasing phosphatase and tensin homolog (PTEN) contents (Song et al. (2007), which reduces phosphatidylinositol phosphate-3 (PIP_3)-mediated AKT phosphorylation. These events occur downstream of PI-3K, therefore it was important to determine a DM related consequence that requires PI-3K activity to determine where the interruption in the IGF-1→PI-3K→AKT→GSK-3β pathway may occur.

DM increased UCP-3 protein in the myocardium. UCP-3 was elevated significantly in DM tissues, a result shown in previous reports (Depre et al., 2000; Hidaka et al., 1999), whereas exercise training did not result in increased quantities of UCP-3, also previously reported in exercised rats (Cortright et al., 1999). IGF-1 has been shown to regulate UCP-3 (Gustafsson et al., 2004a) through activation of PI-3K (Gustafsson et al., 2004b). The latter report indicated that specific inhibition of PI-3K reduced UCP-3 contents, but did not inhibit AKT phosphorylation. By comparison, specific AKT inhibitors have been

shown to act without inhibiting PI-3K. Since AKT is inhibited by PTEN, which is regulated by PI-3K (Fernandez et al., 2008), a subtle multifaceted regulation emerges from the initial IGF-1R activation. Therefore, IGF-1 activation of both PI-3K and AKT in the DM myocardium is not a set-action response; that is, activation of IGF-1 does not guarantee that all members downstream will be phosphorylated, a result that delineates a refined divergence in the IGF-1→PI-3K→AKT→GSK-3β in severe DM. Taken together, the present study showed a lack of GSK-3β phosphorylation, requiring inactive AKT, but demonstrated increased UCP-3, requiring PI-3K activity. Hence, DM uniquely influenced the regulation of downstream kinases and proteins in the myocardium, the consequence of which may be demonstrated by considering the expression of additional proteins and altered stress-related products.

Oxidative stress is reduced in DM animals following exercise. Regardless of how oxidative stress was expressed (absolute or relative to body- or lean-mass), exercise reduced isoprostanes in DM animals. Absolute values for isoprostanes indicated that DM animals did not experience greater oxidative stress than Non-DM animals, however this does not agree with most other reports in STZ-DM rats (Guo et al., 2007; Montero et al., 2000; Palmer et al., 1998). Since absolute values are expressed relative to blood volume it is possible that the weight of the DM animals, relative to Non-DM animals, is disproportionate to the total volume of blood. Indeed, Sato et al. (1985) showed that an increase in body mass was not matched by an equal increase of blood volume in the rat. Based on the slope calculated from data reported by Sato et al. (1985), body mass values in the present study (DM = ~350 g; Non-DM = ~535 g) would predict a blood volume of ~20 ml for DM animals and ~25 ml for Non-DM animals, indicating a larger blood

volume per gram of mass in DM animals. Hence, a smaller animal may produce a larger quantity of isoprostanes per gram of tissue while obtaining a similar quantity per ml of serum.

Since free radicals are produced primarily in the mitochondria (predominantly in lean tissue) it seemed appropriate to express isoprostanes relative to lean mass. Expressed relative to lean mass, DM animals experienced greater oxidative stress than Non-DM animals, and 4 weeks of exercise was able to normalize levels of oxidative stress. Interestingly, oxidative stress was reduced to below normal values with 8 weeks of exercise in DM animals, regardless of how values were expressed. This observation suggests that factors specific to exercise augmented oxidative stress reduction. UCP-3 was uniquely induced by DM and its expression has been correlated with fatty acid oxidation and reduced ROS production (Bevilacqua et al., 2005; MacLellan et al., 2005; reviewed by Schrauwen et al., 2006). In fact, UCP-3 itself may be activated by fatty acids (Jaburek et al., 1999), so its ability to reduce oxidative stress may be expected due to increased reliance on fatty acid metabolism in DM animals. However, it was only the exercised DM animals that reduced isoprostanes, hence, if UCP-3 was responsible for reduced oxidative stress it required activation by both exercise and fatty acids. This is possible since specific quantities of purine nucleotides (e.g. ATP, GTP, cAMP) can inhibit fatty acid activation of uncoupling proteins (Barclay et al., 2008). ADP is a stronger inhibitor than ATP (Echtay et al., 2001). Early reports showed reduced ATP levels in rat skeletal muscle (Moore et al., 1983), deranged nucleotide regulation (Garber, 1980) in rat skeletal muscle, and increased ADP in DM rat lungs (Bakhle and Chelliah, 1983). Moreover, Dudzinska and Hlynczak (2004) showed generally disrupted purine

metabolism and DM dephosphorylated adenine nucleotides in DM erythrocytes. In addition, Mokhtar et al. (1993) showed that DM reduced phosphocreatine levels in the rat myocardium. Therefore, if exercise was able to improve purine metabolism in the present study then improved activation of significant quantities of UCP-3 in DM muscle may have contributed to decreased whole body oxidative stress.

The diabetic myocardium maintained sensitivity of the cellular stress response. Stress- and exercise-induced HSP70 has long been associated with providing myocardial protection against subsequent bouts of stress, such as ischemia reperfusion (Currie et al., 1998; Locke et al., 1995). There are conflicting reports regarding both HSP70 and HSF-1 protein contents in the diabetic myocardium in sedentary and exercised rats. DM reduced constitutive and exercise-induced myocardial HSF-1 and HSP70 protein content in rats (Atalay et al., 2004), in contrast to Najemnikova et al. (2007) that showed unchanged constitutive myocardial contents of HSF-1 and HSP70 protein. In agreement with Najemnikova et al. (2007), the present study showed unchanged myocardial HSP70 contents in sedentary DM animals, a result also shown by Chen et al. (2006).

Our observation that exercise-induced HSP70 is preserved in DM animals is supported in part by Atalay et al., (2004), as they reported increased HSP70 in the diabetic myocardium following exercise, but HSP70 induction was significantly lower in DM animals compared to Non-DM animals. Unlike Atalay et al., (2004), the present study employed a regime of multiple low-dose injections of STZ, which has been shown to prevent the loss of circulating growth hormone (GH) associated with a single higher-dose bolus injection of STZ (Kim et al., 2006). Interestingly, after the addition of GH, heat shock-induced HSP70 was increased in cultured sea bream blood cells (Deane and

Woo, 2005), but was decreased in hepatic tissue (Deane et al., 1999). Moreover, neuropeptide Y has been shown to influence HSP70 expression and is also differentially affected between the low- and high-dose STZ injection regimes (Zhong et al., 2003). Hence, hormone balance between different models may affect HSP70 expression in a tissue-specific manner. In addition, the present study employed a faster running speed than Atalay et al. (2004), which may be critical to myocardial expression of HSP70 (Milne and Noble, 2002), especially if DM animals experienced reduced intensity due to reduced body mass. As a myocardial event is the primary cause of death resulting from DM, the finding that HSP70 is increased following exercise in the diabetic myocardium indicates that protection may still be conferred, even though exercise was unable to maintain or restore normal intracellular signaling pathways in DM animals.

HSP70 is regulated by the DNA binding of HSF-1. We found elevated HSF-1 protein in all DM animals. Zuo et al. (1995) reported that over-expression of HSF-1 can induce constitutive transcriptional activation without the need for an activating stress; however, over-expression of HSF-1 does not typically result in elevated levels of HSPs (Mivechi et al., 1995). In the present study, elevated HSF-1 by DM may be a compensatory mechanism to preserve the cellular stress response and subsequent exercise-induction. Increased HSF-1 expression was an unexpected finding, since it is contrary to the few investigations that report its content in STZ-treated rat myocardium (Atalay et al., 2004; Najemnikova et al., 2007). Unchanged quantities reported by Najemnikova et al. (2007) may reflect their shorter experimental duration (4 weeks) and a single high-dose of STZ, whereas altered HSF-1 quantities between groups reported by

Atalay et al. (2004) may reflect sampling variability due to very large, non-specific bands shown in their western blots.

The current theory regarding upstream signaling of HSF-1 and previous reports indicated that active/unphosphorylated GSK-3β reduced transcriptional activity of HSF-1 and expression of HSP70 (Bijur and Jope, 2000; He et al., 1998; reviewed by Hooper and Hooper, 2008), whereas GSK-3β inhibition led to increased HSF-1 activation (He et al., 1998; Xavier et al., 2001) and increased HSF-1 protein (Chung et al., 2008) following heat stress. Our findings indicate that un-phosphorylated (active) GSK-3β did not prevent the constitutive accumulation of HSF-1 protein or exercise induced HSP70 expression. However, the ability of GSK-3β to inhibit HSF-1 is dependent, and subsequent, to ERK-1 (mitogen activated protein kinase family) phosphorylation of HSF-1 (Chu et al., 1996). ERK-1 activity has been shown to be either increased (Strniskova et al., 2003) or decreased (Ekladous et al., 2008) by DM in the rat myocardium depending on the disease duration. Indeed, work by Naito et al. (2003) showed that increased myocardial ERK-1 activity following DM induction was markedly inhibited with prolonged DM duration (after 20 weeks). Hence, our results continue to suggest that these changes may represent a long term adaptation to DM.

Structural and functional proteins are altered by DM. The myocardium, composed predominantly (50-55%) of myosin (Dreizen, 1971) demonstrated a massive increase in the slow contracting β-MHC I in DM animals, a well-established phenomenon (Depre et al., 2000; Dillmann, 1980), and this was not preventable or reversible by exercise (Paulson et al., 1992). The impetus for isoform shifting is unknown, but a functional consequence includes reduced ATP consumption per unit of force generated by β-MHC I

(Rundell et al., 2004). Nascent expression of β-MHC I may be inherently part of a re-emerging fetal gene program, long associated with heart disease and stress (reviewed by Rajabi et al., 2007). STZ-induced DM is associated with marked bradycardia, also observed in the present study (data not shown). The cause of the bradycardia, although uncertain, may be related to impaired fatty acid transport from reduced carnitine (Malone et al., 2007) or atrial node dysfunction (Li et al., 1989). Up-regulation of the slower contracting β-MHC I would allow for force generation over a prolonged time interval, which may be more appropriate for the lower basal heart rate.

It is possible that IGF-1R signaling influenced β-MHC I expression. For example, overexpressing IGF-1R in normal mouse hearts induced hypertrophy through PI-3K, maintained normal phenotype and improved systolic function, but was associated with abnormally elevated contents of β-MHC I (McMullen et al., 2004). Interestingly, the over-expression of activated GSK-3β in mouse hearts inhibited hypertrophy, but also blocked expression of β-MHC I (Antos et al., 2002). Although the present study observed the over-expression of both IGF-1R and active GSK-3β, the previously observed inhibition of β-MHC I via active GSK-3β was not found. Hence, in the present study, altered IGF-1R signaling may have increased GSK-3β activity (impaired myocardial growth) and contributed to elevated β-MHC I through mechanisms independent of GSK-3β (Cheng et al., 2005; Nagai et al., 2003; Sack et al., 1997).

In addition to β-MHC I expression, re-emergence of the fetal phenotype and the effects of DM are evident in changes to the myocardial extracellular matrix. The matrix, predominantly composed of collagen types I and III, showed markedly increased collagen type III in all DM animals, supported by previous reports (Xia et al., 2007; Gurusamya et

al., 2006; Tschöpe et al., 2004). Exercise was not able to prevent or reverse this change in collagen. Woodiwiss et al. (1996) did not measure collagen type, but did show that exercise decreased myocardial stiffness in DM rats, but did not improve interstitial collagen contents. Similarly, Searls et al. (2004) showed that exercise reduced the cross-sectional surface area of collagen within DM rat myocardia, but did not measure collagen type. As DM generally leads to increased collagen type III without changing type I contents (Candido et al., 2003), it is difficult to determine whether previous results reflect a change in the pathophysiology of diabetic cardiomyopathy as it pertains to collagen type III. Exercise-induced collagen improvements are generally associated with reduced cross-linking rather than changes to collagen type (Thomas et al., 2000; Todaka et al., 1997). Therefore, exercise may reduce fibrosis, but may not prevent or restore DM-altered collagen contents. Consequently, tissue mechanics may still reflect a stress-strain profile intrinsic to collagen type III, thus altering function, regardless of collagen cross-linking.

Changes to the material structure of the extracellular matrix is made manifest by collagen turnover and degradation regulated by matrix metalloproteinases (MMPs) (Mukherjee et al., 2005; reviewed by Bramono et al., 2004). In particular, MMP-3 regulates collagen type III content (Sansilvestri-Morel et al., 2005) and MMP-3 expression and activity is independently associated with both heart disease (Mori et al., 2006; Wu et al., 2005; reviewed by Rockman et al., 2004 and Spinale et al., 2000) and DM-related heart disease (reviewed by Tayebjee et al, 2005). MMP-3 is a target gene of the inflammatory regulator, NF-κB. In particular, the pathological form of NF-κB (p50-p65) is associated with increasing MMP-3 expression (Borghaei et al., 2004). IGF-1

signaling has been shown to increase formation of the transcription repressing p50-p50 form of NF-κB (Knuefermann et al., 2002) and a loss of IGF-1 signaling was correlated with the severity of heart failure (O'Brien et al., 2003). Since a mechanistic link has been previously demonstrated in DM between IGF-1, NF-κB and heart failure (Gupta et al., 2002) and between cardiac inflammation, MMP activity and cardiac disease (Westermann et al., 2007), it is possible that DM-altered collagen type III contents are regulated through IGF-1 signaling through the activities of NF-κB and MMP-3. Hence, the apparent disruption in IGF-1R signaling may be partly instrumental in manifesting the altered matrix-collagen phenotype in DM animals.

Figure 5.14 Intracellular signaling pathway in Non-DM and DM myocardia. ***IGF-1R***: insulin-like growth factor-1 receptor; ***HSP60***: heat shock protein 60; ***p50-p50***: transcription repressing, constitutively bound homodimer of NF-κB; ***p50-p65***: pathology-associated, transcriptionally active heterodimer of NF-κB; ***MMP-3***: matrix metalloproteinase-3; ***collagen III***: extracellular matrix collagen type III; ***PI-3K***; Phosphoinositide 3-kinase; ***PTEN***: phosphatase and tensin homolog; PIP_3 phosphatidylinositol phosphate-3; ***AKT***: AK-transforming kinase or protein kinase B; ***GSK-3β***: glycogen synthase kinase-3beta; ***β-MHC I***: beta-myosin heavy chain I; ***α-MHC*** I: alpha-myosin heavy chain I; ***UCP-3***: uncoupling protein-3; ***ROS***: reactive oxygen species; ***ERK-1***: extracellular-regulated protein kinase-1; ***HSF-1***: heat shock factor-1; ***HSP70***: heat shock protein-70; ***phos***: phosphorylation of substrate; P_i: inorganic phosphate.

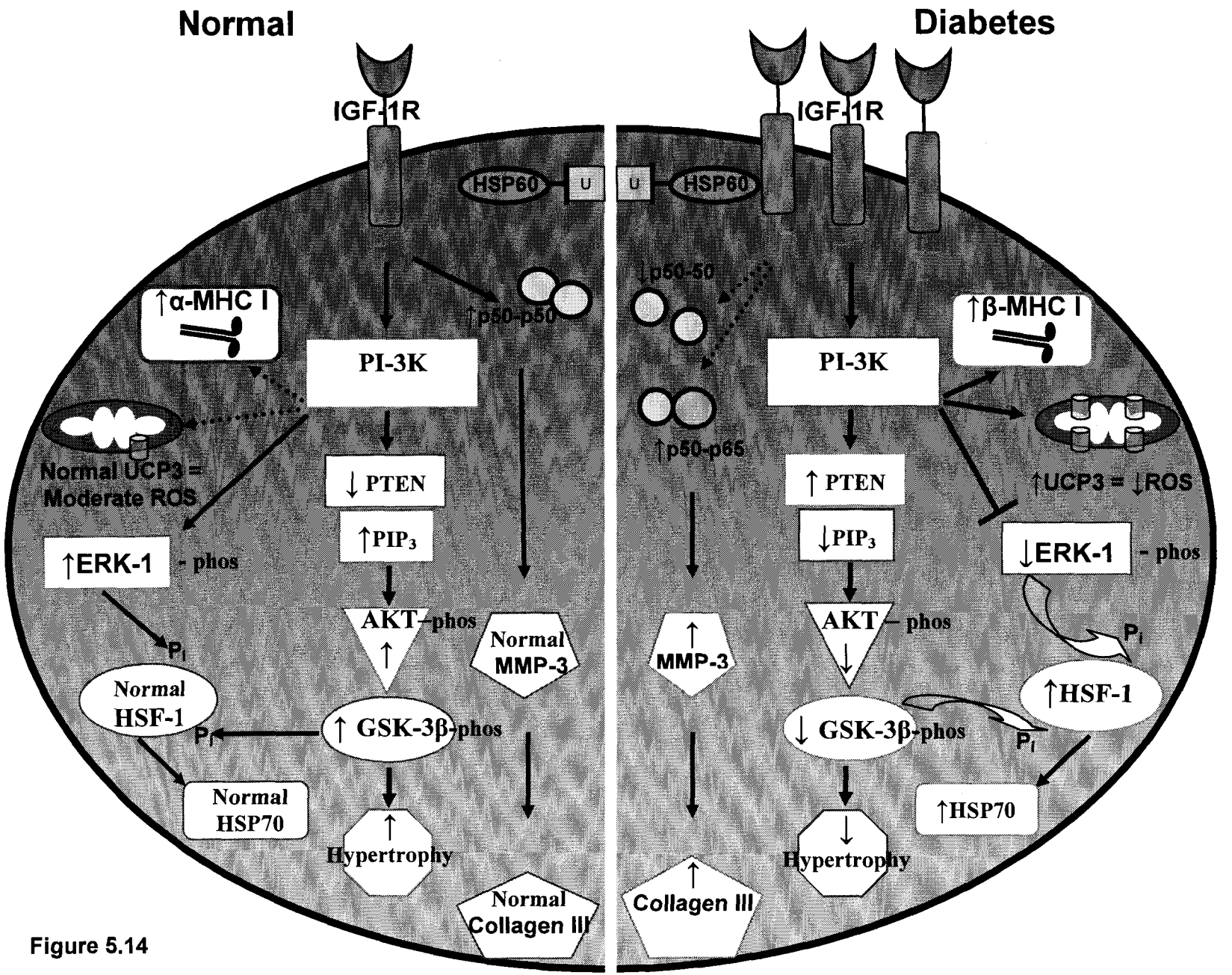

Normal
Diabetes
IGF-1R
IGF-1R
HSP60
U
U
HSP60
↑α-MHC I
↑β-MHC I
↑p50-p50
↓p50-50
PI-3K
PI-3K
↑p50-p65
Normal UCP3 = Moderate ROS
↑UCP3 = ↓ROS
↓ PTEN
↑ PTEN
↑PIP3
↓PIP3
↑ERK-1 - phos
↓ERK-1 - phos
AKT-phos
AKT - phos
Normal MMP-3
↑ MMP-3
Pi
Pi
Normal HSF-1
↑HSF-1
↑ GSK-3β-phos
↓ GSK-3β-phos
Pi
Pi
Normal HSP70
↑HSP70
↑ Hypertrophy
↓ Hypertrophy
Normal Collagen III
↑ Collagen III

Figure 5.14

5.5 CONCLUSION

A lack of interaction between DM and exercise was generally observed in the diabetic myocardium. Distinct consequences on intracellular signaling and protein expression affected by DM were not altered by exercise; however, exercise retained the capacity to induce the cardio-protective protein, HSP70 and reduced oxidative stress in DM animals.

Contents of IGF-1R, the keystone regulator of myocardial growth and protein expression, were increased in all DM animals, but phosphorylation status was similar between all groups. UCP-3 contents were increased in all DM animals, possibly reflecting increased reliance on fatty acid metabolism and indicating altered signaling downstream of IGF-1R via PI-3K signaling. Reduced GSK-3β phosphorylation (increased activity) was found in all DM animals, again indicating altered signaling downstream of IGF-1R via PI-3K→AKT. The minimally expressed myosin protein isoform β-MHC I increased significantly with DM, associated with the fetal program in cardiomyopathy, possibly mediated through IGF-1R→PI-3K signaling. Likewise, the structural protein, collagen type III, was increased by DM, requiring MMP-3 activation, upregulated by NF-κB, potentially regulated by IGF-1 signaling. HSP70 was induced by exercise, and DM increased the expression of the stress response transcription factor, HSF-1.

DM altered intracellular IGF-1R signaling and elevated gene products associated with cardiomyopathy. Although exercise did not demonstrate an ability to normalize cell signaling, structure and function, its induction of the cardio-protective HSP70 and

reduction of whole body oxidative stress encourages its continued prescription and investigations into its biochemical influence.

5.6 REFERENCES

Antos CL, McKinsey TA, Frey N, Kutschke W, McAnally J, Shelton JM, Richardson JA, Hill JA, Olson EN. Activated glycogen synthase-3 beta suppresses cardiac hypertrophy in vivo. *Proc Natl Acad Sci U S A.* 2002; 99(2):907-912.

Antos CL, McKinsey TA, Frey N, Kutschke W, McAnally J, Shelton JM, Richardson JA, Atalay M, Oksala NK, Laaksonen DE, Khanna S, Nakao C, Lappalainen J, Roy S, Hänninen O, Sen CK. Exercise training modulates heat shock protein response in diabetic rats. *J Appl Physiol.* 2004; 97(2):605-611.

Badorff C, Ruetten H, Mueller S, Stahmer M, Gehring D, Jung F, Ihling C, Zeiher AM, Dimmeler S. Fas receptor signaling inhibits glycogen synthase kinase 3 beta and induces cardiac hypertrophy following pressure overload. J Clin Invest. 2002; 109(3):373-81.

Bakhle YS, Chelliah R. Effect of streptozotocin-induced diabetes on the metabolism of ADP, AMP and adenosine in the pulmonary circulation of rat isolated lung. *Diabetologia.* 1983; 24(6):455-459.

Barclay CJ, Woledge RC, Curtin NA. Effects of UCP3 genotype, temperature and muscle type on energy turnover of resting mouse skeletal muscle. *Pflugers Arch.* 2008 Jul 22.

Bevilacqua L, Ramsey JJ, Hagopian K, Weindruch R, Harper ME. Long-term caloric restriction increases UCP3 content but decreases proton leak and reactive oxygen species production in rat skeletal muscle mitochondria. *Am J Physiol Endocrinol Metab.* 2005; 289(3):E429-438.

Bezaire V, Seifert EL, Harper ME. Uncoupling protein-3: clues in an ongoing mitochondrial mystery. *FASEB J.* 2007;21(2): 312-324.

Bijur GN, Jope RS. Opposing actions of phosphatidylinositol 3-kinase and glycogen synthase kinase-3beta in the regulation of HSF-1 activity. *J Neurochem.* 2000; 75(6):2401-2408.

Bilim O, Takeishi Y, Kitahara T, Arimoto T, Niizeki T, Sasaki T, Goto K, Kubota I. Diacylglycerol kinase zeta inhibits myocardial atrophy and restores cardiac dysfunction in streptozotocin-induced diabetes mellitus. Cardiovasc Diabetol. 2008 Feb 4;7:2.

Borghaei RC, Rawlings Jr. PL, Javadi M, Woloshin J. NF-κB binds to a polymorphic repressor element in the MMP-3 promoter. *Biochem Biophys Res Com.* 2004; 316:182–188.

Bornfeldt KE, Skottner A, Arnqvist HJ. In-vivo regulation of messenger RNA encoding insulin-like growth factor-I (IGF-I) and its receptor by diabetes, insulin and IGF-I in rat muscle. *J Endocrinol.* 1992;135: 203–211.

Bradford MM. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72:248-254.

Bramono DS, Richmond JC, Weitzel PP, Kaplan DL, Altman GH. Matrix metalloproteinases and their clinical applications in orthopaedics. Clin *Ortho Rel Res.* 2004; 428:272–285.

Candido R, Forbes JM, Thomas MC, Thallas V, Dean RG, Burns WC, Tikellis C, Ritchie RH, Twigg SM, Cooper ME, Burrell LM. A breaker of advanced glycation end products attenuates diabetes-induced myocardial structural changes. *Circ Res.* 2003; 92(7):785-792.

Chen HS, Jia J, Su HF, Lin HD, Chen JW, Lin SJ, Yang JY, Lai HC, Mestril R, Wang PH.Downregulation of the constitutively expressed Hsc70 in diabetic myocardium is mediated by insulin deficiency. *J Endocrinol.* 2006; 190(2):433-440.

Chen HS, Shan YX, Yang TL, Lin HD, Chen JW, Lin SJ, Wang PH. Insulin Deficiency Downregulated Heat Shock Protein 60 and IGF-1 Receptor Signaling in Diabetic Myocardium. *Diabetes* 2005; 54: 175–181.

Cheng TH, Shih NL, Chen CH, Lin H, Liu JC, Chao HH, Liou JY, Chen YL, Tsai HW, Chen YS, Cheng CF, Chen JJ. Role of mitogen-activated protein kinase pathway in reactive oxygen species-mediated endothelin-1-induced beta-myosin heavy chain gene expression and cardiomyocyte hypertrophy. *J Biomed Sci.* 2005; 12(1):123-133.

Chu B, Soncin F, Price BD, Stevenson MA, Calderwood SK Sequential phosphorylation by mitogen-activated protein kinase and glycogen synthase kinase 3 represses transcriptional activation by heat shock factor-1. *J Biol Chem.* 1996; 271(48):30847-30857.

Chung J, Nguyen AK, Henstridge DC, Holmes AG, Chan MH, Mesa JL, Lancaster GI, Southgate RJ, Bruce CR, Duffy SJ, Horvath I, Mestril R, Watt MJ, Hooper PL, Kingwell BA, Vigh L, Hevener A, Febbraio MA. HSP72 protects against obesity-induced insulin resistance. *Proc Natl Acad Sci U S A.* 2008; 105(5):1739-1744.

Cortright RN, Zheng D, Jones JP, Fluckey JD, DiCarlo SE, Grujic D, Lowell BB, Dohm GL. Regulation of skeletal muscle UCP-2 and UCP-3 gene expression by exercise and denervation. *Am J Physiol.* 1999; 276:E217-221.

Currie, R. W., M. Karmazyn, M. Kloc, and K. Mailer. Heat-shock response is associated with enhanced postischemic ventricular recovery. *Circ. Res.* 1988; 63:543-549.

Deane EE, Kelly SP, Lo CK, Woo NY. Effects of GH, prolactin and cortisol on hepatic heat shock protein 70 expression in a marine teleost *Sparus sarba. J Endocrinol.* 1999; 161(3):413-21.

Deane EE, Woo NY. Growth hormone increases hsc70/hsp70 expression and protects against apoptosis in whole blood preparations from silver sea bream. *Ann N Y Acad Sci.* 2005; 1040:288-292.

Depre C, Young ME, Ying J, Ahuja HS, Han Q, Garza N, Davies PJ, Taegtmeyer H. Streptozotocin-induced changes in cardiac gene expression in the absence of severe contractile dysfunction. *J Mol Cell Cardiol.* 2000; 32(6):985-96.

Dillmann WH. Diabetes mellitus induces changes in cardiac myosin of the rat. *Diabetes.* 1980; 29(7):579-82.

Dreizen P. Structure and function of the myofibrillar contractile proteins. Annu Rev Med. 1971; 22:365-390.

Dudzinska W, Hlynczak AJ. Purine nucleotides and their metabolites in erythrocytes of streptozotocin diabetic rats. *Diabetes Metab.* 2004; 30(6):5575-67.

Echtay KS, Winkler E, Frischmuth K, Klingenberg M. Uncoupling proteins 2 and 3 are highly active H(+) transporters and highly nucleotide sensitive when activated by coenzyme Q (ubiquinone). *Proc Natl Acad Sci U S A.* 2001; 98(4):1416-1421.

Ekladous D, Mehdi MZ, Costa M, Srivastava AK, Chiasson JL, Coderre L. Tissue- and fibre-specific modifications of insulin-signalling molecules in cardiac and skeletal muscle of diabetic rats. *Clin Exp Pharmacol Physiol.* 2008; 35(8):971-978.

Fernandez S, Garcia-Garcia M, Torres-Aleman I. Modulation by insulin-like growth factor I of the phosphatase PTEN in astrocytes. Biochim Biophys Acta. 2008: 1783(5):803-812.

Garber AJ. The impact of streptozotocin-induced diabetes mellitus on cyclic nucleotide regulation of skeletal muscle amino acid metabolism in the rat. *Clin Invest.* 1980; 65(2):478-487.

Gerber LK, Aronow BJ, Matlib MA. Activation of a novel long-chain free fatty acid generation and export system in mitochondria of diabetic rat hearts. *Am J Physiol Cell Physiol.* 2006; 291:C1198–C1207.

Gomez-Cabrera MC, Domenech E, Viña J. Moderate exercise is an antioxidant: upregulation of antioxidant genes by training. *Free Radic Biol Med.* 2008; 44(2):126-131.

Gottlob K, Majewski N, Kennedy S, Kandel E, Robey RB, Hay N. Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. *Genes Dev.* 2001; 15(11):1406-1418.

Guo Z, Xia Z, Jiang J, McNeill JH. Downregulation of NADPH oxidase, antioxidant enzymes, and inflammatory markers in the heart of streptozotocin-induced diabetic rats by N-acetyl-L-cysteine. *Am J Physiol Heart Circ Physiol.* 2007; 292(4):H1728-17236.

Gupta S, Purcell NH, Lin A, Sen S. Activation of nuclear factor-B is necessary for myotrophin-induced cardiac hypertrophy. *J Cell Biol.* 2002; 159(6):1019-1028.

Gurusamya N, Watanabea K, Maa M, Prakasha P, Hirabayashia K, Zhangb S, Muslinb AJ, Kodamac M, Aizawac Y. Glycogen synthase kinase 3b together with 14-3-3 protein regulates diabetic cardiomyopathy: Effect of losartan and tempol *FEBS Letters* 2006; 580:1932–1940.

Gustafsson H, Soderdahl T, Jonsson G, Bratteng JO, Forsby A. Insulin-Like Growth Factor Type 1 Prevents Hyperglycemia-Induced Uncoupling Protein 3 Down-Regulation and Oxidative Stress. *J Neurosci Res.* 2004a; 77:285–291.

Gustafsson H, Tamm C, Forsby A. Signalling pathways for insulin-like growth factor type 1-mediated expression of uncoupling protein 3. *J Neurochem.* 2004b; 88(2):462-468.

Han HJ, Park SH. Alteration of the gene and protein levels of insulin-like growth factors in streptozotocin-induced diabetic male rats. *J Vet Med Sci.* 2006; 68(5):413-419.

He B, Meng YH, Mivechi NF. Glycogen synthase kinase 3beta and extracellular signal-regulated kinase inactivate heat shock transcription factor 1 by facilitating the disappearance of transcriptionally active granules after heat shock. *Mol Cell Biol.* 1998; 18(11):6624-6633.

Hidaka S, Kakuma T, Yoshimatsu H, Sakino H, Fukuchi S, Sakata T. Streptozotocin treatment upregulates uncoupling protein 3 expression in the rat heart. *Diabetes.* 1999; 48(2):430-435.

Hill JA, Olson EN. Activated glycogen synthase-3 beta suppresses cardiac hypertrophy in vivo. *Proc Natl Acad Sci U S A.* 2002; 99:907-912.

Hirotani S, Zhai P, Tomita H, Galeotti J, Marquez JP, Gao S, Hong C, Yatani A, Avila J, Sadoshima J. Inhibition of glycogen synthase kinase 3beta during heart failure is protective. *Circ Res.* 2007; 101(11):1164-1174.

Hollander J, Fiebig R, Gore M, Ookawara T, Ohno H, Ji LL. Superoxide dismutase gene expression is activated by a single bout of exercise in rat skeletal muscle. *Pflugers Arch.* 2001; 442:426–434.

Holzenberger M, Leroith D, Abel ED. IGF-1 Receptor Signaling is Required for Exercise-Induced Cardiac Hypertrophy. Mol Endocrinol. 2008

Hooper PL, Hooper PL. Inflammation, heat shock proteins, and type 2 diabetes. Cell Stress Chaperones. 2008 Aug 22.

Jaburek M, Varecha M, Gimeno RE, Dembski M, Jezek P, Zhang M, Burn P, Tartaglia LA, Garlid KD: Transport function and regulation of mitochondrial uncoupling proteins 2 and 3. *J Biol Chem* 274:26003–26007, 1999

Ji LL. Antioxidants and oxidative stress in exercise. *Proc Soc Exp Biol Med.* 1999; 222(3):283-292.

Kajstura J, Fiordaliso F, Andreoli AM, Chimenti LB, Medow S Limana MS Nadal-Ginard F, Anversa B, Leri P. IGF-1 overexpression inhibits the development of diabetic cardiomyopathy and angiotensin II mediated oxidative stress. *Diabetes.* 2001;50(6):1414–1424.

Kang JH, Chae YM, Park KK, Kim CH, Lee IS, Chang YC. Suppression of mesangial cell proliferation and extracellular matrix production in streptozotocin-induced diabetic rats by Sp1 decoy oligodeoxynucleotide in vitro and in vivo. *J Cell Biochem.* 2008; 103(2):663-674.

Kim E, Sohn S, Lee M, Jung J, Kineman RD, Park S. Differential responses of the growth hormone axis in two rat models of streptozotocin-induced insulinopenic diabetes. *J Endocrinol.* 2006; 188(2):263-270.

Kim J, Wende AR, Sena S, Theobald HA, Soto J, Sloan C, Wayment BE, Litwin SE, Holzenberger M, LeRoith D, Abel ED. Insulin-like growth factor I receptor signaling is required for exercise-induced cardiac hypertrophy. *Mol Endocrinol.* 2008; 22(11):2531-2543.

Knuefermann P, Chen P, Misra A, Shi SP, Abdellatif M, Sivasubramanian N. Myotrophin/V-1, a protein up-regulated in the failing human heart and in postnatal cerebellum, converts NFkappa B p50-p65 heterodimers to p50-p50 and p65-p65 homodimers. *J Biol Chem.* 2002; 277(26):23888-23897.

Konhilas JP, Watson PA, Maass A, Boucek DM, Horn T, Stauffer BL, Luckey SW, Rosenberg P, Leinwand LA. Exercise can prevent and reverse the severity of hypertrophic cardiomyopathy. *Circ Res.* 2006; 98(4):540-854.

Kozikowski AP, Sun H, Brognard J, Dennis PA. Novel PI analogues selectively block activation of the pro-survival serine/threonine kinase Akt. *J Am Chem Soc.* 2003; 125(5):1144-1145.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970; 227:680-685.

Lai HC, Liu TJ, Ting CT, Yang JY, Huang L, Wallace D, Kaiser P, Wang PH. Regulation of IGF-I receptor signaling in diabetic cardiac muscle: dysregulation of cytosolic and mitochondria HSP60. *Am J Physiol Endocrinol Metab.* 2007; 292(1):E292-297.

Lajoie C, Calderone A, Trudeau F, Lavoie N, Massicotte G, Gagnon S, Béliveau L. Exercise training attenuated the PKB and GSK-3 dephosphorylation in the myocardium of ZDF rats. *J Appl Physiol.* 2004; 96(5):1606-1612.

Li XS, Tanz RD, Chang KS. Effect of age and methacholine on the rate and coronary flow of isolated hearts of diabetic rats. *Br J Pharmacol.* 1989; 97(4):1209-1217.

Locke M, Tanguay RM, Klabunde RE, Ianuzzo CD. Enhanced postischemic myocardial recovery following exercise induction of HSP 72. *Am J Physiol.* 1995; 269:H320-325.

MacLellan JD, Gerrits MD, Gowing A, Smith PJS, Wheeler MB, Harper ME. Physiological increases in uncoupling protein 3 augment fatty acid oxidation and decrease reactive oxygen species production without uncoupling respiration in muscle cells. *Diabetes* 2005; 54:2343–2350.

Malone MA, Schocken DD, Hanna SK, Liang X, Malone JI. Effect of age and methacholine on the rate and coronary flow of isolated hearts of diabetic rats Diabetes-induced bradycardia is an intrinsic metabolic defect reversed by carnitine. *Metabolism.* 2007; 56(8):1118-1123.

McMullen JR, Shioi T, Huang WY, Zhang L, Tarnavski O, Bisping E, Schinke M, Kong S, Sherwood MC, Brown J, Riggi L, Kang PM, Izumo S. The insulin-like growth factor 1 receptor induces physiological heart growth via the phosphoinositide 3 kinase(p110alpha) pathway. *J Biol Chem.* 2004; 279(6):4782-4793.

Milne KJ, Noble EG. Exercise-induced elevation of HSP70 is intensity dependent. *J Appl Physiol.* 2002; 93(2):561-568.

Mivechi NF, Shi XY, Hahn GM. Stable overexpression of human HSF-1 in murine cells suggests activation rather than expression of HSF-1 to be the key regulatory step in the heat shock gene expression. *J Cell Biochem.* 1995; 59(2):266-280.

Mokhtar N, Rousseau-Migneron S, Tancrède G, Nadeau A. Partial correction of impaired creatine kinase activity in diabetic rat heart by physical training. *Metabolism.* 1992 Sep;41(9):1004-1008.

Mokhtar N, Rousseau-Migneron S, Tancrède G, Nadeau A. Physical training attenuates phosphocreatine and long-chain acyl-CoA alterations in diabetic rat heart. *J Appl Physiol.* 1993; 74(4):1785-1790.

Montero A, Munger KA, Khan RZ, Valdivielso JM, Morrow JD, Guasch A, Ziyadeh FN, Badr KF. F(2)-isoprostanes mediate high glucose-induced TGF-beta synthesis and glomerular proteinuria in experimental type I diabetes. *Kidney Int.* 2000; 58(5):1963-1972.

Moore RD, Munford JW, Pillsworth TJ Jr. Effects of streptozotocin diabetes and fasting on intracellular sodium and adenosine triphosphate in rat soleus muscle. J Physiol. 1983; 338:277-294.

Mori S, Gibson G, McTiernan CF. Differential Expression of MMPs and TIMPs in Moderate and Severe Heart Failure in a Transgenic Model. *J Cardiac Failure*. 2006; 12 (4): 314-325.

Morimoto R.I., Jurivich DA, Kroeger PE, Mathur SK, Murphy SP, Nakai A, Sarge K, Abravaya K, Sistonen LT. Regulation of heat shock gene transcription by a family of heat shock factors. *In*: The biology of heat shock proteins and molecular chaperones (ed. R.I. Morimoto, A. Tissieres and C. Georgopoulos). 1994; Cold Spring Harbor Laboratory Press, Cold Spring: 417-456.

Mukherjee R, Bruce JA, McClister DM Jr., Allen CM, Sweterlitsch SE, Saul JP. Time-dependent changes in myocardial structure following discrete injury in mice deficient of matrix metalloproteinase-3. *J Mol Cell Cardiol*. 2005; 39 259–268.

Nagai M, Kamide K, Rakugi H, Takiuchi S, Imai M, Kida I, Matsukawa N, Higaki J, Ogihara T. Role of endothelin-1 induced by insulin in the regulation of vascular cell growth. *Am J Hypertens*. 2003; 16(3):223-228.

Nahrendorf M, Frantz S, Hu K, von zur Mühlen C, Tomaszewski M, Scheuermann H, Kaiser R, Jazbutyte V, Beer S, Bauer W, Neubauer S, Ertl G, Allolio B, Callies F. Effect of testosterone on post-myocardial infarction remodeling and function. *Cardiovasc Res*. 2003; 57(2):370-378.

Naito Z, Takashi E, Xu G, Ishiwata T, Teduka K, Yokoyama M, Yamada N, Sugisaki Y, Asano G. Different influences of hyperglycemic duration on phosphorylated extracellular signal-regulated kinase 1/2 in rat heart. *Exp Mol Pathol*. 2003; 74(1):23-32.

Najemnikova E, Rodgers CD, Locke M. Altered heat stress response following streptozotocin-induced diabetes. *Cell Stress Chaperones*. 2007; 12(4):342-352.

O'Brien RJ, Loke I, Davies JE, Squire IB, Ng LL. Myotrophin in Human Heart Failure. *J Am Col Cardiol*. 2003; 42(4):719-725.

Palfi A, Toth A, Hanto K, Deres P, Szabados E, Szereday Z, Kulcsar G, Kalai T, Hideg K, Gallyas F Jr, Sumegi B, Toth K, Halmosi R. PARP inhibition prevents postinfarction myocardial remodeling and heart failure via the protein kinase C/glycogen synthase kinase-3beta pathway. J Mol Cell Cardiol. 2006; 41(1):149-159.

Palmer AM, Gopaul N, Dhir S, Thomas CR, Poston L, Tribe RM. Endothelial dysfunction in streptozotocin-diabetic rats is not reversed by dietary probucol or simvastatin supplementation. *Diabetologia*. 1998; 41(2):157-164.

Paulson DJ, Gupta M, Zak R, Zhao J. Effects of exercise training and diabetes on cardiac myosin heavy chain composition. *Mol Cell Biochem.* 1992; 117:175-179.

Paulson DJ, Mathews R, Bowman J, Zhao J. Metabolic effects of treadmill exercise training on the diabetic heart. *J Appl Physiol.* 1992; 73(1):265-271.

Puduvalli VK, Sampath D, Bruner JM, Nangia J, Xu R, Kyritsis AP. TRAIL-induced apoptosis in gliomas is enhanced by Akt-inhibition and is independent of JNK activation. *Apoptosis.* 2005; 10(1):233-243.

Rajabi M, Kassiotis C, Razeghi P, Taegtmeyer H. Return to the fetal gene program protects the stressed heart: a strong hypothesis. *Heart Fail Rev.* 2007; 12(3-4):331-343.

Rockman MV, Hahn MW, Soranzo N, Loisel DA, Goldstein DB, Wray GA. Positive selection on *MMP3* regulation has shaped heart disease risk. *Cur Biol.* 2004; 14:1531–1539

Rundell VL, Geenen DL, Buttrick PM, de Tombe PP. Depressed cardiac tension cost in experimental diabetes is due to altered myosin heavy chain isoform expression. *Am J Physiol Heart Circ Physiol.* 2004; 287(1):H408-413.

S, Sherwood MC, Brown J, Riggi L, Kang PM, Izumo S. The insulin-like growth factor 1 receptor induces physiological heart growth via the phosphoinositide 3-kinase (p110alpha) pathway. *J Biol Chem.* 2004; 279(6):4782-4793.

Sack MN, Disch DL, Rockman HA, Kelly DP. A role for Sp and nuclear receptor transcription factors in a cardiac hypertrophic growth program. *Proc Natl Acad Sci U S A.* 1997; 94(12):6438-6443.

Salminen A, Vihko V. Lipid peroxidation in exercise myopathy. *Exp Mol Pathol.* 1983; 38:380–388.

Samelman TR. Heat shock protein expression is increased in cardiac and skeletal muscles of Fischer 344 rats after endurance training. *Exp Physiol.* 2000; 85(1):92-102.

Sansilvestri-Morel P, Rupin A, Jullien ND, Lembrez N, Mestries-Dubois P, Fabiani JN, Verbeuren TJ. Decreased production of collagen type III in cultured smooth muscle cells from varicose vein patients is due to a degradation by MMPs: possible implication of MMP-3. *J Vasc Res.* 2005; 42:388–398.

Satomi-Kobayashi S, Kawashima S, Sakoda T, Ueyama T, Hirase T, Kawai M, Toh R, Iwai K, Yokoyama M. Glycogen Synthase Kinase-3 is Involved in the Process of Myocardial Hypertrophy Stimulated by Insulin-Like Growth Factor-1. *Circ J* 2004; 68: 247 –253.

Schrauwen P, Hoeks J, Hesselink MK. Putative function and physiological relevance of the mitochondrial uncoupling protein-3: involvement in fatty acid metabolism? *Prog Lipid Res.* 2006; 45(1):17-41.

Schreck R, Rieber P, Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J.* 1991; 10(8):2247-2258.

Searls YM, Smirnova IV, Fegley BR, Stehno-Bittel L. Exercise attenuates diabetes-induced ultrastructural changes in rat cardiac tissue. *Med Sci Sports Exerc.* 2004; 36(11):1863-1870.

Shan YX, Yang TL, Mestril R, Wang PH. Hsp10 and Hsp60 Suppress Ubiquitination of Insulin-like Growth Factor-1 Receptor and Augment Insulin-like Growth Factor-1 Receptor Signaling in Cardiac Muscle. *J Biol Chem.* 2003;278(46): 45492–45498.

Singleton JR, Dixit VM, Feldman EL. Type I insulin-like growth factor receptor activation regulates apoptotic proteins. *J Biol Chem.* 1996; 271, 31791–31794.

Song P, Wu Y, Xu J, Xie Z, Dong Y, Zhang M, Zou MH. Reactive nitrogen species induced by hyperglycemia suppresses Akt signaling and triggers apoptosis by upregulating phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) in an LKB1-dependent manner. *Circulation.* 2007; 116(14):1585-1595.

Spinale FG, Coker ML, Bond BR, Zellner JL. Myocardial matrix degradation and metalloproteinase activation in the failing heart: a potential therapeutic target. *Cardio Res.* 2000; 46 225–238.

Strniskova M, Barancik M, Neckar J, Ravingerova T. Mitogen-activated protein kinases in the acute diabetic myocardium. Mol Cell Biochem. 2003 Jul;249(1-2):59-65.

Tayebjee MH, Lip GYH, MacFadyen RJ. What role do extracellular matrix changes contribute to the cardiovascular disease burden of diabetes mellitus? *Diabet Med.* 2005; 22: 1628–1635.

Thomas DP, Zimmerman SD, Hansen TR, Martin DT, McCormick RJ. Collagen gene expression in rat left ventricle: interactive effect of age and exercise training. *J Appl Physiol.* 2000; 89(4):1462-1468.

Todaka K, Wang J, Yi GH, Knecht M, Stennett R, Packer M, Burkhoff D. Impact of exercise training on ventricular properties in a canine model of congestive heart failure. *Am J Physiol.* 1997 Mar;272(3 Pt 2):H1382-1390.

Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Biotechnology.* 1979; 24:145-149.

Tschöpe C, Walther T, Königer J, Spillmann F, Westermann D, Escher F, Pauschinger M, Pesquero JB, Bader M, Schultheiss HP, Noutsias M. Prevention of cardiac fibrosis and left ventricular dysfunction in diabetic cardiomyopathy in rats by transgenic expression of the human tissue kallikrein gene. *FASEB J.* 2004; 18(7):828-835.

Tseng AS, Engel FB, Keating MT. The GSK-3 inhibitor BIO promotes proliferation in mammalian cardiomyocytes. *Chem Biol.* 2006; 13: 957-963.

Westermann D, Rutschow S, Jäger S, Linderer A, Anker S, Riad A, Unger T, Schultheiss HP, Pauschinger M, Tschope C. Contributions of inflammation and cardiac matrix metalloproteinase activity to cardiac failure in diabetic cardiomyopathy. *Diabetes* 2007; 56(3):641-646.

Wilkins BJ, Dai YS, Bueno OF, Parsons SA, Xu J, Plank DM, Jones F, Kimball TR, Molkentin JD. Calcineurin/NFAT coupling participates in pathological, but not physiological, cardiac hypertrophy. *Circ Res.* 2004; 94(1):110-118.

Woodiwiss AJ, Kalk WJ, Norton GR. Habitual exercise attenuates myocardial stiffness in diabetes mellitus in rats. *Am J Physiol.* 1996; 271:H2126-2133.

Wu TC, Leu HB, Lin WT, Lin CP, Lin SJ, Chen JW. Plasma matrix metalloproteinase-3 level is an independent prognostic factor in stable coronary artery disease. *Eur J Clin Inves.* 2005; 35:537–545.

Xavier IJ, Mercier PA, McLoughlin CM, Ali A, Woodgett JR, Ovsenek N. Glycogen synthase kinase 3beta negatively regulates both DNA-binding and transcriptional activities of heat shock factor 1. *J Biol Chem.* 2000; 275(37):29147-29152.

Xia Z, Kuo KH, Nagareddy PR, Wang F, Guo Z, Guo T, Jiang J, McNeill JH. N-acetylcysteine attenuates PKCbeta2 overexpression and myocardial hypertrophy in streptozotocin-induced diabetic rats. *Cardiovasc Res.* 2007; 73(4):770-782.

Zhong WD, Zeng GQ, Hu JB, Cai YB, Liu JK, Huang SH, Chen MS, Wei HA. Stimulation of neuropeptide Y on heat shock protein expression in renal vascular smooth muscle and the inhibition thereon by losartan. *Zhonghua Yi Xue Za Zhi.* 2003; 83(6):515-517.

Zuo J, Rungger D, Voellmy R. Multiple layers of regulation of human heat shock transcription factor 1. *Mol Cell Biol.* 1995; 15(8):4319-4330.

CHAPTER 6

Exercise does not prevent dysfunction or restore dysfunction in the streptozotocin-induced diabetic myocardium

6.1 INTRODUCTION

The myocardium features an array of contractile and structural proteins aligned externally circumferential and internally furrowed, creating internal chambers that accommodate a volume of blood proportional to the needs of the body and proportional to the functional capacity and efficiency of the heart. The myocardium expresses proteins that are the housing structure for a specific volume of blood and the contractile apparatus that propels the blood into circulation. The quantity of muscle relative to the chamber volume and shape is tightly controlled and has significant implications on cardiac compliance, force generation and blood volume displacement. Cardiomyopathy alters protein expression, changing the architectural design, material compliance and disrupting normal function. Echocardiographic evidence reveals that diabetic cardiomyopathy involves changes to both systolic and diastolic contraction (Hoit et al., 1999), ventricular filling (Wichi et al., 2007), myocardial performance (Borges et al., 2006), wall thickness (Fiordaliso et al., 2004), internal chamber dimensions (Nemoto et al., 2006) and myocardial growth (Wichi et al., 2007).

The pathophysiology of diabetes mellitus (DM)-induced cardiomyopathy ensues from elevated blood glucose and lost insulin signaling, leading to altered metabolism, disrupted intracellular signaling, and deranged structure and function of the heart. Indeed, the ability to control hyperglycemia is associated with improved intracellular signaling and reduced cardiovascular complications (Fang et al., 2004; Severson, 2004; reviewed by Olasnski, 2004 and Davidson, 2004).

Exercise improves cardiovascular physiology, metabolism and structure in normal and diabetic myocardia (reviewed by Li et al., 2003). Although, the exercise induced

improvement is generally associated with improving risk factors and glucose control (reviewed by Saraceni and Broderick, 2007), exercise also improves myocardial performance independent of reduced blood glucose in streptozotocin- (STZ) induced DM (Loganathan et al., 2007). Hence, exercise may act as an insulin surrogate to restore downstream signaling of protein expression independent of a role in glucose disposal. The importance of uncovering the capacity of exercise to normalize myocardial structure and function in DM is emphasized by the frequent finding that patients with type I DM obtain normalized levels of glucose and often show better lipid profiles than Non-DM individuals (Wadwa et al., 2005), and yet all-cause mortality remains 3-4 times greater (Soedamah-Muthu et al., 2006), with cardiomyopathy accounting for the majority cause of death. Exercise may instigate intracellular events analogous to those present within the normal myocardium, and may thereby help preserve the myocardium.

Therefore, the purpose of the present study was to determine whether exercise can prevent or reverse the altered myocardial structure and function normally associated with DM. It was hypothesized that the cardiovascular benefits normally conferred by exercise would be extended to protecting and restoring the structure and function in the diabetic myocardium.

6.2 METHODS AND MATERIALS

Animal Characteristics and Experimental Groups. All procedures were approved by the Animal Care Subcommittee at The University of Western Ontario. Sixty male Sprague-Dawley rats (~220-250g; Charles River Laboratories, Quebec, Canada) were maintained on a 12-hour dark/light cycle, housed at 20 ±1°C, 50% relative humidity, and

provided with standard rat chow and water ad libitum. Animals were divided into DM and non-DM groups. Animals from each group (n=10 per group) were assigned to 1 of 3 treatments: (1) sedentary (SED); (2) 4-weeks sedentary followed by 4-weeks of exercise (EX-4); or (3) 8-weeks of exercise (EX-8).

DM Induction. Animals received a low-dose injection of STZ (20mg/kg; i.p.; Sigma-Aldrich) within 5 minutes of dissolving in citrate buffer (0.1 M, pH 4.5) on 5 consecutive days. Non-DM animals were injected with citrate buffer only. DM was confirmed by measuring a blood glucose level >15 mmol. Whole body animal weights were monitored weekly and blood glucose values were monitored weekly via blood draw from the saphenous vein using the One Touch Ultra 2 Blood Glucose Monitoring System (Lifescan Canada Ltd, Burnaby, BC, Canada) and One Touch test strips (Lifescan Canada; range = 0–600 mg/dl).

Exercise Training. Rats were familiarized to treadmill running on 2 occasions with brief, 10-minute exposures at 15m/min (5 & 3 days prior to acute run). At the beginning of the acute training, rats were run at progressing intensities with unchanging duration and frequency (one hour/day, 5 days/week) starting at 19m/min for 5 days (week 1), followed by 23m/min for 5 days (week 2), 27m/min for 5 days (week 3), and continued thereafter at a running speed of 27m/min (the final intensity corresponding with a work rate of ~75% VO_{2max}). Continuous running during the exercise session was encouraged by an electronically activated puff of compressed air blowing at right angles onto the rat haunches. This air jet served to act as a stimulus to encourage running and to act as a warning that the end of the belt was approaching. All animals were able to adjust to increasing intensities and completed the training protocol.

Echocardiography. Echocardiography was performed under isoflurane anesthesia (2–5% with 1 L O2/min). Transthoracic echocardiography was performed in rats using an echocardiographic system equipped with a 17.5-MHz transducer (Vevo 770, VisualSonics, Toronto, ON, Canada). Values were measured over at least four consecutive cardiac cycles and performed by the advanced cardiac analysis software on the Vevo 770. Posterior and anterior wall thickness (PWT, AWT), end-diastolic and end-systolic left ventricular (LV) diameters (LVIDd and LVIDs) and LV ejection time were acquired by a two-dimensional short-axis view of the LV at the level of the papillary muscle (M-mode). A modified relative wall thickness (RWT) was calculated by dividing the sum of PWT and AWT by LVID. A long axis view at the level of the aortic valve was obtained to record B-mode tracings and used to calculate LV flow values. LV fractional shortening (FS%) was calculated as the ratio of (LVIDd–LVIDs)/LVIDd. Cardiac output was determined by the product of stroke volume (calculated from the posterior wall Doppler blood flow measurements of the aorta velocity time integral, and the B-Mode acquisition of the ventricular outflow tract length) and heart rate. Similarly, ejection fraction (EF%) was determined using calculated volumes based on the LVIDd and LVIDs. Posterior wall circumferential fibre shortening velocity (V_{cf}) was calculated by dividing the difference between end-diastolic and end-systolic internal diameters by the ejection time. Doppler mode was used to acquire and calculate values for isovolumic contraction and relaxation times, and LV filling velocities reflected by E-wave and A-wave velocities. LV mass was calculated by estimating total LV volume based on wall thicknesses and internal diameter in diastole, multiplied by the density constant (1.053 g/ml) of rat heart tissue (Vinnakota and Bassingthwaighte, 1994).

Lean Mass and Tibial Length. Lean mass was acquired via whole body CT scan and was assumed to correspond to a density of 1.06 g/cm^3 reflected on a plotted histogram. Likewise, tibial length was acquired by CT scan by estimating the distance between the medial malleolus and the tibial plateau using an electronic tracer on a full scale image of the skeleton.

Statistical Analysis. A two-way ANOVA (SPSS) was performed using DM and exercise as conditions for each group. A Bonferonni post hoc was used to determine significant differences between groups. Differences between groups were considered statistically significant at a level of $P < 0.05$. Data are expressed as the mean ± SEM.

6.3 RESULTS

Blood Glucose. Initial glucose values (Figure 6.1) for Non-DM and DM animals were not significantly different (6.45 ± 0.17 mmol/L and 6.92 ± 0.30 mmol/L, respectively). Four weeks following DM induction, a significant difference ($P < 0.05$) was found between Non-DM and DM animals (5.6 ± 0.13 mmol/L and 24.2 ± 1.14 mmol/L, respectively). Final glucose values (week 8) also showed significant difference ($P < 0.05$) between Non-DM and DM animals (6.9 ± 0.34 mmol/L and 29.5 ± 0.83 mmol/L, respectively). No differences between exercised and non-exercised animals within either group were found. However, for DM animals, the final glucose value was greater than the glucose value obtained at week 4, suggesting increased DM severity over time.

Animal Characteristics. Initial values for body mass (Figure 6.2*A*) did not show a difference between Non-DM and DM animals (333 ± 3.0 g vs. 331 ± 2.5 g, respectively).

Thereafter, Non-DM animals grew at a faster rate than DM animals (4 wks: 461 ± 9.8 g vs. 373 ± 6.1 g, respectively; 8 weeks: 521 ± 7.4 g vs. 362 ± 7.2 g, respectively). Final values for lean mass (Figure 6.2*B*) were significantly greater ($P < 0.05$) in Non-DM animals compared with DM animals (414 ± 6.1 g vs. 289 ± 6.8 g, respectively). When standardized to tibial length (Yin et al., 1982), LV mass was significantly greater ($P < 0.05$) in all Non-DM animals compared with DM animals (0.27 ± 0.004 g/cm vs. 0.22 ± 0.005 g/cm, respectively). LV mass was not significantly altered by exercise (Figure 6.4).

Myocardial Structure. PWT and AWT (Table 6.1) were significantly greater ($P < 0.05$) in Non-DM animals, and a significantly greater ($P < 0.05$) RWT was observed in Non-DM animals compared to DM animals (0.42 ± 0.01 vs. 0.34 ± 0.01, respectively), indicating reduced myocardial tissue relative to LV chamber volume (shown in Figure 6.5).

Systolic Function. DM animals showed a significant bradycardia, regardless of exercise. Exercise in Non-DM animals did not show a significant change in heart rate from DM controls (Figure 6.3). FS% was significantly greater ($P < 0.05$) in Non-DM myocardia (0.46 ± 0.01 vs. 0.40 ± 0.01, respectively) and was not normalized by exercise (Figure 6.6). Similarly, the EF% was significantly reduced ($P < 0.05$) in DM animals (0.75 ± 0.01 vs. 0.68 ± 0.01, respectively and exercise had no effect on this (Figure 6.7). Isovolumic contraction time (time between mitral valve closure and aortic valve opening) was significantly lower ($P < 0.05$) in Non-DM compared to DM myocarida (23.4 ± 0.74 ms vs. 29.8 ± 0.63 ms, respectively) and is shown in Figure 6.8. Likewise, the measure of myocardial contractility (V_{cf}) was significantly reduced ($P < 0.05$) in all DM animals (60.8 ± 2.3 mm/s vs. 43.4 ± 1.5 mm/s, respectively). When expressed in absolute values, Non-DM myocardial demonstrated significantly greater ($P < 0.05$) cardiac output

compared with DM myocardia (96.9 ± 3.14 ml/min vs. 79.6 ± 2.03 ml/min, respectively). When standardized to lean mass, cardiac output was significantly greater in the diabetic myocardia and exercise did not influence this difference (Figures 6.9*A* and 6.9*B*).

Diastolic Function. Ventricular filling is achieved by the early phase (E-wave) passive filling and the late contribution of atrial contraction (A-wave). Changes in the ratio between MVE (peak velocity of the E-wave at the mitral valve) and MVA (peak velocity of the A-wave at the mitral valve) reflect alterations in ventricular filling during diastole. As shown in Figure 6.10, normal myocardia demonstrated significantly lower MVE/MVA compared to DM animals (1.59 ± 0.05 mm/s vs. 1.76 ± 0.07 mm/s, respectively), suggesting a greater contribution to ventricular filling by the early phase in diabetic animals.

These results indicate that DM attenuated LV mass and reduced the percentage of mass responsible for a given volume of blood, and altered systolic and diastolic function determined by reduced FS%, EF%, increased isovolumic contraction time and MVE/MVA. Exercise was not able to alter these measures of structure and function.

Table 6.1. Structural Geometry From Echocardiography.

Group	AWT (mm)	PWT (mm)	ID (mm)	LV Vol (μl)
		Systole		
Control	3.01 ± 0.13	3.24 ± 0.04	4.32 ± 0.18	38.5 ± 10.5
Ex 4	2.90 ± 0.10	3.36 ± 0.08	4.58 ± 0.23	38.4 ± 5.5
Ex 8	2.94 ± 0.13	3.32 ± 0.12	5.06 ± 0.22	40.3 ± 7.7
DM	2.53 ± 0.12[a]	2.75 ± 0.10[a]	5.09 ± 0.12	43.0 ± 6.3
DM Ex 4	2.42 ± 0.07[a]	2.71 ± 0.09[a]	5.22 ± 0.15	31.6 ± 2.3
DM Ex 8	2.29 ± 0.08[a]	2.61 ± 0.09[a]	5.49 ± 0.20	31.7 ± 3.0
		Diastole		
Control	1.45 ± 0.07	2.09 ± 0.07	8.42 ± 0.12	388 ± 12
Ex 4	1.48 ± 0.06	2.06 ± 0.04	8.50 ± 0.18	396 ± 18
Ex 8	1.63 ± 0.05	2.08 ± 0.09	8.79 ± 0.11	426 ± 12
DM	1.25 ± 0.06[a]	1.70 ± 0.07[a]	8.81 ± 0.09[a]	427 ± 10[a]
DM Ex 4	1.23 ± 0.04[a]	1.74 ± 0.06[a]	8.61 ± 0.15[a]	407 ± 15[a]
DM Ex 8	1.28 ± 0.05[a]	1.68 ± 0.05[a]	8.93 ± 0.14[a]	441 ± 15[a]

Values are means ± SEM (n=10); AWT: anterior wall thickness; PWT: posterior wall thickness; ID: left ventricular internal diameter; LV Vol: left ventricular volume; [a]Significantly different ($P<0.05$) from Non-DM (two-way anova).

Table 6.2. Contractile velocity, stroke volume and diastolic LV volume-relative to heart diameter.

Group	V_{cf} (mm/s)	SV (μl)	Vol-to-Diameter (ml/mm)
Control	66.0 ± 3.3	301 ± 11	32.4 ± 0.8
Ex 4	60.1 ± 4.4	296 ± 14	32.8 ± 1.0
Ex 8	56.3 ± 4.0	301 ± 15	34.0 ± 0.7
DM	45.5 ± 2.1[a]	303 ± 5	36.3 ± 0.6[a]
DM Ex 4	42.9 ± 2.1[a]	275 ± 9	35.1 ± 1.1[a]
DM Ex 8	41.7 ± 3.4[a]	292 ± 8	37.0 ± 0.9[a]

Values are means ± SEM; n=10/group. [a]Significantly different ($P<0.05$) from Non-DM (Two-way anova). V_{cf}: velocity of circumferential fibre shortening; SV: stroke volume. Vol-to-Diameter: ratio of end-diastolic volume-to-short axis diameter of whole heart.

Figure 6.1. Blood glucose values measured prior to DM induction, at week 4 and during the final week of the experiments. Initial blood samples demonstrated no difference between animals assigned to Non-Dm and DM groups. Week 4 glucose values were significantly greater ($P < 0.05$) in DM animals and final glucose values (week 8) showed that DM animals obtained significantly greater ($P < 0.05$) blood glucose values compared to Non-DM animals and were also significantly greater ($P < 0.05$) than DM animals at week 4. Data expressed as mean ± SEM (n = 30). *Significantly greater ($P < 0.05$) than Non-DM animals and pre-injection values. †Significantly greater ($P < 0.05$) than values measured at all other time points.

Figure 6.1

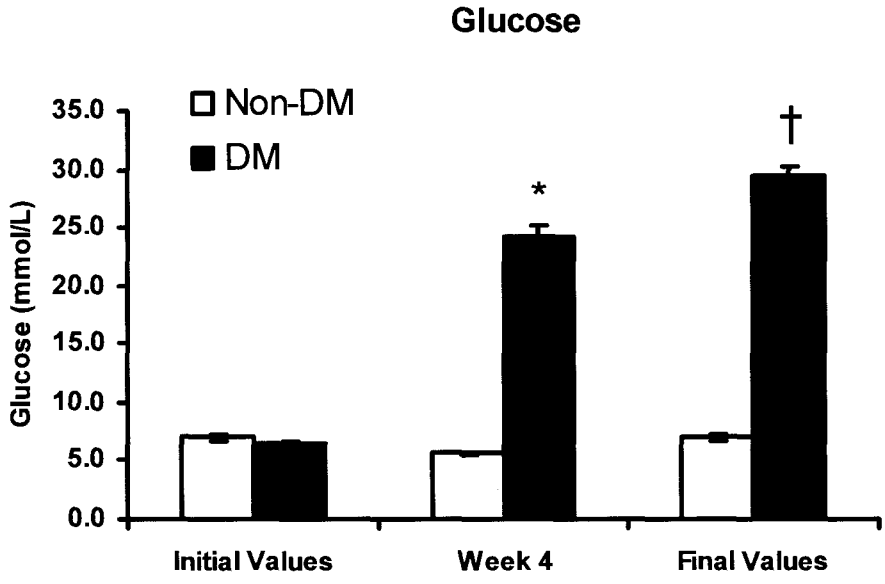

Glucose
Non-DM
DM
Glucose (mmol/L)
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
*
†
Initial Values
Week 4
Final Values

Figure 6.2. Total and lean animal body mass.

A: Total body mass.at week 0, week 4 and week 8 and final lean mass. While non-DM animals significantly increased body mass throughout the experimental period, DM animals did not increase body mass beyond 4 weeks of DM. Diabetic animals significantly increased ($P < 0.05$) body mass after 4 weeks and did not significantly increase its mass above week 4 values when measured at week 8. However, Non-DM animals demonstrated significantly increased body mass at each successive time point compared to DM animals. Data expressed as mean ± SEM (n = 30 per group). *Significantly greater ($P < 0.05$) than pre-treatment values. †Significantly greater ($P < 0.05$) than pre-treatment and DM animals. §Significantly greater ($P < 0.05$) than pre-treatment and DM animals.

B. Final lean mass values. Non-DM animals significantly increased ($P < 0.05$) lean mass after 8 weeks. Data expressed as mean ± SEM (n = 30). *Significantly greater ($P < 0.05$) than values obtained for DM animals.

Figure 6.2*A*

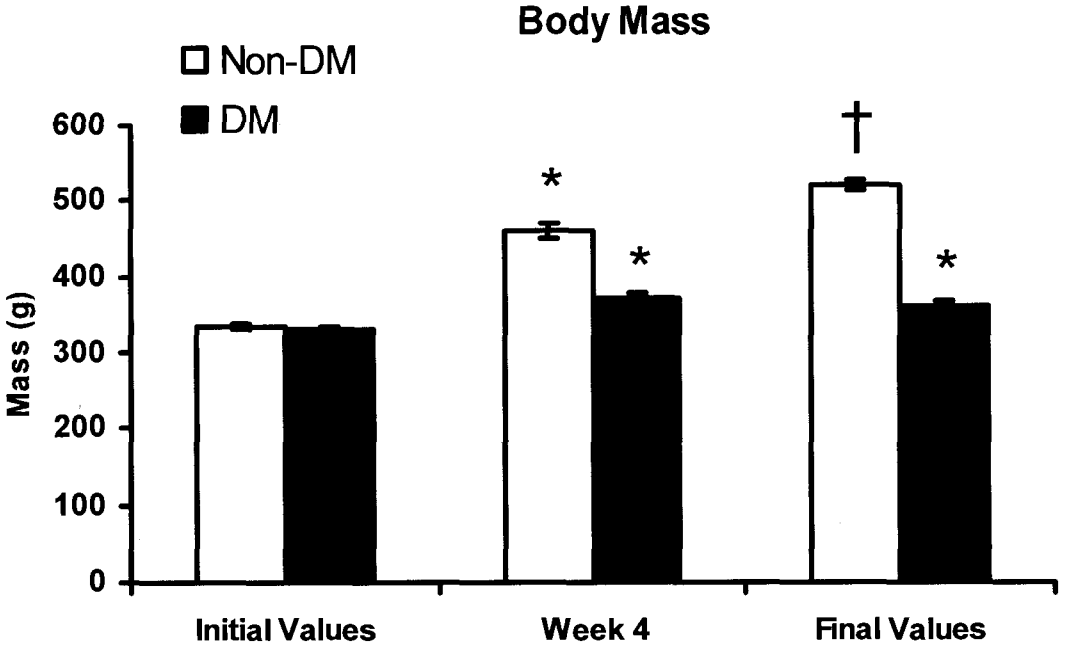


Figure 6.2*B*

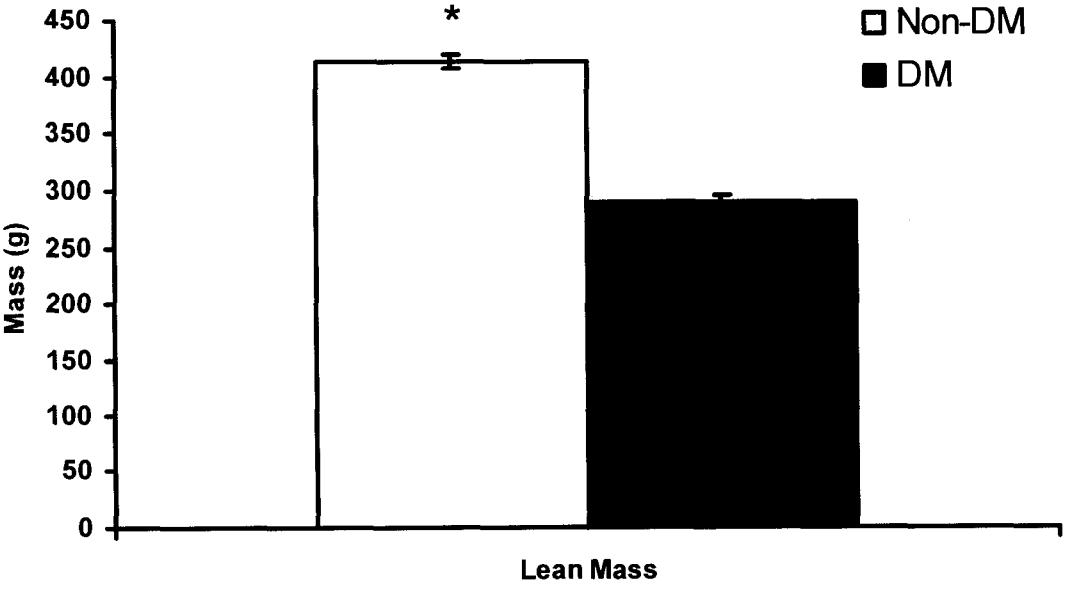


Figure 6.3. Altered heart rate in diabetic animals. DM resulted in significantly reduced ($P < 0.05$) resting heart rate compared to Non-DM animals. Exercise did not normalize heart rate in DM animals, nor did it produce a significant bradycardia in Non-DM animals. Data expressed as mean beats per minute (BPM) ± SEM (n = 10 per group). *Significantly less ($P < 0.05$) than values obtained for Non-DM animals.

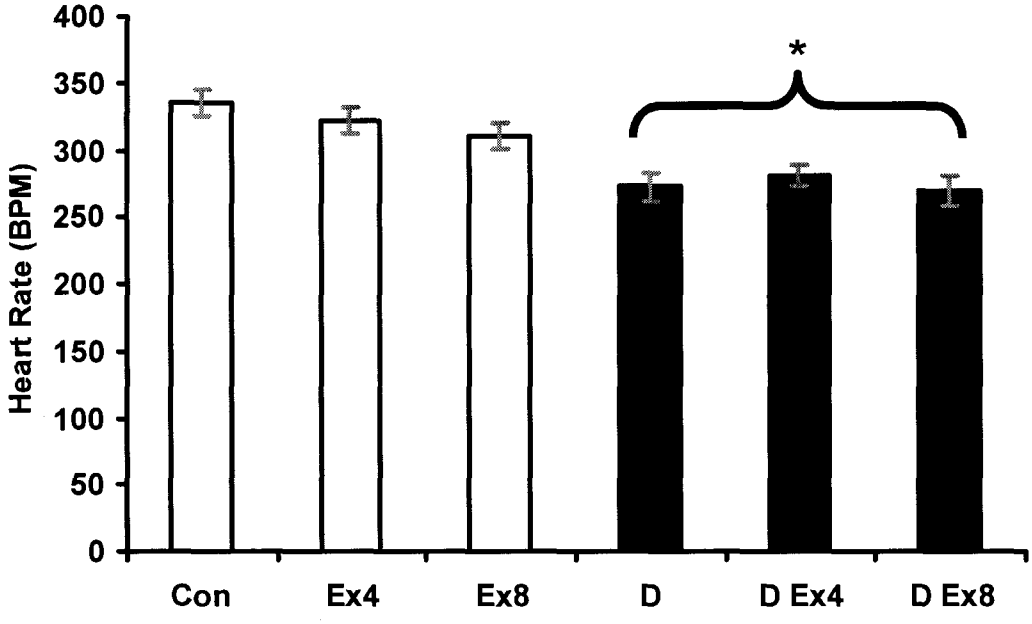

400
350
300
250
200
150
100
50
0
Heart Rate (BPM)
*
Con
Ex4
Ex8
D
D Ex4
D Ex8

Figure 6.4. Left ventricular (LV) mass standardized to tibial length. At the end of the experimental protocol, LV mass-to-tibial length was significantly greater ($P < 0.05$) in Non-DM animals compared to DM animals. Exercise did not normalize LV mass-to-tibial length. Data expressed as mean ± SEM (n = 30). *Significantly less ($P < 0.05$) than values obtained for Non-DM animals.

Figure 6.4

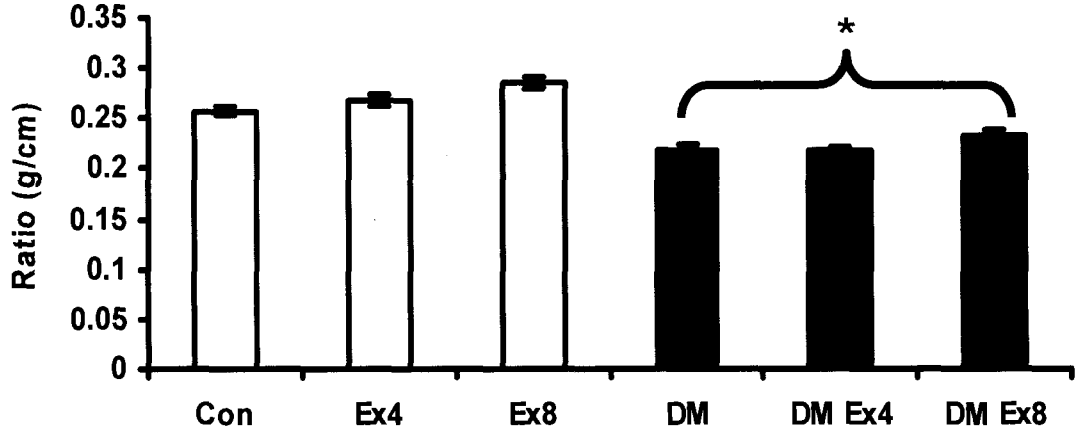


Figure 6.5. Relative wall thickness (RWT) measured at week 8. At the end of the experimental protocol, the proportion of wall thickness-to-internal diameter was significantly reduced ($P < 0.05$) in DM myocardia. Exercise was not able to increase wall thickness relative to internal diameter in DM animals. Data expressed as mean ± SEM (n = 30). *Significantly less ($P < 0.05$) than values obtained for Non-DM animals.

Figure 6.5

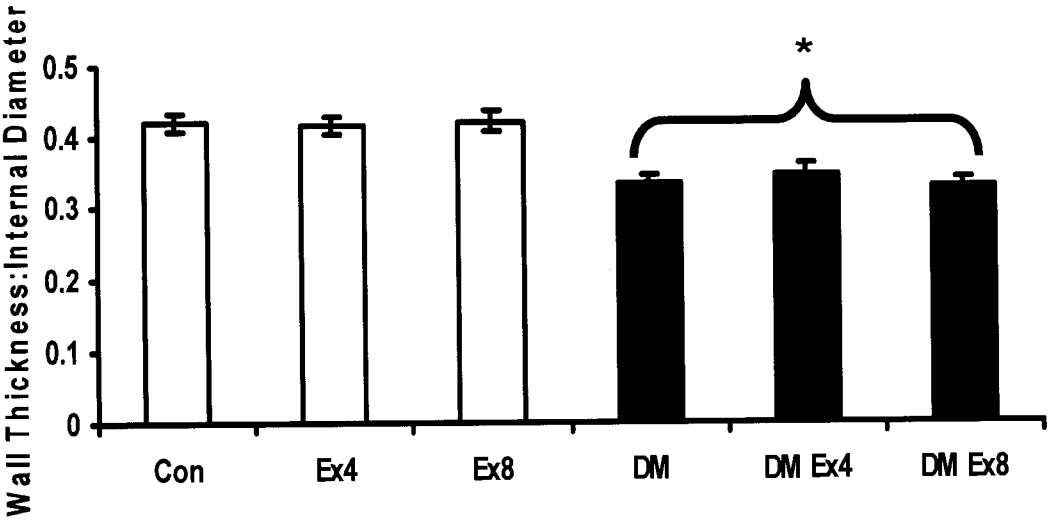

Wall Thickness:Internal Diameter
0.5
0.4
0.3
0.2
0.1
0
*
Con
Ex4
Ex8
DM
DM Ex4
DM Ex8

Figure 6.6. Left ventricular fractional shortenting (FS%) following exercise and/or DM derived from the fractional difference between LV internal diameter measured at systole and diastole. DM significantly reduced ($P < 0.05$) FS% in DM myocardia. Exercise was not able to alter the shortening percentage in DM animals. Data expressed as mean ± SEM (n = 30). *Significantly less ($P < 0.05$) than values obtained for Non-DM animals.

Figure 6.6

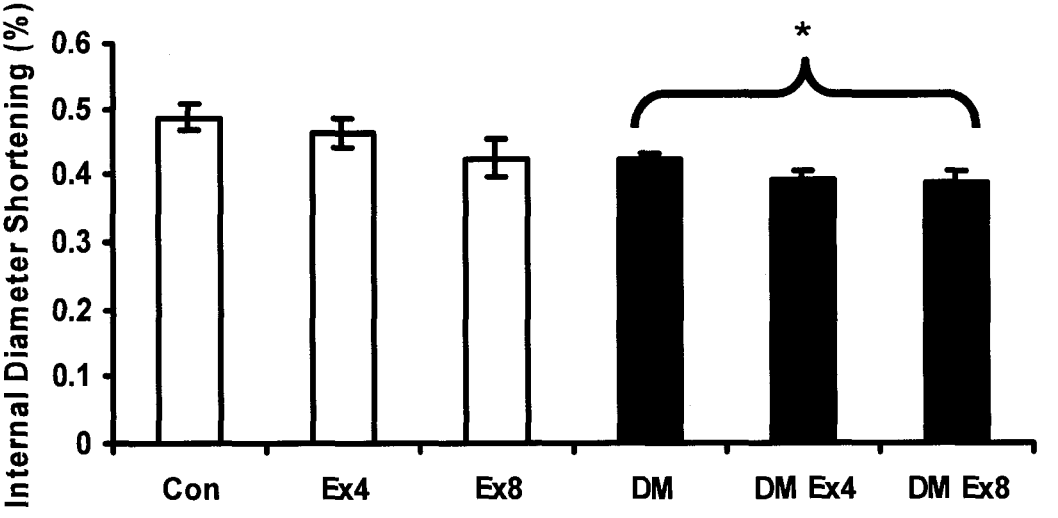

Internal Diameter Shortening (%)
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Con
Ex4
Ex8
DM
DM Ex4
DM Ex8

Figure 6.7. Left ventricular ejection fraction (EF%) following exercise and/or DM. EF% was determined by calculating (LV $volume_{systole}$ - LV $volume_{diastole}$)/ LV $volume_{systole}$. DM significantly reduced ($P < 0.05$) EF% in DM myocardia. Exercise was not able to alter the EF% to normal levels in DM animals. Data expressed as mean ± SEM (n = 30). *Significantly less ($P < 0.05$) than values obtained for Non-DM animals.

Figure 6.7

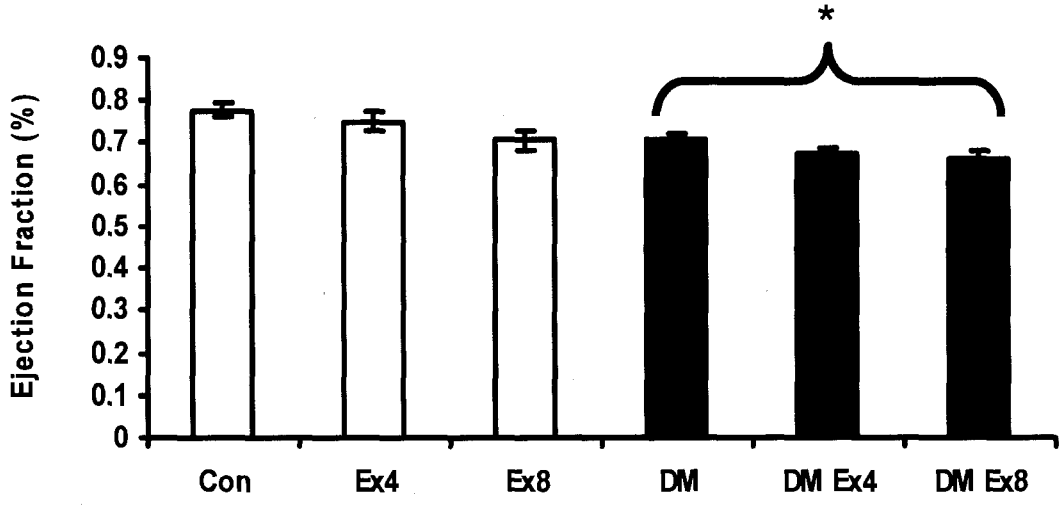

*
Ejection Fraction (%)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Con
Ex4
Ex8
DM
DM Ex4
DM Ex8

Figure 6.8. LV isovolumic contraction time (time interval between mitral valve closure and aortic valve opening) following exercise and/or DM. DM significantly elevated ($P < 0.05$) isovolumic contraction time in DM myocardia. Exercise was not able to restore or preserve the isovolumic contraction time to normal levels in DM animals. Data expressed as mean ± SEM (n = 30). *Significantly greater ($P < 0.05$) than values obtained for Non-DM animals.

Figure 6.8

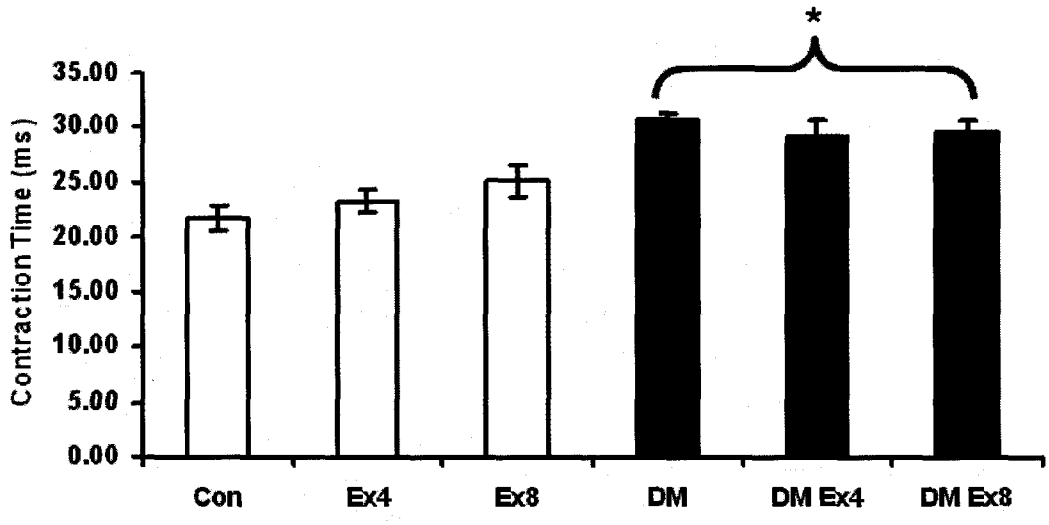

*
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Contraction Time (ms)
Con
Ex4
Ex8
DM
DM Ex4
DM Ex8

Figure 6.9 Cardiac output values for control, exercise and DM animals.

A. Absolute cardiac output values obtained at 8 weeks. Cardiac output was significantly diminished ($P < 0.05$) in DM animals. Exercise did not prevent reduced absolute values nor restore absolute values to normal levels. Data expressed as mean ± SEM (n = 30). *Significantly less ($P < 0.05$) than values obtained for Non-DM animals.

Figure 6.9*B*. Cardiac output standardized to lean mass (Output Ratio). DM significantly increased ($P < 0.05$) standardized cardiac output. Eight weeks of exercise did not alter values in Non-DM animals, but restored values to control levels in DM animals. Data expressed as mean ± SEM (n = 30). *Significantly greater ($P < 0.05$) than standardized cardiac output values in Non-DM animals.

Figure 6.9*A*

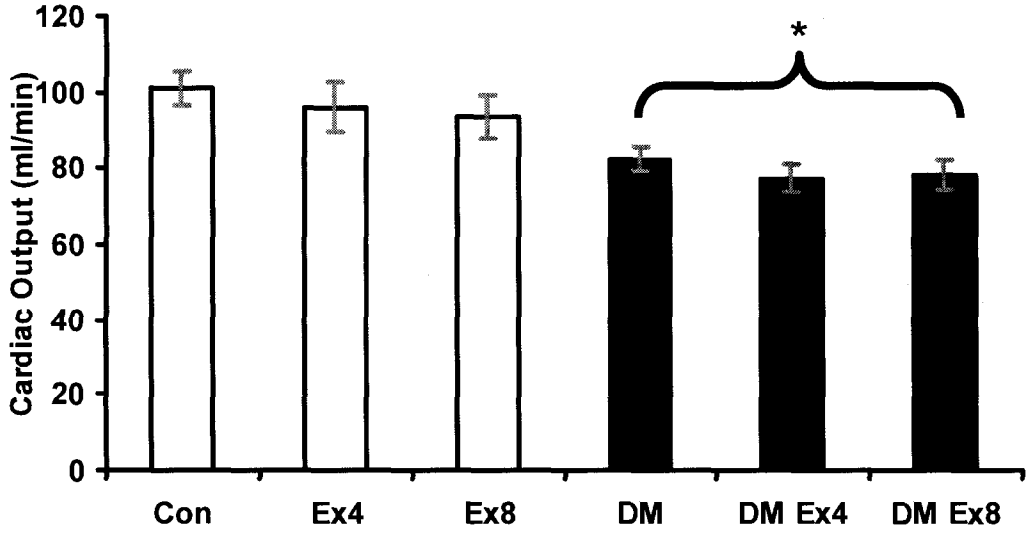


Figure 6.9*B*

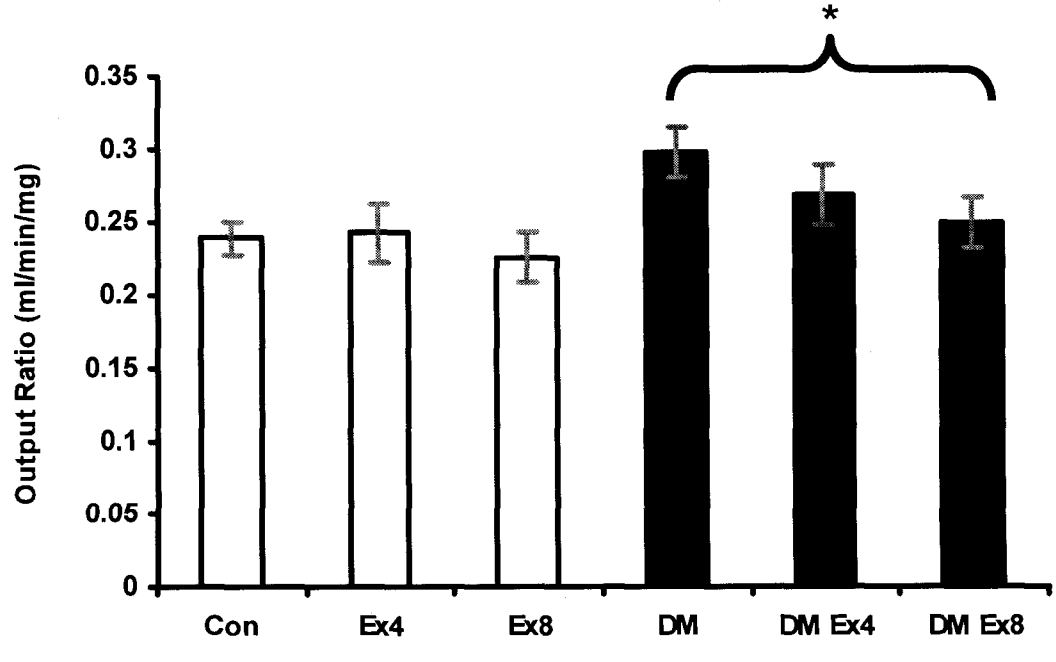
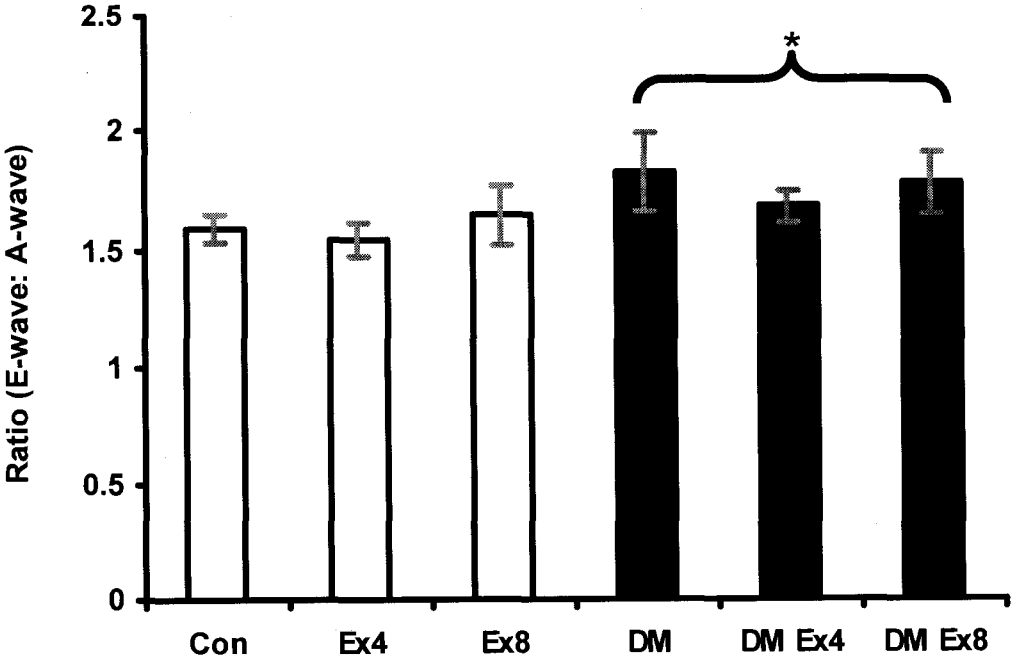


Figure 6.10. MVE-to-MVA ratio (peak wave velocity of early filling relative to peak wave velocity from atrial contraction) obtained after 8 weeks of DM and/or exercise. With or without exercise, MVE/MVA values were significantly greater ($P < 0.05$) in DM animals. Data expressed as mean ± SEM (n = 30). *Significantly greater ($P < 0.05$) than values obtained for Non-DM animals.

Figure 6.10



6.4 DISCUSSION

The primary goal of treatment intervention for DM is to improve glucose control, which is strengthened by the observation that improvements in cardiovascular physiology following exercise prescription is invariably correlated with reduced risk factors and improved glucose regulation (reviewed by Saraceni and Broderick, 2007). Nonetheless, the present study was grounded in the assertion that exercise initiates physical actions that independently command intracellular signals, and may be able to confer physiological benefits independent of insulin, hyperglycemia and the metabolic shift implicit in DM.

It was demonstrated that myocardial geometry and function were disrupted by DM. Exercise introduced concurrent with DM-induction, or 4 weeks after established DM, did not preserve or restore myocardial architecture or function. There were three main findings in the present study. First, LV mass was reduced by DM and was associated with significant wall thinning. Reduced wall thickness was disproportionate to the myocardium as reflected by a diminished RWT. Second, systolic function was altered by DM, demonstrated by reduced FS%, EF%, heart rate, increased isovolumic contraction time and normalized cardiac output. Third, diastolic function was affected by DM, as the contribution of early-phase to ventricular filling increased over the contribution of the late-phase-atrial-contraction to LV filling (elevated MVE/MVA ratio).

Myocardial structural. Myocardial growth and development depends on appropriate hypertrophic signaling, material substrate and physical demands placed on the heart. Myocardial remodeling is observed in both athletes and diseased populations as a response to forces and volume loads placed on the heart. For instance, endurance

activities increase pre-loads (increased LV blood volume at end-diastole) as increased venous return elevates diastolic volume loads, whereas strength athletes experience greater after-loads (increased force required to open aortic valve) due to a contraction-mediated elevation in peripheral pressure resistance. Increased pre-load is passively resisted and stretches the myocardial walls, whereas increased after-load elicits a greater contraction force. Overload stretching increases the length of the muscle by adding sarcomeres in series, whereas after-load induced hypertrophy creates greater girth by increasing sarcomeres in parallel (reviewed by Opie, 2004). The longer, thinner muscle in the endurance-trained heart is described as eccentric hypertrophy (also observed in dilated cardiomyopathy and systolic dysfunction), whereas the thicker, stronger muscle in the strength trained heart is considered concentric hypertrophy (also induced by hypertension and congestive heart failure; reviewed by Mihl et al., 2008). As there is considerable mechanistic overlap between physiological and pathological heart adaptations it is important to consider the magnitude of remodeling as well as the potential mechanisms responsible for pathological remodeling and dysfunction specific to the diabetic myocardium.

The present study demonstrated structural abnormalities in the diabetic myocardium. Thinning anterior and posterior walls were not a simple reflection of attenuated growth in comparison to normal myocardia, as reduced RWT in DM animals revealed that the proportion of wall thickness-to-internal dimensions did not match the proportions of Non-DM animals. This pattern of myocardial re-structuring, along with reduced heart mass, is commonly reported in STZ-treated animals (Wichi et al., 2007; Nemoto et al., 2006; Fiordaliso et al., 2004). As described previously, wall thinning with

increased chamber dimensions reflects structural adaptations akin to eccentric hypertrophy. Hence, conditions generally associated with inducing eccentric hypertrophy in athletes or disease populations may be demonstrated in the present study; elevated pre-load or greater end-diastolic LV volume may be measurably indicative of volume overload and may be an initiating factor for the phenotypic adaptation (reviewed by Mihl et al., 2008). Indeed, the present study showed that end-diastolic volume, relative to the heart diameter, was significantly greater in DM animals, with or without exercise, which may provide evidence of overload-induced eccentric hypertrophy.

Eccentric hypertrophy via endurance exercise induced by overload is explained by increased venous return, but in DM animals, volume overload cannot be explained by increased venous return. Nonetheless, elevated end-diastolic volumes relative to the heart diameter in DM animals suggests that pre-load volumes are disproportionately greater in the diabetic myocardium, as previously reported (Cosyns et al., 2007). Although the origin of volume overload in DM animals may be idiopathic, echocardiographic evidence indicates that volume-overload may originate from inherent systolic dysfunction.

Ejection fraction (EF%) was significantly reduced in DM animals (Figure 6.7), as is commonly found in STZ-treated rats (Nemoto et al., 2006; Akula et al., 2003). Reduced ejection fraction would leave a larger relative volume of blood in the LV at end-systole. Combined with normalized venous return the resultant end-diastolic volume may lead to overload and initiate eccentric hypertrophy. Although reduced ejection fraction may be quantifiably rationalized to contribute to volume overload, it may not explain the original dysfunction leading to reduced volume ejection. Mechanically, the displacement of internal blood volume is performed by reducing the space within the LV via wall

contraction and measured by fractional differences in internal diameter between diastole and systole (i.e., fractional shortening, FS%). The amount of FS% obtained by DM animals was much reduced relative to Non-DM animals, which is also a commonly reported phenomenon (Wichi et al., 2007; Nemoto et al., 2006; Akula et al., 2003; Hoit et al., 1999). Hence, the inability of the diabetic myocardium to appropriately contract to such a degree as to expel a normal quantity of blood may lead to volume overload and initiate the phenotypic eccentric hypertrophy generally observed in diabetic animals.

It is uncertain what reduces FS%, but one possibility includes impaired myocardial contraction. It has been shown previously that Ca^{2+} handling in the DM myocardium is impaired, with lower Ca^{2+} transient amplitude and decreased Ca^{2+} re-uptake (Lacombe et al., 2007). The circumferential fibre shortening velocity (V_{cf}) is a strong echocardiographic indicator of contractility (Gorscan et al., 1997) and was significantly reduced in all DM animals. Therefore, in the present study, impaired Ca^{2+} handling may have reduced myocardial contractility, which may be related to impaired glucose uptake. Indeed, LV function and strain rate were positively correlated with glucose levels (Andersen et al., 2008) and myocardial contractile force was improved by increasing glucose uptake (Pogátsa and Dubecz, 1977). Moreover, it has long been established that the ability of insulin to increase contractility is a function of its ability to increase Ca^{2+} movement (Imanaga, 1976). Both insulin-dependent and non-insulin-dependent DM are implicated in this reduced inotropic phenomenon, as high fat diets were also associated with both contractile dysfunction (Ouwens et al., 2005) and diminished insulin-regulation of Ca^{2+}-mediated contractile force generation (Ouwens et al., 2007). Therefore, the hypoinsulinemia and massive hyperglycemia in the present

study, which likely reflects, in part, reduced myocardial glucose uptake (Kainulainen et al., 1994), may have redeuced Ca^{2+} movement leading to a weakened contractility and diminished FS%, ejection fraction and increased volume overload.

A striking modification in myocardial function was a marked bradycardia in DM animals. Heart rate is associated with myocardial contractility via the Bowditch effect (Bowditch, 1871), as contractile force is positively correlated with rate change (Mulieri et al., 1993; Blinks and Koch-Wesler, 1961), possibly due to accumulating myocardial sodium that increases cytosolic calcium. This effect, reproduced in the rat myocardium (Kassiri et al., 2000), may implicate the markedly reduced heart rate in DM animals as a contributor to reduced FS% and volume overload. Moreover, systolic dysfunction is more likely caused by intrinsic contractile mechanisms rather than reduced force capacity due to wall thinning, since the time-course of contractile dysfunction (Hankiewicz et al., 2008; Zhong et al., 2001) typically precedes that seen for reduced wall thickness.

Diastolic Dysfunction. The diastolic phase of myocardial function allows for ventricular filling and is accomplished predominantly by early filling (E-wave) upon mitral valve opening with a smaller contribution by late-phase atrial contraction (A-wave) prior to mitral valve closure. Changes to the E/A ratio are indicative of changes to diastolic function. The present study showed a significantly increased E/A ratio in DM animals, with or without exercise. Wichi et al. (2007) found a reduced E/A ratio in DM rats after 30 days. A reduced ratio generally precedes an augmented E/A ratio, and is an earlier sign of diastolic dysfunction. Hence, our longer duration and augmented E/A ratio may reflect a more advanced dysfunction. Indeed, an increased E/A ratio is described as a *restrictive filling pattern* (Nishimura et al., 1999) and identifies severe progression of

diastolic complications with poor prognosis and is observed in numerous cardiomyopathies, including diabetic cardiomyopathy (reviewed by Cosson and Kevorkian, 2003). Increased stiffness and decreased compliance are generally associated with a markedly increased E/A ratio (Opie and Perlroth, 2004), both of which occur in STZ-induced DM (Liu et al., 2003; Shimizu et al., 1993). Alternatively, as the E/A ratio is calculated based on peak filling velocities across the mitral valve it is possible that the shape of the myocardium may influence the filling velocities (Riordan et al., 2008) and contribute to increasing the E/A ratio independent of LV stiffness or compliance. Eccentric hypertrophy-associated elongation of the myocardium may have influenced the filling rate of the E-wave and increased the ratio. Regardless of the etiology, diastolic function was imparied and reflects the sum of altered structure and contractile and material function.

Exercise and the diabetic myocardium. Changes to heart structure and function leading to cardiac dysfunction were attributable to DM and the contribution of exercise to preventing or reversing these changes was insignificant. It may be important to note that structural changes associated with endurance exercise (volume overload) typically lead to reduced wall thickness, increased LV volume and reduced ejection fraction (Petersen et al., 2005), albeit in different proportions. Hence, it may be unreasonable to expect aerobic exercise to prevent wall thinning, chamber dilation and to increase ejection fraction when the direction of its phenotypic and functional provisions match the pathophysiology of diabetic cardiomyopathy. However, it may be critical to consider that Fiordaliso et al. (2004) reported reduced cardiomyocyte number (~27%) with a concomitant increase in cardiomyocyte size (~20%) in STZ treated rats. Since the only way a myocyte increases

its cross-sectional area is by increasing sarcomeres in parallel then the diabetic heart experiences concentric hypertrophy and may only resemble eccentric hypertrophy; that is, wall thinning may reflect reduced fibre number via necrosis or apoptosis, and increased myocyte size may reflect a hypertrophic response to increased mechanical demands. If so, then a pruning of fibre numbers in the diabetic heart requires elevated force production from the remaining fibres, which, through a retained capacity for concentric hypertrophy, increases myocyte size. Of course, a combination of eccentric and concentric hypertrophy may coincide.

Exercise did not increase LV mass or improve structural or functional measures impaired by DM. Therefore, any preserved capacity to respond positively to exercise-stimulation did not match the negative consequences of DM. In agreement, previous reports indicate that voluntary running in DM rats did not improve myocardial function (Howarth et al., 2007). However, a moderate-intensity (18m/min) training program did improve FS% or calcium-induced muscle contractions (Howarth et al., 2008) in DM rats. Moreover, Loganathan et al. (2007) showed increased EF% and end-diastolic fraction following an 8-week training program (20m/min, 60min/day). However, they reported that end-diastolic function was reduced in DM animals, in opposition to most reports, including the present study. The discrepancy may reflect differences in measurement techniques, as most non-invasive measures employ echocardiography which approximates volumes based on dimensional assumptions, whereas Loganathan et al. (2007) employed a hyper-accurate 3-D cine-MRI technique. Hence, improved measures based on volume may have occurred in the present study that escaped detection.

Nonetheless, other reports show exercise-induced improvements based on echocardiograpic evidence in DM rats. Impressively, Bidasee et al. (2008) showed improved EF% and reduced end-systolic diameter when exercise was introduced 4 weeks after DM-induction. However, these investigators included only those animals that required no encouragement to run, potentially biasing their exercise group with a sub-population of rats with an intrinsically strong exercise capacity and possibly pre-disposed to exercise-induced improvements. The remaining reports that demonstrate exercise improvements in DM rats are generally limited to showing that exercise reduces myocardial stiffness (Woodiwiss et al., 1996) without improving collagen content. Since DM is known to increase collagen type III (Candido et al., 2003; see chapter 5), and collagen type III is more compliant than collagen type I (Silver et al., 2001), reduced stiffness via exercise may include reduced fibrosis (Searls et al., 2004), but may reflect reduced stiffness inherent to collagen type III. Therefore, although some measurable improvements may be conferred by exercise, the progression of diabetic cardiomyopathy may proceed despite preserved sensitivity to exercise.

Sensitivity to endurance exercise would likely include evidence of improved metabolism. Resting VO_2 values (absolute or relative to body mass) are known to be higher, with lower maximum VO_2 values, in STZ-treated rats (Murphy et al., 1981). Since resting VO_2 is most closely correlated with lean mass (Sprynarov et al., 1987) then cardiac output relative to lean mass should reflect whole body O_2 uptake at rest. Relative cardiac output values in the present study demonstrated that DM animals obtained a significantly greater cardiac output relative to lean mass, suggesting greater O_2 requirements in diabetic tissue. Increased reliance on fatty acid metabolism may help

explain this observation, since lipid metabolism uses O_2 less efficiently than does glucose metabolism (Krogh and Lindhard, 1920). In the present study, exercise demonstrated a trend towards normalizing these values; however significance was not reached when all groups were included together in the data analysis. Smirnova et al. (2006) reported improved heart rate, respiratory and EKG recovery from an exercise stress test in DM rats following an 8-week, low-intensity (20m/min, 60 min/day) training program. However, these investigators also reported reduced glucose levels in exercised diabetic rats, hence, improvements conferred by exercise may have been related to improved glucose control.

6.5 CONCLUSION

The present study demonstrated that 8 weeks of DM reduced heart mass, body mass, lean body mass, altered myocardial structure (reduced wall thickness, RWT, increased internal diameter), impaired systolic function (reduced EF%, %FS, V_{cf}, heart rate, increased isovolumic contraction time), and altered diastolic function (increased MVE/MVA). Exercise introduced at either the onset of DM or 4 weeks after the onset of DM was not able to prevent or reverse these structural or functional changes. Since exercise is generally prescribed for DM in order to help improve glucose regulation and its control is correlated with improved cardiovascular function it follows that exercise may be of limited benefit independent of improved glucose regulation. In the present study the severity of DM was maintained (unaltered blood glucose) in all DM animals and may have prevented an overt display of exercise-derived benefits. The intracellular signaling events normally initiated by exercise may still remain in DM animals, but

physiological improvements provided by exercise may only be actualized in concert with glucose uptake and specific cell signaling made possible by insulin.

6.6 REFERENCES

Akula A, Kota MK, Gopisetty SG, Chitrapu RV, Kalagara M, Kalagara S, Veeravalli KK, Gomedhikam JP. Biochemical, histological and echocardiographic changes during experimental cardiomyopathy in STZ-induced diabetic rats. *Pharmacol Res.* 2003; 48(5):429-435.

Andersen NH, Bojesen A, Christiansen JS, Gravholt CH. Glycemia, lipidemia and systolic left ventricular function evaluated by myocardial strain rate: a tissue Doppler echocardiographic study. *Ultrasound Med Biol.* 2008; 34(1):151-154.

Bidasee KR, Zheng H, Shao CH, Parbhu SK, Rozanski GJ, Patel KP. Exercise training initiated after the onset of diabetes preserves myocardial function: effects on expression of beta-adrenoceptors. *J Appl Physiol.* 2008; 105(3):907-914.

Blinks JR, Koch-Wesler J Analysis of the effects of changes in rate and rhythm upon myocardial contractility. J Pharmacol Exp Ther. 1961; 134:373-389.

Borges GR, de Oliveira M, Salgado HC, Fazan R Jr. Myocardial performance in conscious streptozotocin diabetic rats. Cardiovasc Diabetol. 2006 Dec 4;5:26.

Bowditch HP: Ueber die Eigenthumlichkeiten der Reizbarkeit, welche die Muskelfasern des Herzens zeigen. *Arb Physiol Anst Leipzig.* 1871; 6:139-176.

Candido R, Forbes JM, Thomas MC, Thallas V, Dean RG, Burns WC, Tikellis C, Ritchie RH, Twigg SM, Cooper ME, Burrell LM. A breaker of advanced glycation end products attenuates diabetes-induced myocardial structural changes. *Circ Res.* 2003; 92(7):785-792.

Cosson S, Kevorkian JP. Left ventricular diastolic dysfunction: an early sign of diabetic cardiomyopathy? *Diabetes Metab.* 2003; 29(5):455-466.

Cosyns B, Droogmans S, Weytjens C, Lahoutte T, Van Camp G, Schoors D, Franken PR. Effect of streptozotocin-induced diabetes on left ventricular function in adult rats: an in vivo Pinhole Gated SPECT study. *Cardiovasc Diabetol.* 2007; 6:30.

Davidson JA. Treatment of the patient with diabetes: importance of maintaining target HbA(1c) levels. *Curr Med Res Opin.* 2004; 20:1919–1927.

Fang ZY, Prins JB, Marwick TH. Diabetic cardiomyopathy: evidence, mechanisms, and therapeutic implications. *Endocr Rev.* 2004; 25(4): 543–567.

Fiordaliso F, Bianchi R, Staszewsky L, Cuccovillo I, Doni M, Laragione T, Salio M, Savino C, Melucci S, Santangelo F, Scanziani E, Masson S, Ghezzi P, Latini R. Antioxidant treatment attenuates hyperglycemia-induced cardiomyocyte death in rats. *J Mol Cell Cardiol.* 2004; 37(5):959-968.

Gorcsan J 3rd, Strum DP, Mandarino WA, Gulati VK, Pinsky MR. Quantitative assessment of alterations in regional left ventricular contractility with color-coded tissue Doppler echocardiography. Comparison with sonomicrometry and pressure-volume relations. *Circulation.* 1997; 95(10):2423-2433.

Hankiewicz JH, Goldspink PH, Buttrick PM, Lewandowski ED. Principal strain changes precede ventricular wall thinning during transition to heart failure in a mouse model of dilated cardiomyopathy. *Am J Physiol Heart Circ Physiol.* 2008; 294(1):H330-336.

Hoit BD, Castro C, Bultron G, Knight S, Matlib MA. Noninvasive evaluation of cardiac dysfunction by echocardiography in streptozotocin-induced diabetic rats. *J Card Fail.* 1999; 5(4):324-333.

Howarth FC, Almugaddum FA, Qureshi MA, Ljubisavijevic M. Effects of varying intensity exercise on shortening and intracellular calcium in ventricular myocytes from streptozotocin (STZ)-induced diabetic rats. *Mol Cell Biochem.* 2008; 317(1-2):161-167.
Imanaga I. Effects of insulin on mammalian cardiac muscle. *Recent Adv Stud Cardiac Struct Metab.* 1976; 11:441-450.

Kainulainen H, Breiner M, Schürmann A, Marttinen A, Virjo A, Joost HG. In vivo glucose uptake and glucose transporter proteins GLUT1 and GLUT4 in heart and various types of skeletal muscle from streptozotocin-diabetic rats. *Biochim Biophys Acta.* 1994; 1225(3):275-282.

Kassiri Z, Myers R, Kaprielian R, Banijamali HS, Backx PH. Rate-dependent changes of twitch force duration in rat cardiac trabeculae: a property of the contractile system. J Physiol. 2000; 524:221-231.

Krogh A, Lindhard J. The Relative Value of Fat and Carbohydrate as Sources of Muscular Energy: With Appendices on the Correlation between Standard Metabolism and the Respiratory Quotient during Rest and Work. *Biochem J.* 1920; 14(3-4):290-363.

Lacombe VA, Viatchenko-Karpinski S, Terentyev D, Sridhar A, Emani S, Bonagura JD, Feldman DS, Györke S, Carnes CA. Mechanisms of impaired calcium handling underlying subclinical diastolic dysfunction in Diabetes. *Am J Physiol Regul Integr Comp Physiol.* 2007;

Li S, Culver B, Ren J. Benefit and risk of exercise on myocardial function in diabetes. *Pharmacol Res.* 2003; 48(2):127-132.

Liu J, Masurekar MR, Vatner DE, Jyothirmayi GN, Regan TJ, Vatner SF, Meggs LG, Malhotra A. Glycation end-product cross-link breaker reduces collagen and improves cardiac function in aging diabetic heart. *Am J Physiol Heart Circ Physiol.* 2003; 285: H2587–H2591.

Loganathan R, Bilgen M, Al-Hafez B, Zhero SV, Alenezy MD, Smirnova IV. Exercise training improves cardiac performance in diabetes: in vivo demonstration with quantitative cine-MRI analyses. *J Appl Physiol.* 2007; 102(2):665-672.

Mihl C, Dassen WR, Kuipers H. Cardiac remodelling: concentric versus eccentric hypertrophy in strength and endurance athletes. *Neth Heart J.* 2008; 16(4):129-133.

Mulieri LA, Leavitt BJ, Martin BJ, Haeberle JR, Alpert NR. Myocardial force-frequency defect in mitral regurgitation heart failure is reversed by forskolin. Circulation. 1993 Dec;88(6):2700-2704.

Murphy RD, Vailas AC, Tipton CM, Matthes RD, Edwards JG. Influence of streptozotocin-induced diabetes and insulin on the functional capacity of rats. *J Appl Physiol.: Respirat Environ Exercise Physiol.* 1981; 50(3):482-486.

Nemoto O, Kawaguchi M, Yaoita H, Miyake K, Maehara K, Maruyama Y. Left ventricular dysfunction and remodeling in streptozotocin-induced diabetic rats. *Circ J.* 2006; 70(3):327-334.

Nishimura RA, Abel MD, Hatle LK, Tajik AJ. Assessment of diastolic function of the heart: background and current applications of Doppler echocardiography. *Mayo Clin Proc.* 1989;64:181-203.

Olansky L. Advances in diabetes for the millennium: chronic microvascular complications of diabetes. *Med Gen Med.* 2004; 9(6):14.

Opie LH. Overload hypertrophy and its molecular biology. In: Opie HO, eds. Heart physiology - from cell to circulation. Philadelphia: Lippincott Williams & Wilkins, 2004: 402-427.

Opie LH, Perlroth MG. Ventricular function. In: Opie HO, eds. Heart physiology - from cell to circulation. Philadelphia: Lippincott Williams & Wilkins, 2004: 355-401.

Ouwens DM, Boer C, Fodor M, de Galan P, Heine RJ, Maassen JA, Diamant M. Cardiac dysfunction induced by high-fat diet is associated with altered myocardial insulin signalling in rats. *Diabetologia.* 2005; 48(6):1229-1237.

Ouwens DM, Diamant M, Fodor M, Habets DD, Pelsers MM, El Hasnaoui M, Dang ZC, van den Brom CE, Vlasblom R, Rietdijk A, Boer C, Coort SL, Glatz JF, Luiken JJ. Cardiac contractile dysfunction in insulin-resistant rats fed a high-fat diet is associated with elevated CD36-mediated fatty acid uptake and esterification. *Diabetologia.* 2007; 50(9):1938-1948.

Petersen SE, Selvanayagam JB, Francis JM, Myerson SG, Wiesmann F, Robson MD, Östman-Smith I, Casadei B, Watkins H, Neubauer S. Differentiation of athlete's heart from pathological forms of cardiac hypertrophy by means of geometric indices derived from cardiovascular magnetic resonance. J Cardio Mag Res. 2005; 7:551–558.

Pogátsa G, Dubecz E. The direct effect of hypoglycaemic sulphonylureas on myocardial contractile force and arterial blood pressure. *Diabetologia.* 1977; 13(5):515-519.

Rewers M. Awareness and treatment of dyslipidemia in young adults with type 1 diabetes. *Diabetes Care.* 2005; 28(5):1051-1056.

Riordan MM, Kovács SJ. Elucidation of spatially distinct compensatory mechanisms in diastole: radial compensation for impaired longitudinal filling in left ventricular hypertrophy. J Appl Physiol. 2008 Feb;104(2):513-20. Epub 2007 Nov 21.

Saraceni C, Broderick TL. Cardiac and metabolic consequences of aerobic exercise training in experimental diabetes. *Curr Diabetes Rev.* 2007; 3(1):75-84.

Searls YM, Smirnova IV, Fegley BR, Stehno-Bittel L. Exercise attenuates diabetes-induced ultrastructural changes in rat cardiac tissue. *Med Sci Sports Exerc.* 2004; 36(11):1863-1870.

Severson DL. Diabetic cardiomyopathy: recent evidence from mouse models of type 1 and type 2 diabetes. *Can J Physiol Pharmacol.* 2004; 82(10):813–823.

Shimizu M, Umeda K, Sugihara N, Yoshio H, Ino H, Takeda R, Okada Y, Nakanishi I. Collagen remodelling in myocardia of patients with diabetes. *J. Clin. Pathol.* 1993; 46;32-36.

Silver FH, Horvath I, Foran DJ. Mechanical implications of the domain structure of fiber-forming collagens: comparison of the molecular and fibrillar flexibilities of the alpha1-chains found in types I-III collagen. *J Theor Biol.* 2002; 216(2):243-254.

Smirnova IV, Kibiryeva N, Vidoni E, Bunag R, Stehno-Bittel L. Abnormal EKG stress test in rats with type 1 diabetes is deterred with low-intensity exercise programme. *Acta Diabetol.* 2006; 43:66–74.

Soedamah-Muthu SS, Fuller JH, Mulnier HE, Raleigh VS, Lawrenson RA, Colhoun HM All-cause mortality rates in patients with type 1 diabetes mellitus compared with a nondiabetic population from the UK general practice research database, 1992–1999. *Diabetologia.* 2006; 49(4):660–666.

Sprynarov S, Pafizkova J, Bunc V. Relationships between body dimensions and resting and working oxygen consumption, in boys aged 11 to 18 years. *Eur J Appl Physiol.* 1987; 56:725-736.

Vinnakota KC, Bassingthwaighte JB. Myocardial density and composition: a basis for calculating intracellular metabolite concentrations. *Am J Physiol Heart Circ Physiol.* 2004; 286: H1742–H1749.

Wadwa RP, Kinney GL, Maahs DM, Snell-Bergeon J, Hokanson JE, Garg SK, Eckel RH, Wichi R, Malfitano C, Rosa K, De Souza SB, Salemi V, Mostarda C, De Angelis K, Irigoyen MC. Noninvasive and invasive evaluation of cardiac dysfunction in experimental diabetes in rodents. *Cardiovasc Diabetol.* 2007; 6:14.

Woodiwiss, Angela J., William J. Kalk, and Gavin R. Norton. Habitual exercise attenuates myocardial stiffness in diabetes mellitus in rats. *Am J Physiol.* 1996; 271(40):H2126-H2133.

Yin FCP, Spugeon HA, Rakusan K, Weisfeldt ML, Laratte EG. Use of tibial length to quantify, cardiac hypertrophy: application in the aging rat. *Am J Physiol.* 1982; 243 (12):H941-947.

Zhong Y, Ahmed S, Grupp IL, Matlib MA. Altered SR protein expression associated with contractile dysfunction in diabetic rat hearts. *Am J Physiol Heart Circ Physiol.* 2001; 281(3):H1137-147.

7.0 CONCLUSIONS

7.1 Summary

The production of energy is indispensable to cellular function, and a profound modification of metabolism is tantamount to modifying energy and functional capacity. DM not only eradicates a primary method of energy production by restricting glucose utilization, but also limits intracellular insulin signaling imperative to regulating protein production and function. This invariably leads to a deranged cellular phenotype and physiology. Nonetheless, the founding tenet of the current project was that exercise bestows health and vitality, even under pathological conditions. It was hypothesized that exercise could compensate for those intracellular signals lost or deranged by hypoinsulinemia and hyperglycemia. While an expectation of complete restoration of intracellular signaling, structure and function was not anticipated, a measurable benefit of exercise was expected.

Herein, severe, uncompensated DM complications were observed throughout the whole animal body and manifested through hypoinsulinemia, hyperglycemia, dyslipidemia, glycated serum proteins, elevated oxidative stress, organ hypertrophy, skeletal and cardiac muscle atrophy, inflammatory dysregulation, osteopenia, intracellular signaling derangements, myocardial remodeling and dysfunction. Limited benefit was observed when moderate aerobic exercise training was introduced at the onset of, or 4 weeks following, DM induction. However, oxidative stress was significantly reduced by exercise in DM animals only and was proportional to the number of exercise sessions (0 vs. 20 vs. 40), indicating that exercise reduces DM-related oxidative stress in a volume-dependent manner. The cellular stress response remained

sensitive to exercise, as the cardio-protective HSP70 protein was increased similarly by exercise in both Non-DM and DM myocardia.

Despite maintaining an exercise-sensitive stress response and increased HSP70, associated with preserving cellular structure and function (Locke et al., 1995), myocardial structure was apparently altered (evidenced by wall thinning, chamber dilation, volume overload and reduced mass) and its function was diminished (reduced contractility, FS%, EF%, cardiac output and increased MVE/MVA). Metabolism likely was altered in favor of fatty acid oxidation, as cardiac output-to-lean mass was greater in DM animals. Exercise promoted the normalization of cardiac output by reducing this trend, but when all groups were analyzed together variability and reduced statistical power resulted in no discernable improvements.

It was discovered that an intricate intracellular signaling network within cardiac muscle responds to the applied stress of DM by increasing the contents and activity of specific structural and functional proteins while decreasing the contents and activity of others. Specifically, protein contents of the signaling receptor (IGF-1R) were significantly elevated, while its phosphorylation status (activation) was maintained. However, its downstream kinase activity (largely via the IGF-1R→PI-3K pathway) appeared to be altered, which influenced inflammatory, metabolic, cellular stress and hypertrophic regulators. The formation and activity of the pathological protein combination (p50-p65) of the inflammatory regulator, NF-κB, appeared to increase, evidenced by increased IκB-α and collagen Type III. The mitochondrial protein UCP-3 was increased and may be partly responsible for exercise-attenuated oxidative stress. Re-emergence of the fetal phenotype was observed by the accumulation of β-MHC I, while

normal growth and development was attenuated, possibly mediated by GSK-3β. In addition, contents of the cellular stress response regulator, HSF-1, was increased, possibly through a lack of ERK-1/GSK-3β inhibition, allowing for preserved expression of its target gene, HSP70.

Taken together, the proposed DM-specific pathway does not clearly indicate impairment or lost signaling, but rather suggests alternative signaling, since both increased and decreased protein contents and kinase activities were observed and are mediated through the same regulators. Hence, if DM had simply reduced or increased IGF-1R or PI-3K activity then the resultant protein contents and cellular phenotype would likely have reflected that direction and magnitude of change in signaling. As it was, the signaling pathway was refined and re-directed, which advocates for an abnormal or compensatory response, unilaterally commanded by insulin ablation, hyperglycemia and/or hyperlipidemia. Regardless of intracellular signaling modification, exercise was able to reduce oxidative stress and increase cardio-protective HSP70, hence, the potency of exercise was maintained. Therefore, the progressive remodeling of organ, bone, skeletal muscle and myocardial structure may suggest that the actions of insulin are irreplaceable.

7.2 Physiological Significance of Current Research

The present study was motivated in large part by the observation that the majority cause of mortality in diabetic patients is heart disease, regardless of DM type (Soedamah-Muthu et al., 2006; Stone et al., 1995). This suggests that even if insulin can be replaced (as with DM type I), with its associated glucose clearance and normalized lipidemia,

there are factors that control cellular fate that require a more sophisticated regulation than can be provided by a bolus injection of insulin. This further suggests that DM symptoms and traditional risk factors correlated with disease complications may still perform harmful deeds even when macroscopic measures demonstrate improvements. However, it cannot be discounted that improved health is associated with reducing risk factors (Hsieh and Wang, 2005); therefore, interventions that improve extracellular risk factor profiles may concurrently improve intracellular signaling through either common or uncommon mediators.

Since exercise is a non-pharmacological treatment associated with reduced risk factors and improved health, it was expected that the contents and activity of key intracellular regulators of cellular structure, function and protection noticeably disturbed by DM would be normalized by exercise. The position stand of both the American Diabetes Association and the American College of Sports Medicine recommends exercise prescription for patients with DM at levels similar to those recommended for normal individuals (ECDCDM, 2002; Kesaniemi et al., 2001). Consistent with the goals of traditional treatment interventions, the benefits of exercise for individuals with DM and cardiovascular disease are correlated with a reduction in cardiovascular risk factors and DM symptoms, such as improved lipids, inflammatory markers and glycemic control (Hsieh and Wang, 2005; Lindstrom et al. 2003; Hersey et al., 1994). Hence, it is difficult to expect normalized intracellular signaling and externalized function without a noticeable improvement in these variables. Nonetheless, since exercise previously improved function independent of overt improvement of risk factors, it was asserted that intracellular signaling can be manipulated by exercise and the active members identified.

It is difficult to indisputably delineate biochemical mechanisms in whole animals without the use of kinase-specific inhibitors or transgenic animals. Many mechanistic studies employ cell cultures to avoid the confounding variables that are uncontrollably inherent to *in vivo* models. The present study employed a multiple low-dose STZ injection regime to induce hyperglycemia by selectively ablating pancreatic β-cells and insulin production. It was expected that this would most closely approximate an *in vitro* preparation, which would allow for selective removal of insulin while introducing the defining DM variable of hyperglycemia. Any subsequent dyslipidemia or cellular dysfunction would progress as a direct result of manipulating those 2 variables while avoiding high serum levels of adipokines, cytokines, lipids and cellular dysfunction induced by long term adiposity inherent to type 2 DM or high fat diets (reviewed by Fantuzzi, 2005).

7.3 Limitations and Future Directions

Type 2 DM comprises 90-95% of all DM cases in the general population (ADA, 2006), whereas the present study employed a model of Type 1 DM. Hence, the findings herein are limited to a specific DM category with a much lower prevalence rate. A model similar to Type 2 DM may expose intracellular mechanisms distinctive from those delineated in the present study.

Secondly, our model did not provide a STZ-group that received insulin replacement therapy in order to normalize blood glucose and tease-out physiological adaptations due to pharmacological side-effects from the effects of hyperglycemia and hypoinsulinemia. This decision was made based on the fact that normal insulin secretion

follows an oscillating, circadian pattern of release (Ahlersová et al., 1984) in addition to responding rapidly and directly from blood glucose (Sturis et al., 1991; reviewed by Froy, 2007). Since it was not possible to infuse animals with insulin with the quantity and timing associated with normal physiology it was concluded that glucose clearance and attenuated changes by insulin replacement would generally satisfy the criteria for successful normalization, but any outcome variable not normalized by insulin treatments would be erroneously attributed to STZ cytotoxicity (Johnston et al., 2007).

Thirdly, some variables determined with echocardiography are based on single-dimension measurements and calculated with specific geometric assumptions. If DM causes structural abnormalities it is possible that the shape of the myocardium would bias the accuracy of the assumed shape. Nonetheless, measures obtained by echocardiography are in general agreement with direct invasive measures (Zamorano et al., 1997) and avoid restricting the ability to exercise train the animals following cardiac function testing. Future access to hyper-accurate MRI able to capture images throughout the heart cycles may provide more precise measurements of wall thickness, chamber dimensions and blood flow.

Finally, the present study employed young Sprague-Dawley rats to approximate the age of disease-onset seen in humans. Moreover, only males were used in our study, reducing the need to measure and consider fluctuating hormone levels in female rats as contributors to myocardial dysfunction. This reduces a broad application of the present findings and glaringly ignores the fact that diabetic females have a 4-fold increase in CVD mortality compared to 2-fold increase in males (Kannel and McGee, 1979); therefore, determining pathophysiological distinctions between males and female animals

and the mechanisms for these sex-differences may help focus treatments for DM-related complications. In particular, exploring the role of estrogen in influencing the cellular stress response and restricting accumulation of the cardio-protective HSP70 (reviewed by Milne and Noble, 2008) could uncover potential mechanisms contributing to myocardial remodeling and increased myocardial mortality in individuals with DM.

7.4 REFERENCES

Ahlersová E, Ahlers I, Milárová R, Datelinka I, Toropila M. Circadian oscillations of thyroid hormones, insulin and glucagon in the blood of laboratory rats in the course of the year. *Physiol Bohemoslov.* 1984;33(4):309-319.

American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care.* 2006; 29(Suppl 1):S43–S48.

Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (ECDCDM) American Diabetes Association: clinical practice recommendations 2002. *Diabetes Care.* 2002; 25(Suppl 1): S1–S147.

Fantuzzi G. Adipose tissue, adipokines, and inflammation. *J Allergy Clin Immunol.* 2005; 115(5):911-919.

Froy O. The relationship between nutrition and circadian rhythms in mammals. *Front Neuroendocrinol.* 2007; 28(2-3):61-71.

Hersey WC 3rd, Graves JE, Pollock ML, Gingerich R, Shireman RB, Heath GW, Spierto F, McCole SD, Hagberg JM. Endurance exercise training improves body composition and plasma insulin responses in 70- to 79-year-old men and women. *Metabolism.* 1994; 43:847–854.

Hsieh CJ, Wang PW. Effectiveness of weight loss in the elderly with type 2 diabetes mellitus. *J Endocrinol Invest.* 2005; 28:973–977.

Johnston AP, Campbell JE, Found JG, Riddell MC, Hawke TJ. Streptozotocin induces G2 arrest in skeletal muscle myoblasts and impairs muscle growth in vivo. *Am J Physiol Cell Physiol.* 2007; 292(3):C1033-1040.

Kannel WB, McGee DL. Diabetes and cardiovascular disease. The Framingham study. *JAMA.* 1979; 241:2035–2038.
Kesaniemi YK, Danforth E,Jr, Jensen MD, Kopelman PG, Lefebvre P, Reeder BA Dose–response issues concerning physical activity and health: an evidence-based symposium. *Med Sci Sports Exerc.* 2001; 33:S351–S358.

Lindstrom J, Louheranta A, Mannelin M, Rastas M, Salminen V, Eriksson J, Uusitupa M, Tuomilehto J, Finnish Diabetes Prevention Study Group. The Finnish Diabetes Prevention Study (DPS): lifestyle intervention and 3-year results on diet and physical activity. *Diabetes Care.* 2003; 26:3230–3236.

Locke M, Tanguay RM, Klabunde RE, Ianuzzo CD. Enhanced postischemic myocardial recovery following exercise induction of HSP 72. *Am J Physiol.* 1995; 269(1 Pt 2):H320-325.

Milne KJ, Noble EG. Response of the myocardium to exercise: sex-specific regulation of hsp70. *Med Sci Sports Exerc.* 2008; 40(4):655-663.

Soedamah-Muthu SS, Fuller JH, Mulnier HE, Raleigh VS, Lawrenson RA, Colhoun HM. All-cause mortality rates in patients with type 1 diabetes mellitus compared with a nondiabetic population from the UK general practice research database, 1992–1999. *Diabetologia.* 2006; 49(4):660–666.

Stone PH, Muller JE, Hartwell T, York BJ, Rutherford JD, Parker CB, Turi ZG, Strauss HW, Willerson JT, Robertson T. The effect of diabetes mellitus on prognosis and serial left ventricular function after acute myocardial infarction: contribution of both coronary disease and diastolic left ventricular dysfunction to the adverse prognosis. The MILIS Study Group. *J Am Coll Cardiol.* 1989; 14:49–57.

Sturis J, Polonsky KS, Mosekilde E, Van Cauter E. Computer model for mechanisms underlying ultradian oscillations of insulin and glucose. *Am J Physiol.* 1991; 260(5 Pt 1):E801-809.

Zamorano J, Wallbridge DR, Ge J, Drozd J, Nesser J, Erbel R. Non-invasive assessment of cardiac physiology by tissue Doppler echocardiography. A comparison with invasive haemodynamics. *Eur Heart J.* 1997; 18(2):330-339.

Bruce Frier Curriculum Vitae

Education

University of Western Ontario	(PhD. completed)	2003-2009
University of Toronto	(*Master of Science*)	2001-2003
University of Toronto	(Undergrad/Grad Exercise Science)	2000-2001
Augustana University	(Science)	1999
University of Alberta	(*Bachelor of Education*)	1987-1989
Concordia University	(Education)	1985-1987

Focus of Degrees

B.Ed. Physical Education and Music major; English and Psychology minor

M.Sc. Muscle Biochemistry: Skeletal Muscle Hypertrophy; Satellite Cell Activation; Heat Shock Proteins

Ph.D. Muscle Biochemistry: Diabetic Skeletal and Cardiac Muscle Hypertrophy and Atrophy;
Muscle-specific Alterations to NF-κB Expression and DNA Binding in Diabetes
Heat Shock Protein Regulation of Inflammation (NF-kB Activation)
Influence of Exercise on Diabetes-induced Protein Expression and Signaling in Skeletal and Cardiac Muscle
Influence of Exercise on Diabetes-induced Cardiomyopathy

Employment

Teaching Assistant

Anatomy (UWO)	2004-Present
Physiology (UWO)	2003-2005
Exercise Physiology (UWO)	2004-Present
Exercise Physiology (U of T)	2001-2003
Biophysical Sciences (U of T)	2001-2003

School Teacher

Elementary Music, Jr. High Phys Ed., Band (Edmonton)	1996-1998
Gr. 7-12 Language Arts, Drama, Math, Social Studies (Wabasca Reserve)	1992-1995
Gr. 1-12 Substitute Teacher (Edmonton)	c.1990-1992
Grade 10-12 Music, Social Studies, Phys.Ed. (Edmonton)	1991

Publications

Frier BC, Noble EG, Locke M. Diabetes induced atrophy is associated with a muscle-specific alteration in NF-κB activation and expression. *Cell Stress & Chaperones* 2008 Sep;13(3):287-296.

Yang P, Frier BC, Goodman L, Duffin J. Respiratory Muscle Training and the Performance of a Simulated Anti-G Straining Maneuver. *Aviat Space Environ Med.* 2007; 78: 1035-1041.

Frier BC, Locke M. Heat stress inhibits skeletal muscle hypertrophy. *Cell Stress & Chaperones* (2007) 12: 132-141.

Frier BC, Yang P, Taylor AW. Diabetes, aging and physical activity *Eur Rev Aging Phys Act* (2006) 3: 63–73.

Frier BC, Locke M. Preservation of heat stress induced myocardial hsp 72 in aged animals following caloric restriction *Experimental Gerontology* (2005) 40: 615–617.

Frier BC, Taylor AW. Osteoarthritis, aging and physical activity. *Eur Rev Aging Phys Activity* (2005) 3:47-56.

In Preparation

Frier BC, Noble EG. Anthropomorphic and bone density alterations with exercise in streptozotocin-induced diabetic animals.

Frier BC, Noble EG. Locke M. Exercise training and diabetes similarly increases NF-κB-DNA binding in the rat myocardium but may be uniquely regulated by upstream effectors.

Frier BC, Williams D, Wright DC. Apelin induces mitochondrial biogenesis in rat skeletal muscle.

Frier BC, Noble EG. IGF-1 receptor signaling in the diabetic myocardium.

Frier BC, Karmazyn M, Noble EG. Exercise does not prevent or restore myocardial dysfunction in the streptozotocin-induced diabetic myocardium.

Frier BC, Locke M. Transcriptional regulation of nuclear factor-kappa B by Hsp72 and Hsp90 in skeletal muscle.

Frier BC, Locke M. Induced skeletal muscle hypertrophy is attenuated independent of satellite cell replication following heat shock.

Abstracts

Frier BC, Williams D, Wright DC. Apelin induces mitochondrial biogenesis in rat skeletal muscle. International Biochemistry of Exercise Conference, Guelph, Ontario, Canada, June 1-4, 2009.

Frier BC, Locke M, Noble EG. Heat stress prevents overload-induced hypertrophy of myosin heavy chain type I fiber area, but does not alter structural damage or localized patterns of cellular replication. Experimental Biology (FASEB), San Diego, California, April 5-9, 2008

Frier BC, Yang P, Noble EG, Locke M. Tissue-specific changes to NF-kB activation & expression in diabetic skeletal muscle. *9th International Congress of the Immunology of Diabetes Society and American Diabetes Association Research Symposium* Miami, Florida on November 14-18, 2007

Yang P, Frier BC, Oh P. Daily walking distance is the best predictor of peak aerobic capacity in patients with coronary artery disease following one year of cardiac rehabilitation. *Accepted for presentation at the Canadian Association of Cardiac Rehabilitation Conference, Vancouver BC, October 21, 2006.*

Frier BC, Locke M. Preservation of the myocardial stress response in aged animals following caloric restriction. *JAPA.* 6th World Congress on Aging and Physical Activity. 2004.

Frier BC, Locke M. Heat shock attenuates hypertrophy independent of satellite cell replication. *APS Intersociety Meeting*, Austin, Texas, October, 2004.

Yang P, Frier BC, Goodman L. Six-weeks of respiratory muscle training increases peak systolic blood pressure during the valsalva maneuver. *APS Intersociety Meeting*, Austin, Texas, October 9, 2004.

Yang P, Frier BC, Goodman L. Six-weeks of respiratory muscle training increases peak systolic blood pressure during the valsalva maneuver. *Canadian society for exercise physiology.* Saskatoon, Saskatchewan, October 16, 2004.

Awards

Special University Scholarship: Department of Kinesiology ($10,000; 2005-2007, UWO).

Special University Scholarship: Department of Kinesiology ($8,000; 2004-2005, UWO).

Special University Scholarship: Department of Kinesiology ($6,000; 2003-2004, UWO).

Ph.D. Research Poster Competition Award ($250 prize at the 6th World Congress on Activity and Aging. London, ON; August, 2004).

Department of Exercise Science Academic Fellowship ($12,000; 2002-2003, University of Toronto)

Department of Exercise Science Academic Fellowship ($8,000; 2001-2002, University of Toronto)