ALTERATIONS IN CARDIAC CONTRACTILE PERFORMANCE AND SARCOPLASMIC RETICULUM FUNCTION AND ITS REGULATION IN HIGH SUCROSE-FED RATS IS ASSOCIATED WITH INSULIN RESISTANCE

By

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A thesis submitted to the

University of Manitoba

In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Physiology & Faculty of Medicine

Institute of Cardiovascular Sciences

St. Boniface General Hospital Research Centre

Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Alterations in Cardiac Contractile Performance and Sarcoplasmic Reticulum Function and its Regulation in High Sucrose-fed Rats is Associated with Insulin Resistance

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

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MASTER OF SCIENCE

ACKNOWLEDGEMENTS

My special thanks and appreciation go to Dr. Thomas Netticadan who accepted the role of my supervisor as well as of my guardian. Dr. Netticadan gave me an opportunity to pursue my research interest in diabetes mellitus, a disease that has greatly impacted my immediate family. As a mentor, Dr. Netticadan shared his breadth of knowledge in diabetes mellitus and cardiovascular physiology, offered me insight into pursing a career in scientific research, as well as provided me direction and feedback with respect to my research project. As a guardian and friend, Dr. Netticadan gave me a wealth of support and encouragement. Dr. Netticadan, I wish you all the best in your future endeavours.

I would like to acknowledge my advisory committee members Dr. Ian Dixon, Dr. Elissavet Kardami, and Dr. Jeffrey Wigle. I greatly appreciate your guidance and support over the last couple of years and I thank you for your diligent review of my thesis.

My sincere thanks go to my colleagues with whom I have had the privilege of working, collaborating, and making friends. My time in R3042 would not have been as lively and memorable without all of you: Andrea Babick, Elliott Cantor, Dr. Xing-Hai Yao, Dr. Satyajeet Rathi, Fuwu Bai, Raja Singh, Punam Chohan, Rishi Seth, Jason Greville, John Babick, Mellissa Moyen, and Danijel Juric. I have also enjoyed getting to know my other colleagues at the Institute of Cardiovascular Sciences.

My academic experience at the Institute of Cardiovascular Sciences is invaluable. I am honoured to have had the opportunity to conduct my research amongst one of the world's leading scientists in diabetic cardiomyopathy, Dr. Naranjan S. Dhalla.

I would also like to acknowledge the University of Manitoba's Department of Physiology. Thank you to the Department heads Dr. Peter Cattini and Dr. Janice Dodd for your kind words of encouragement and many thanks to Gail McIndless and Judith Olfert for answering my questions and handling my paperwork.

A special thank you goes to my family and friends for their unwavering care and encouragement throughout my time in Winnipeg. I am very fortunate to have you all in my life.

Lastly, my deepest gratitude goes to my mom and dad. Mom, you have been my pillar of strength, giving me your unconditional love and support. Dad, I dedicate my thesis to you. You showered me with your love, empowered me to achieve my goals, and taught me the value of hard work. You are and will always be my inspiration. I wish that I could share this with you but I know that you are always with me. I hope that you are proud of me and I will continue make you proud. I love you and I miss you.

ABSTRACT

Diabetes mellitus (DM) causes the development of a specific heart muscle disease known as diabetic cardiomyopathy that results from the metabolic derangements present in DM. Diabetic cardiomyopathy is characterized by early diastolic dysfunction preceding systolic damage. While the cardiac contractile dysfunction in type 1 DM has been linked to defects in the function and regulation of the sarcoplasmic reticulum (SR), the primary regulator of intracellular calcium (Ca^{2+}) handling essential in mediating cardiac contraction and relaxation, very little is known about SR function in type 2 DM. Resistance to the biological actions of insulin in glucose and lipid metabolism is the core defect in type 2 DM and develops years before disease diagnosis. Accordingly, this study examined cardiac contractile performance and SR function and its regulation in the prestage of type 2 DM (i.e. during insulin resistance). High sucrose feeding was used to induce whole body insulin resistance while plasma metabolite levels were measured to monitor disease progression. In addition, cardiac contractile performance was assessed by *in vivo* echocardiography and SR function was measured by SR Ca^{2+} uptake. Rats fed a high sucrose diet exhibited hyperinsulinemia, hyperglycemia, hyperlipidemia, and increased body weights relative to control rats. Serial echocardiographic assessments in the sucrose-fed rats revealed early abnormalities in diastolic function followed by late systolic dysfunction and concurrent alterations in myocardial structure. The hearts of the 10-week sucrose-fed rats showed depressed SR function demonstrated by a significant reduction in SR Ca^{2+} uptake. The decline in SR Ca^{2+} uptake may be due primarily to a significant decrease in the cAMP-dependent protein kinase (PKA) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II)-mediated phosphorylation of phospholamban

(PLB). The reduction in SR Ca^{2+} uptake in the sucrose hearts may be exacerbated by a marginal decrease in the expression of sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA2a), the SR membrane transport protein responsible for resequestration of Ca^{2+} into the SR, and concomitant slight increase in the expression of its regulatory protein PLB. Our results show that abnormalities in cardiac contractile performance and SR function and its regulation occur at an insulin resistant stage before the manifestation of overt type 2 DM.

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LIST OF ABBREVIATIONS

DM – Diabetes mellitus

SR – Sarcoplasmic reticulum

Ca^{2+} – Calcium

cAMP – Cyclic adenosine monophosphate

PKA – cAMP-dependent protein kinase

CaMK II – Ca^{2+}/calmodulin-dependent protein kinase

PLB – Phospholamban

ATP – Adenosine triphosphate

SERCA2a – Sarcoendoplasmic reticulum Ca^{2+}-ATPase

CVD – Cardiovascular disease

PP1 – Protein phosphatase 1

PP2B – Protein phosphatase 2B

GDM – Gestational diabetes mellitus

FFA – Free fatty acid

PPAR-γ – Peroxisome proliferator-activated receptor-γ

IRS-1 – Insulin receptor substrate-1

PI 3-kinase – Phosphatidylinositol 3-kinase

FA-CoAs – Fatty acyl coenzyme As

DAG – Diacylglycerol

PKC – Phospholipid-dependent protein kinase

PAI-1 – Plasminogen activator inhibitor-1

TNF-α – Tumor necrosis factor-α

IL-6 – Interleukin-6

MODY – Maturity-onset diabetes of the young

RNA – Ribonucleic acid

IAPP – Islet amyloid polypeptide

GIP – Gastric inhibitory peptide

GLP-1– Glucagon-like peptide-1

AGE – Advanced glycosylation end product

TZD – Thiazolidinedione

GLUT4 – Glucose transporter 4

LV – Left ventricular or left ventricle

IVRT – Isovolumic relaxation time

E/A – Ratio of the peak early to late diastolic filling velocity

NCX – Sodium-calcium exchanger

DHPR – Dihydropyridine receptor

RyR2 – Ryanodine receptor

kDa – Kilodalton

FKBP-12.6 – FK-506 binding protein-12.6

mAKAP – Muscle A-kinase anchoring protein

PP2A – Protein phosphatase 2A

PP – Protein phosphatase

V_{max} – Maximal velocity

cGMP – Cyclic guanosine monophosphate

STZ – Streptozotocin

cp/cp – Corpulent

db/db – Diabetic

OLETF – Otsuka Long-Evans Tokushima fatty

ZDF – Zucker diabetic fatty

IVS – Interventricular septal dimensions

LVPW – Left ventricular posterior wall dimensions

LVID – Left ventricular internal dimensions

IVSd – Interventricular septal dimensions at diastole

LVPWd – Left ventricular posterior wall dimensions at diastole

LVIDd – Left ventricular internal dimensions at diastole

IVSs – Interventricular septal dimensions at systole

LVPWs – Left ventricular posterior wall dimensions at systole

LVIDs – Left ventricular internal dimensions at systole

FS – Fractional shortening

EF – Ejection fraction

CO – Cardiac output

EDV – End diastolic volume

ESV – End systolic volume

E – Peak early diastolic filling velocity

A – Peak late diastolic filling velocity

E decel – E wave deceleration time

ET – Aortic ejection time

SE – Standard error

HW/BW – Heart weight to body weight

AKAPS – A-kinase anchoring proteins

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I. INTRODUCTION

Diabetes mellitus is a serious health problem that is reaching epidemic proportions in the United States and throughout the world (1). The global figure of people with DM is expected to rise from the current estimate of 150 million to 221 million in 2010, and 300 million in 2025 (2,3). DM is a syndrome that comprises a heterogeneous group of disorders characterized by abnormally high levels of glucose in the blood (4). There are two main forms of DM type 1 (insulin-dependent) and type 2 (non-insulin-dependent). Type 1 DM is caused by destruction of the pancreatic β-cells, often immune-mediated, that leads to the loss of insulin secretion and absolute insulin deficiency (4). The prevalence of type 1 DM is low relative to that of type 2 DM which accounts for 90% to 95% of cases globally (4). Type 2 DM results from insulin resistance and abnormal insulin secretion, either of which may predominate (5). The current diabetes epidemic relates particularly to type 2 DM primarily because of increases in the prevalence of a sedentary lifestyle and obesity in developed and developing nations (6).

Cardiovascular disease (CVD) is the major cause of mortality in diabetic patients (7). Moreover, the development of a specific heart muscle disease that is independent of coronary artery, hypertensive, or valvular heart disease represents a major complication of DM (8). This diabetic cardiomyopathy is characterized by both structural and functional abnormalities in the heart that result from the metabolic derangements present in DM (9). Furthermore, prediabetic metabolic derangements may produce abnormalities in cardiac structure and function before the development of overt DM (10).

The SR is an intracellular membranous network in cardiac cells that plays a critical role in regulating the intracellular concentration of Ca^{2+} thereby controlling

cardiac contraction and relaxation (11). SERCA2a is the Ca^{2+} pump responsible for a species-specific amount of intracellular Ca^{2+} reuptake into the SR (70% to 92%) facilitating cardiac relaxation, and thereby making Ca^{2+} available for the next wave of contraction (12). SERCA2a, therefore, is considered to be a key determinant of cardiac contractility in the mammalian heart. The function of SERCA2a can be modulated through its physical interaction with the phosphoprotein PLB in that phosphorylation of PLB alters the PLB-SERCA2a interaction. PLB in its unphosphorylated state inhibits SERCA2a activity while PKA-mediated phosphorylation of the amino acid residue serine-16 or CaMK II-mediated phosphorylation of the amino acid residue threonine-17 relieves this inhibition (11). The stimulatory effects of Ca^{2+} transport can be reversed by SR-associated protein phosphatase 1 (PP1) which dephosphorylates both the PKA and CaMK II sites on PLB (13). In addition, protein phosphatase 2B (PP2B), also known as calcineurin, has been shown to reduce SR Ca^{2+} transport through dephosphorylation of PLB at threonine-17 (14). While it has been well established that type 1 DM results in a cardiomyopathy associated with alterations in SR function and its regulation (15-26), very little is known about cardiac SR function in type 2 DM.

High sucrose feeding has been demonstrated to induce whole body insulin resistance in male rats (27-36), a condition intimately associated with the metabolic syndrome, obesity, and the early stages of type 2 DM in humans. Moreover, previous studies have shown that high sucrose feeding in male rats leads to the development of cardiac contractile dysfunction (28-32, 34, 36). However, no study to date has determined the impact of insulin resistance on the function and regulation of cardiac SR vesicles.

Accordingly, this study examined cardiac contractile performance, SR Ca^{2+} cycling and its regulatory mechanisms in sucrose-induced insulin resistant rats.

II. REVIEW OF LITERATURE

Diabetes Mellitus

2.1 Definition of Diabetes Mellitus

Diabetes mellitus is a syndrome that comprises a heterogeneous collection of disorders with different aetiologies, although their pathological effects after onset of the disease may be similar (4). *Diabetes* literally means "syphon" or "running through," a reference to the large urine volume associated with this condition; *mellitus* means "sweet," a reference to the sweetness of the urine resulting from the excess blood glucose that spills into the urine (37). DM is characterized by abnormally high levels of glucose in the blood due to a deficiency of insulin secretion, or to the resistance of the body's cells to the action of insulin, or to a combination of these two factors (4). There are often disturbances of carbohydrate, fat, and protein metabolism in diabetic patients (4).

2.2 Classification of Diabetes Mellitus

The most recent classification system proposed by the American Diabetes Association distinguishes two principle forms of DM termed *type 1 DM* and *type 2 DM* (4). This classification system also includes a category termed *other types of diabetes*, in which the causes could be attributed to known factors, such as endocrinopathies or drug- or chemical-induced pancreatic changes. This category comprises approximately 1% to 2% of people with DM (4). Gestational DM (GDM) comprises a fourth class recognized as a condition of carbohydrate intolerance resulting in hyperglycemia of variable severity with onset or first recognition during pregnancy (38). The prevalence of GDM ranges between 1% and 14% of all pregnancies (39).

2.3 Type 1 Diabetes Mellitus

Type 1 DM results from the destruction of the insulin-secreting β-cells of the islets of Langerhans in the pancreas, leading to the total loss of insulin secretion and an absolute insulin deficiency (4). There are two subclasses of type 1 DM type 1A, which is immune mediated, and type 1B, which is idiopathic (4). Type 1A is an autoimmune disease in which pancreatic β-cells are targeted for destruction by an aberrant host immune system. This process involves the generation of islet-specific T-lymphocyte reactivity, as well as autoantibodies directed against islet cell antigens (4). Autoantibodies are markers for the immune-mediated attack of the pancreatic β-cells with approximately 90% of newly diagnosed type 1A patients having antibodies to one or more of three principal autoantigens: insulin, glutamic acid decarboxylase 65, and islet antigen 2 (4). The development of type 1A is influenced by genetic and environmental factors. The causes of β-cell destruction in type 1B are not understood but are not thought to be immune mediated (4).

Type 1 DM comprises approximately 5% to 10% of all DM cases. This type of DM is more prevalent among children and adolescents giving rise to the former age-related designation *juvenile-onset DM*. However, onset of type 1 DM in adults is not uncommon.

2.3.1 Genetics of Type 1 Diabetes Mellitus

Alleles or genetic variants associated with type 1 DM either predispose to or protect against the disease (40). The strongest genetic determinants for type 1A are the human leukocyte antigen genes, specifically the DR and DQ alleles, within the major histocompatibility complex on chromosome 6 (designated IDDM1) (41). The variable

number of tandem repeats region located upstream of the insulin gene on chromosome 11 (designated IDDM2) also makes an important contribution to diabetes susceptibility (41). Twin studies have confirmed the strong genetic contribution to the development of type 1 DM, indicating a risk of approximately 50% for monozygotic twin siblings and less than 10% for dizygotic twin or non-twin siblings of patients (42). Nonetheless, as the concordance rate in monozygotic twin siblings is not 100%, it has been suggested that environmental factors also play a role in the development of type 1 DM.

2.3.2 Environmental Factors

Several environmental agents have been associated with an increased risk of developing type 1 DM. These include viral infections, dietary factors in early infancy, vaccination, toxins (e.g. nitrates, nitrites or nitrosamines), stress (41), and seasonality (40, 43). These environmental factors may promote β-cell destruction via different mechanisms or may interact with genetic determinants influencing disease risk (41). Viruses for which there is extensive evidence for association with type 1 DM include rubella and enteroviruses, specifically coxsackie A, B, and echoviruses (43). The cytomegalovirus, Epstein-Barr virus, mumps, retrovirus, and rotavirus have also been linked with type 1 DM, although to a lesser degree (43). Dietary factors associated with risk to develop type 1 DM include cow's milk (40) and vitamin D3 and its receptor (44).

2.4 Type 2 Diabetes Mellitus

Type 2 DM results from disorders of insulin action and insulin secretion, either of which may be the predominant feature and both of which are usually present when the disease becomes clinically manifest (38). Type 2 DM is caused by a combination of genetic and environmental factors.

Type 2 DM affects approximately 90% to 95% of people with DM (4). It is the more common form among adults and was formerly termed *adult-onset DM*. However, there is a growing incidence of type 2 DM in children from high-risk populations, such as individuals of Hispanic, Asian, South Asian, or African descent (45).

2.4.1 Physiology and Defects of Insulin Action

The major sites of insulin action are skeletal and cardiac muscle, adipose tissue, and liver (46). The effects of insulin on these target tissues include the stimulation of glucose uptake into muscle and adipose cells and the inhibition of lipolysis in adipose tissue. Insulin also inhibits the production and release of glucose by the liver through inhibition of gluconeogenesis and glycogenolysis (46). The loss of insulin-mediated actions in these insulin-sensitive target tissues–that is, a condition of reduced insulin sensitivity or insulin resistance–may be the first lesion in the development of type 2 DM and may be due to genetic as well as environmental factors. The primary defect in insulin resistance appears to be the inability of insulin to stimulate glucose uptake in muscle (46). In addition, insulin resistance leads to increased lipolysis and free fatty acid (FFA) release by adipose tissue as well as increased gluconeogenesis and glucose release by the liver. The pancreatic β-cells increase basal and postprandial insulin secretion to compensate for the insulin resistance, which further aggravates insulin resistance.

2.4.1.1 Genetics of Insulin Resistance

The importance of genetic factors in the development of insulin resistance and type 2 DM can be recognized both at the individual level (family history) and at the population level (ethnic background). Evidence for familial inheritance comes from studies in monozygotic twin siblings which have shown concordance rates of 50% to

75% for insulin resistance (46) and 50% up to close to 100% for type 2 DM (47). Further evidence for the role of genetic factors comes from the study of populations with different genetic backgrounds living in the same environment. In Canada, the prevalence of type 2 DM is three to five times higher in Aboriginal communities than that of the general population (45). Despite the evidence that susceptibility for type 2 DM is inherited, the specific susceptibility genes and their mode of inheritance have yet to be elucidated. Candidate genes that have been found to be associated with the development of insulin resistance and type 2 DM are described as follows.

A common amino acid polymorphism (Pro12Ala) in peroxisome proliferator-activated receptor-γ (PPAR-γ) has been linked with type 2 DM (48). Homozygous carriers of the Pro12 allele are more insulin resistant than those having one Ala12 allele and have a 1.25-fold increased risk of developing DM (48, 49). Furthermore, there is evidence for an interaction between this polymorphism and FFAs, thereby linking this gene with diet (50). Genetic variation in the gene encoding calpain-10 has also been associated with a threefold increased risk of type 2 DM (51) through affects on both insulin action in muscle and adipose tissue and on normal β-cell function (49). Additional genes implicated in the development of insulin resistance include those involving the insulin receptor, the insulin receptor substrate 1 (IRS-1), the glycogen synthase genes (52), and candidate genes from the adipocyte associated with obesity (53). However, currently identified gene mutations account for less than 5% of all cases of insulin resistance, suggesting that genetically determined insulin resistance is not a dominant cause for the development of type 2 DM (52).

2.4.1.2 Environmental Factors

The environmental or acquired factors associated with the development of insulin resistance and type 2 DM are aging, physical inactivity, and obesity (52).

Insulin resistance and type 2 DM are associated with aging (54). This can be attributed to loss of muscle and an increase in adipose tissue (54). Additional factors that may contribute to age-related insulin resistance include decreased mobility, decreased physical activity, dietary factors, hypertension, and medications (4).

Physical inactivity is an independent risk factor for insulin resistance and type 2 DM (55). Potential mechanisms by which physical inactivity directly contributes to insulin resistance and type 2 DM include reductions in skeletal muscle oxidative phosphorylation, glucose uptake, transcription of metabolic and mitochondrial genes, and insulin signalling as well as increased lipid accumulation in skeletal muscle (55). Accordingly, exercise improves mitochondrial function, glucose metabolism, and insulin action in skeletal muscle in individuals with insulin resistance and type 2 DM (55).

Obesity, in particular increased accumulation of fat in the central abdominal cavity, termed *visceral adiposity*, is negatively correlated with insulin resistance (56). The mechanisms by which adiposity may affect the normal cellular actions of insulin and contribute to insulin resistance involve factors released by adipose tissue, specifically FFAs and adipocytokines.

The adipocytes of obese nondiabetic individuals and type 2 diabetics are markedly resistant to the inhibitory effect of insulin on lipolysis resulting in elevated plasma FFA concentrations (57). Chronically increased plasma FFAs and increased lipid deposition in tissues cause insulin resistance and promote β-cell failure by a process

termed *lipotoxicity* (57). The mechanism by which FFAs cause insulin resistance was first formulated by Randle *et al.* based upon experiments performed in rat diaphragm and heart muscle (58). His group proposed that increased FFA oxidation restrains glucose oxidation in muscle by altering the redox potential of the cell and by inhibiting key glycolytic enzymes (58). Recent studies conducted in human skeletal muscle implicate mechanisms of FFA-induced insulin resistance in addition to those proposed by Randle. Elevated plasma FFAs reduce insulin-stimulated glucose transport activity through diminished insulin-stimulated IRS-1–associated phosphatidylinositol 3-kinase (PI 3-kinase) activity (59).

In addition to the increase in plasma FFA levels, obese nondiabetic individuals and type 2 diabetics have increased stores of triglycerides in muscle and liver (57). Triglycerides in muscle and liver are in a state of constant turnover, and the intracellular metabolites of triglycerides and FFAs (i.e. fatty acyl coenzymes A (FA-CoAs), ceramides, and diacylglycerol (DAG)) impair insulin action in both muscle and liver (60). One attractive hypothesis is that elevated plasma FFA concentrations lead to the accumulation of long-chain FA-CoAs and DAG; these then activate phospholipid-dependent protein kinase (PKC) θ, leading to phosphorylation of serine sites on IRS-1, which reduces IRS-1–associated PI 3-kinase activity (59). A direct effect of long-chain FA-CoAs on glucose phosphorylation (61) and glycogen synthesis (62) has also been demonstrated. Furthermore, increased muscle ceramide levels, secondary to increased long-chain FA-CoAs, inhibit glycogen synthesis in muscle through inhibition of the insulin-stimulated protein kinase B pathway (63, 64). In the liver increased FFAs activate

some key gluconeogenic enzymes (65) and increase the activity of glucose-6-phosphate (66) resulting in increased hepatic glucose production and release respectively.

In addition to releasing FFAs, adipose tissue is an endocrine organ that secretes adipocytokines which affect insulin action (67). In obese nondiabetic individuals and type 2 diabetics adipocytes become dysfunctional producing excessive amounts of insulin resistance-inducing, inflammatory, and atherosclerotic-provoking cytokines such as resistin, angiotensinogen, plasminogen activator inhibitor-1 (PAI-1), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and leptin; and fail to secrete normal amounts of insulin-sensitizing adipocytokines such as adiponectin (68).

Lastly, genetic or acquired alterations in glucose metabolism (e.g. FFA-induced impairment in glycolysis or glycogen synthesis) may result in an elevation in intracellular glucose concentration causing intracellular glucose toxicity. This in turn could lead to the increased routing of glucose into the hexosamine biosynthesis pathway, which is capable of desensitizing the glucose transport system to insulin, therefore playing a role in insulin resistance (4).

2.4.1.3 Insulin Resistance Syndrome

The insulin resistance syndrome is characterized by insulin resistance and compensatory hyperinsulinemia, and it is this combination that differentiates it from type 2 DM (4). The combination of insulin resistance and compensatory hyperinsulinemia prevents the development of overt hyperglycemia, but greatly increases the risk of having some degree of glucose intolerance, a high plasma triglyceride and low high-density lipoprotein cholesterol concentration, and essential hypertension. In 1988, it was proposed that individuals exhibiting this cluster of abnormalities associated with insulin

resistance/compensatory hyperinsulinemia were at significantly increased risk of CVD, and furthermore, the cluster of abnormalities was referred to as *syndrome X* (69). More recently the report of the Adult Treatment Panel III of the National Cholesterol Education Program acknowledged the importance of insulin resistance/compensatory hyperinsulinemia as CVD risk factors and proposed specific diagnostic criteria for what they termed the *metabolic syndrome* (70). Other than the inclusion of abdominal obesity, the criteria selected by the Adult Treatment Plan III are those initially proposed to comprise syndrome X. While the designation *metabolic syndrome* provides a descriptive listing of clinical findings that cluster within an individual without implying any mechanistic explanation that accounts for the observed relationship, the term *insulin resistance syndrome* provides a physiological construct to explain why insulin resistant/hyperinsulinemic individuals are at increased CVD risk (71). Over several years, new risk factors were added to the metabolic syndrome including elevated uric acid, increased PAI-1, and central (visceral) obesity (72, 73).

The metabolic syndrome is considered to be a strong prediabetic condition as there is a high rate of conversion from impaired glucose tolerance to type 2 DM (4). Furthermore, the metabolic syndrome has a complex and multifactorial pathophysiology involving genetic and environmental components. Genetic influences on risk factors such as body weight, fat distribution, and fasting insulin level may play a role in developing components of the metabolic syndrome (4). Environmental factors such as a sedentary lifestyle, smoking, and progressive weight gain, particularly central in distribution, can also lead to the metabolic syndrome (4).

2.4.2 Impaired Insulin Secretion

In the development of type 2 DM the progressive decrease in insulin action is followed by an increase in insulin secretion by the pancreatic β-cells to compensate for the insulin resistance. Eventually the β-cells fail to produce enough insulin leading to the development of impaired glucose tolerance or the inability to properly metabolize glucose, and overt type 2 DM (46). Early manifestations of disordered β-cell function include irregularities in glucose transport (glucose is the primary regulator of insulin exocytosis in the β-cell), secretagogue pathways, ion channels, or other processes involved in the synthesis, processing, storage, and release of insulin (74). Genetic factors, glucose toxicity, lipotoxicity, and deposition of amyloid-like material in the islets have also been implicated in β-cell failure in type 2 DM (67).

2.4.2.1 Genetics of Impaired Insulin Secretion

Maturity-onset diabetes of the young (MODY) is a heterogeneous group of disorders characterized by autosomal dominant inheritance, early age of onset, and a primary defect in pancreatic β-cell function (49). MODY can result from mutations in any one of at least six different genes that encode the glycolytic enzyme glucokinase and five transcription factors: glucokinase (MODY 2), hepatocyte nuclear factors-4α, -1α and -1β (MODY 1, MODY 3, and MODY 5 respectively), insulin promoter factor-1 (MODY 4), and neurogenic differentiation 1/β-cell E-box transactivator 2 (MODY 6) (49, 57). MODY may account for 5% of all patients with type 2 DM (46).

In addition to mutations in the nuclear genome, mutations in the mitochondrial genome may also lead to type 2 DM (75). The most common mutation is a substitution of guanidine for adenine at position 3243 of leucine transfer RNA (4). This

mutation results in defects in insulin secretion, specifically failure of glucose to stimulate the insulin secretory response and/or abnormal insulin secretory oscillations (76).

2.4.2.2 Toxicities of the Internal Environment

In the internal environment, toxicities from chronic exposure to elevated glucose and FFA concentrations may directly affect the function of pancreatic β-cells (67). Glucose toxicity is the process wherein chronic exposure to supraphysiological glucose concentrations impairs insulin action and leads to mostly irreversible damage to β-cells. This damage causes defects in insulin synthesis, which in turn leads to decreased insulin content and insulin exocytosis (4). Mechanisms by which glucose toxicity may damage β-cells involve decreased insulin gene expression and chronic oxidative stress (4). There is a tendency to use the terms glucose toxicity, glucose desensitization, and β-cell exhaustion synonymously, however these terms are distinct. β-cell exhaustion refers to the depletion of intracellular insulin stores and is the earlier form of glucose toxicity and is more likely to be reversible (4). In contrast, glucose desensitization is a state of β-cell refractoriness that occurs after repeated or prolonged exposure to high glucose concentrations. This refractoriness is a response by the β-cell to avoid overstimulation by glucose and is readily reversed in a time-dependent manner after restoration of normal glucose concentrations (4).

Lipotoxicity also has a deleterious effect on insulin secretion by the pancreatic β-cells. Normally, FFAs support between 30% and 50% of basal insulin secretion and simultaneously promote glucose-stimulated insulin secretion (52). Therefore, acute exposure of β-cells to physiologic increases in FFAs stimulates insulin secretion (57). In contrast, chronic exposure to elevated FA-CoAs (FFAs are converted

into their FA-CoA derivative within the β-cell) inhibit insulin secretion through operation of the Randle cycle (57) and/or through long-term alterations in the expression of genes encoding proteins involved in glucose-stimulated insulin secretion (60). Increased FA-CoA concentrations within the β-cells also stimulate ceramide synthesis, which augments nitric-oxide synthase. The resultant elevation in nitric oxide increases the expression of inflammatory cytokines, including interleukin-1 and TNF-α, which impair β-cell function and promote β-cell apoptosis (57). There is evidence that chronic hyperlipidemia may be deleterious only in the presence of concomitant hyperglycemia, in contrast to chronic hyperglycemia which can cause β-cell dysfunction in the absence of hyperlipidemia (77).

2.4.2.3 Amyloid Deposition in Islets

Islet amyloid polypeptide (IAPP), also called amylin, has been implicated in progressive β-cell failure in type 2 DM (78, 79, 80). IAPP is a neuroendocrine peptide hormone that is produced and cosecreted with insulin from the pancreatic β-cells (57). Following its secretion, IAPP accumulates extracellularly in close proximity to the β-cell and it is has been suggested that amyloid deposits, formed by fibrillation of amylin, cause β-cell dysfunction (57). The mechanisms responsible for amyloid formation in type 2 DM are not completely understood but may be due to increased secretion of IAPP or pro-IAPP, the IAPP precursor, or due to stored IAPP that remains as a sticky residue after the apoptotic death of β-cells (4).

2.4.2.4 Additional Factors Associated with Impaired Insulin Secretion

A deficiency of or resistance to gut derived hormones termed *incretins* and low birth weight have also been implicated in pancreatic β-cell dysfunction in type 2 DM (57). Two gut derived hormones gastric inhibitory peptide (GIP) and glucagon-like

peptide-1 (GLP-1) are released from endocrine cells of the duodenum and jejunum in response to intraluminal carbohydrate but not in response to circulating glucose and subsequently modulate β-cell insulin secretion. In type 2 diabetic patients, the GIP response to glucose ingestion is normal, suggesting the resistance of β-cells to the incretin-effect of GIP (57). In addition, the GLP-1 response to oral glucose is diminished (57). With regard to low birth weight, developmental studies in animals and humans have shown that poor nutrition and impaired fetal growth are associated with impaired insulin secretion or reduced β-cell mass (57). Lastly, the number of β-cells within the pancreas is an important determinant of the amount of insulin secreted. Some studies suggest that β-cell mass is reduced in type 2 DM (81), which may be due to reduced islet neogenesis and/or accelerated β-cell apoptosis.

2.5 Type 1.5 Diabetes and Latent Autoimmune Diabetes

The classification of patients into one of the two principal categories of DM is sometimes difficult as approximately 10% to 30% of patients with phenotypic type 2 DM have autoantibodies to islet cell antigens (82-84). Accordingly a classification system has been implemented in which autoantibody positive patients who are at least 35 years old at diagnosis and who are phenotypically similar to type 1 patients with a lean body type, but who are initially non-ketotic and non-insulin requiring are categorized as having latent autoimmune diabetes in adults (4). Where as, autoantibody positive patients who are phenotypically similar to type 2 patients with a higher body mass index and evidence of insulin resistance are categorized as type 1.5 patients (4).

2.6 Complications of Diabetes Mellitus

Patients with either type 1 or type 2 DM are vulnerable to long-term complications of the disease. The complications that are specific to DM include macrovascular and microvascular disease, both of which contribute greatly to the morbidity and mortality associated with the disease.

2.6.1 Macrovascular Complications

Macrovascular disease affects the large blood vessels of the body, such as the cardiac, cerebral, and peripheral arteries, and may result in coronary artery disease, stroke, and peripheral vascular disease (85). Macrovascular disease is the major cause of morbidity and mortality in patients with DM, accounting for approximately 50% of deaths in individuals with either type 1 or type 2 DM (4). The increased prevalence of macrovascular disease is due to many factors including obesity, dyslipidemia, hyperinsulinemia, hyperglycemia, hypertension, coagulation abnormalities, platelet abnormalities, and endothelial dysfunction (86).

2.6.2 Microvascular Complications

Microvascular disease affects the small vessels of the body, such as those supplying the retina, nerves, and kidneys. Diabetic retinopathy, a retinal disease in DM, is the leading cause of new cases of blindness in individuals 20 to 74 years of age in the United States (4). Diabetic nephropathy, a kidney disease in DM, is the leading cause of end-stage renal disease, accounting for approximately 35% to 40% of new cases of end-stage renal disease requiring renal dialysis or transplantation (87). Diabetic neuropathy, a peripheral nerve disease in DM, is the leading cause of non-traumatic lower extremity amputations, accounting for 50% of amputations in the United States (87). Microvascular

disease is primarily caused by chronic hyperglycemia, though complications also arise from other metabolic alterations including insulin resistance, hypertension, and dyslipidemia (85). The mechanisms by which hyperglycemia causes damage to the microvasculature include increased polyol (sorbitol/aldose reductase) pathway flux, production of advanced glycosylation end products (AGE), generation of reactive oxygen species, and activation of DAG and PKC isoforms (85).

2.7 Therapeutic Strategies for Diabetes Mellitus

Diabetes mellitus is a potentially devastating disease with a high morbidity and mortality associated with increased risk of macrovascular and microvascular complications. Therapeutic strategies that may be effective in the management of DM and prevent or delay its complications are described as follows.

2.7.1 Type 1 Diabetes Mellitus

Given that type 1 DM is a disease of insulin deficiency replacement of this hormone is central to the treatment of the disease. However, insulin therapy is complex and fraught with difficulties. Given the complexities associated with insulin therapy, additional potential therapies for type 1 DM have emerged including controlling glucose homeostasis, immunotherapy, islet transplantation, gene therapy, and islet cell neogenesis and regeneration (88).

2.7.1.1 Insulin Therapy

How, when, and the type of insulin prescribed is only a small part of this therapeutic approach. Previous means of exogenous insulin administration involved intramuscular, intravenous, peritoneal, or subcutaneous injection. More recent insulin therapy uses more patient-friendly methods including inhaled, buccal, or oral application

(89). The development of insulins in which the structure of the molecule has been altered to change its absorption characteristics has resulted in insulin analogues differing in duration of action, such as fast-acting, intermediate-acting, and slow-acting. Insulin therapy is further complicated by the variability and unpredictability of insulin efficacy as well as by lifestyle factors. Variances of insulin efficacy include differences in insulin absorption in different body sites; differences from one injection to another in degree of absorption; inconsistencies in measuring insulin; counterregulatory hormonal effects on insulin action, storage, or depletion of glycogen reserves; and illness (4). Lifestyle factors that effect insulin treatment include how, when, and in what quantities food and snacks are ingested; the glycemic index of foods and liquids; and the effects of activity and day-to-day life stresses (4).

2.7.1.2 Controlling Glucose Homeostasis

One potential therapy for controlling glucose homeostasis is the use of pramlintide, a human amylin (IAPP) analog, as an adjunct to insulin therapy in type 1 diabetic patients who are amylin deficient. Animal studies with amylin and clinical studies with pramlintide have shown that these compounds suppress postprandial glucagon secretion which reduces hepatic glucose output; regulate gastric emptying which slows the rate of nutrient delivery to the small intestine; and act as satiety agents reducing food intake and body weight (90-94).

The glucoregulatory effects of GLP-1 and exendin-4, a unique lizard peptide that exhibits approximately 50% amino acid identity to mammalian GLP-1 and functions as a potent GLP-1 agonist (95), make them potential attractive antidiabetic compounds. In clinical studies, GLP-1 has been found to inhibit gastric emptying,

suppress glucagon secretion, stimulate insulin secretion, and lower appetite (95). In animal studies, both GLP-1 and exendin-4 have been shown to increase insulin secretion and insulin mRNA as well as decrease adipose tissue and body weight (95).

2.7.1.3 Immunotherapies

As previously mentioned, type 1A DM arises as a consequence of immune-mediated destruction of the pancreatic β-cells, hence the goal of immunotherapies is to arrest the immune destruction. Nonspecific immunosuppressive drugs such as cyclosporine A and azathioprine have been reported to slow the disease process and produce long-term remissions (4, 88). However, β-cell toxicity and side effects associated with these agents have limited their use and have led to the development of newer immunosuppressive drugs such as mycophenolate mofetil or rapamycin (sirolimus) (4). Research has also been conducted on immunotherapy strategies that directly inactivate autoreactive T-lymphocytes or induce regulatory cells or mechanisms that re-establish autoimmune tolerance. Such a strategy is the development of antigen-based therapies given as altered peptide ligands or as vaccines (88). Additional promising immune intervention strategies involve the use of monoclonal antibody therapies, specifically anti-T-lymphocyte monoclonal antibodies (88); and the protection of β-cells from immune-mediated destruction through the use of nicotinamide, antioxidants, and a vitamin D analogue as well as through the removal of cow's milk proteins from the neonatal diet (4).

2.7.1.4 Islet Transplantation

Transplantation of the pancreas as a whole organ allows for long-term graft function with more physiologic control of blood glucose levels and eliminates the

need for exogenous insulin administration. However, the procedure is still associated with surgical complications, graft failure, lack of suitable organ donors (88), and perioperative morbidity (96). Therefore, the replacement of pancreatic islets in patients with type 1 DM offers an appealing therapeutic alternative. Potential advantages of islet transplantation versus whole-organ pancreas transplants include the low antigenic burden (i.e. 1-2 g islet packed cell volume vs. a multicellular whole organ of approximately 100 g); a less invasive, simpler, and low-morbidity transplantation procedure involving infusion of pancreatic islets into the portal vein versus surgical vascularization of the pancreatic allograft; and the ability to manipulate islet cells *in vitro* to alter immunogenicity or to produce gene products that favourably alter the islet microenvironment or protect the islet cells from noxious elements that can result in necrosis or apoptosis (4). The use of islet cell transplants for the treatment of patients with type 1 DM is limited in that the availability of human islets from cadaveric pancreata is a finite source and immunosuppressive drugs are required to prevent transplant rejection. These challenges have led to the expansion of islet research in the areas of xenotransplantation, islet encapsulation, stem cells, and genetically engineered β-cells (88).

2.7.1.5 Gene Therapy

Gene therapy involves modifying the expression of an individual's genes or correcting abnormal genes to treat disease (4). Several gene therapy approaches for type 1 DM currently under investigation include manipulating the immune system to induce antigen-specific tolerance; increasing β-cell resistance to immune effectors (97); shielding islet transplants from immune rejection by gene transfer of immunoregulatory

genes; engineering non-β-cells to be glucose-sensing, insulin-producing cells; manipulating islet precursor cells to develop into β-cells; and promoting growth, maintenance, or extension of life-span of islet precursor cells or early β-cells (98).

2.7.1.6 Islet Cell Neogenesis and Regeneration

Pancreatic β-cells are in a state of constant dynamic flux, with formation through either mitotic division of pre-existing differentiated β-cells (replication) or differentiation from precursor epithelial stem cells or pancreatic ductal tissue (neogenesis) and death through apoptosis (4). Given that the development of type 1 DM involves the immune-mediated destruction of β-cells, understanding the mechanisms by which putative precursor cells proliferate and subsequently differentiate into new islet cells may lead to novel treatments (99). There is encouraging evidence that compounds promoting islet neogenesis such as islet neogenesis-associated protein, GLP-1, and exendin-4, may be potential treatments for patients with type 1 DM (88).

2.7.2 Type 2 Diabetes Mellitus

Conventional treatment of type 2 DM is directed at improving blood glucose control through dietary management and physical exercise. In circumstances in which these measures are insufficient or fail, oral antihyperglycemic agents may be helpful. Current oral agents include compounds that promote the synthesis/secretion of insulin by pancreatic β-cells, compounds that enhance the action of insulin at the level of the target tissues, inhibitors of α-glucosidase activity, and inhibitors of FFA oxidation (100). Gene therapy is also a potential alternative treatment for type 2 DM.

2.7.2.1 Dietary Therapy

The new general approach to the dietary therapy of type 2 DM comprises spreading the nutrient load or lengthening the absorption time of carbohydrates (4). This approach involves increasing food frequency (nibbling vs. gorging), consuming viscous soluble fibres (e.g. guar, pectin, β-glucan, psyllim), consuming low glycemic foods (e.g. dried legumes, barley, pasta), and slowing the rate of carbohydrate absorption by the use of α-glucosidase inhibitors (4). Potential effects of prolonging the absorption time of carbohydrates include reducing postprandial increase in insulin and gut hormones (incretins), prolonging suppression of FFA release, and reducing hepatic cholesterol synthesis (4).

2.7.2.2 Exercise

Regular physical exercise plays an important role in both the prevention and treatment of type 2 DM. The benefits of exercise are many and include reduced body fat, increased insulin sensitivity, improved long-term blood glucose control, improved lipid profiles, decreased blood pressure, and increased cardiovascular fitness (4). Before starting an exercise program, patients with type 2 DM should have a complete medical history and physical examination, with particular attention to complications of DM that may affect exercise safety and tolerance and medications that may affect blood glucose control during or after exercise. Exercise programs should consist of moderate-intensity aerobic exercises that can be sustained for at least 30 minutes and achieve a heart rate of 60% to 70% of the patient's predetermined heart rate, with greater intensities or longer durations depending on the level of physical conditioning (4). Patients should exercise at least three days a week and preferably five to seven days a week (4).

2.7.2.3 Insulin Secretagogues

Oral antihyperglycemic agents that improve pancreatic β-cell insulin secretion are termed *insulin secretagogues* and are described as follows. Sulfonylureas are one class of insulin secretagogues which act on the β-cells to increase insulin secretion (100, 101), with a secondary action of improving hepatic and peripheral insulin sensitivity (100). Specific sulfonylurea drugs include glimepiride, glyburide, glipizide, tolazamide, chlorpropramide, and tolbutamide (4). Repaglinide, a nonsulfonylurea insulin secretagogue of the meglitindie class and nateglinide, a D-phenylalanine derivative, also have the ability to stimulate insulin secretion from β-cells (4). In addition, the modulation of insulin secretion by the major incretins, GIP and GLP-1, make these hormones attractive candidates for the treatment of type 2 DM.

2.7.2.4 Insulin Sensitizers

The antidiabetic agents thiazolidinediones (TZD) and biguanides improve insulin sensitivity. TZDs act by modulating the activity of PPAR-γ, a ligand-activated nuclear transcription factor. The expression of PPAR-γ in insulin responsive tissues (fat and muscle) and pancreatic β-cells is associated with lower levels of insulin resistance and improved insulin secretion (4). Troglitazone was the first TZD approved for clinical use but was withdrawn from the market because of associated hepatoxicity (4, 101). The two TZDs in clinical use today are rosiglitazone and pioglitazone (4). The most widely used biguanide metformin improves insulin sensitivity primarily in the liver by promoting glucose uptake and inhibiting glucose production, and secondarily in muscle by augmenting glucose uptake (4).

2.7.2.5 α-Glucosidase Inhibitors

While the development of type 2 DM is not thought to involve abnormalities in gastrointestinal physiology, the digestive process may be manipulated to neutralize some of the characteristic defects in metabolic regulation (4). In the small intestine, complex carbohydrates are digested to oligosaccharides by amylase and further digested by membrane-bound α-glucosidases. α-glucosidase inhibitors competitively bind to the oligosaccharide binding site of the α-glucosidases preventing the enzymatic hydrolysis of the oligosaccharide. In this way, α-glucosidase inhibition delays the breakdown of carbohydrate and thereby promotes the digestion of carbohydrate throughout the length of the intestine. This alteration reduces postprandial increases in plasma glucose which prevents postprandial hyperglycemia and subsequent hyperinsulinemia (4, 100). α-glucosidase inhibitors include acarbose, the most widely prescribed agent, as well as miglitol, voglibose, and emiglitate (4). In addition to its primary effect on postprandial glucose levels, α-glucosidase inhibition has several important secondary actions such as lowering fasting plasma glucose levels and improving insulin sensitivity and lipid metabolism (4).

2.7.2.6 Free Fatty Acid Oxidation Inhibitors

As stated earlier, FFAs impair muscle and hepatic glucose metabolism as well as stimulate hepatic gluconeogenesis. FFA oxidation inhibitors prevent cells from metabolizing fat and consequently force cells to utilize glucose resulting in lower blood glucose levels (100). FFA oxidation inhibitors include etomoxir (carnitine palmitoyl transferase I inhibitor), long-chain acylcarnitine translocase inhibitors, inhibitors of pyruvate carboxylase, and analogues of nicotinic acid such as acipimox (100).

2.7.2.7 Combination Therapy

All of the modalities available for the treatment of type 2 DM, diet, exercise, oral antihyperglycemic agents, may be used as monotherapy. In circumstances in which monotherapy is inadequate or fails, combination therapy may be necessary. It has been demonstrated that combination therapy involving different oral agents may produce synergistic effects in excess of that seen by discontinuing one agent and starting another (100). In addition, exogenous insulin may be required either in addition to an oral agent or alone.

2.7.2.8 Gene Therapy

Although oral antihyperglycemic agents and insulin therapy are beneficial, these treatments have drawbacks including side effects and daily treatment which over the long-term raises the risk for toxicity and resistance. Current research has therefore focused on the possibility of treating type 2 DM by the use of gene therapy. As type 2 DM results from disorders of insulin action and insulin secretion, gene therapy strategies are aimed at re-establishing insulin sensitivity and insulin secretion respectively. Promising approaches for the restoration of normal insulin sensitivity in insulin-sensitive tissues involve genetic manipulation of the insulin receptor, insulin receptor downstream signal transducers, muscle specific insulin-dependent glucose transporters (GLUT4), PPAR-γ, and adipocytokines such as TNF-α and IL-6 (98). Gene therapy approaches for restoring insulin secretion are similar to those mentioned for type 1 DM (e.g. engineering non β-cells); genetic modification of insulinotropic hormones such as GLP-1 is another alternative. In addition, as obesity is a risk factor for type 2 DM, anti-obesity strategies involve reducing satiety, by manipulating the leptin gene for example, and promoting

biochemical pathways that decrease fat and augment glucose and triglyceride utilization (98).

Diabetic Cardiomyopathy

2.8 Diabetic Cardiomyopathy Defined

Cardiomyopathy is defined as disease of heart muscle (10). DM causes the development of a specific diabetic cardiomyopathy–independent of coronary artery, hypertensive, or valvular heart disease–that results from the metabolic derangements present in DM (9). The existence of a diabetic heart muscle disease was first proposed by Rubler *et al.* (102) on the basis of postmortem findings in four diabetic patients with congestive heart failure that could not be attributed to coronary artery, hypertensive, valvular, neuromuscular, or renal disease, or alcoholism. Support for the existence of diabetic cardiomyopathy comes from experimental, pathological, epidemiological, and clinical studies showing that DM results in cardiac functional and structural changes, independent of hypertension, coronary artery disease, or any other known cardiac disease (103).

2.8.1 Functional Changes in Diabetic Cardiomyopathy

The diabetes-specific myocardial contractile dysfunction has been described as early abnormalities in diastolic performance followed by late impairments in systolic performance (104). Characteristic abnormalities in left ventricular (LV) diastolic function present in diabetic patients include prolonged isovolumic relaxation time (IVRT), delayed mitral valve opening and impairment in early diastolic filling, increased atrial contribution of LV filling, and reduced peak early to late diastolic filling velocity (E/A)

ratio (104). LV systolic dysfunction is described by reductions in fractional shortening, velocity of circumferential shortening (103), peak strain and strain rate (9).

2.8.2 Structural Changes in Diabetic Cardiomyopathy

The most prominent structural alteration in diabetic hearts is fibrosis, which may be perivascular, interstitial, or both (103). Myocardial fibrosis is attributed to replacement fibrosis caused by focal myocyte necrosis and increased interstitial fibrosis due to collagen accumulation (103). Collagen accumulation may be due to impaired collagen degradation caused by the glycosylation of the lysine residues on collagen (103). In addition, collagen accumulation may contribute to diastolic dysfunction, as indicated by a study showing that diabetic rats exhibited prolonged deceleration time and reduced peak early diastolic filling velocity, which was associated with collagen deposition and extracellular fibrosis (105).

2.8.3 Mechanisms of Diabetic Cardiomyopathy

The pathophysiology of diabetic cardiomyopathy remains to be completely elucidated, but includes metabolic disturbances, small vessel disease, and cardiac autonomic neuropathy (103).

2.8.3.1 Metabolic Alterations in the Diabetic Myocardium

Metabolic derangements in the diabetic myocardium include alterations in the supply and use of glucose and FFA as well as abnormalities in Ca^{2+} homeostasis.

Diabetic hearts have decreased myocardial glucose uptake and glucose oxidation and increased FFA oxidation with subsequent inhibition of pyruvate dehydrogenase activity (106). The reduction in glucose transport across the sarcolemmal membrane into the myocardium may be due to a reduction in the number of GLUT4

transporters (107), impaired GLUT4 translocation, or decreased intrinsic activity of GLUT4 (57). Reduced glucose influx into the myocardium subsequently decreases glucose phosphorylation and oxidation. Glucose oxidation is also reduced through the inhibitory effect of FFA oxidation on pyruvate dehydrogenase activity, the primary enzyme regulating glucose oxidation, due to elevated circulating FFAs (103). The elevation in circulating FFAs is due to enhanced adipose tissue lipolysis as well as hydrolysis of augmented myocardial triglyceride stores (103). Furthermore, the increase in FFA oxidation results in increased myocardial oxygen consumption and the intracellular accumulation of toxic intermediates, leading to deleterious effects (108). These effects include the accumulation of long-chain acylcarnitines that can cause free radical-mediated damage to both the sarcolemma and intracellular membranes, which in turn could perturb ion pumping and exchange mechanisms (109).

Hyperglycemia induces the production of reactive oxygen and nitrogen species, leading to oxidative stress in the myocardium (103, 106, 110). The heart is susceptible to oxidative stress because it contains low levels of free radical scavengers (106). Oxidative stress causes abnormal gene expression, altered signal transduction, and activation of pathways leading to apoptosis (103, 106).

Hyperglycemia also leads to the nonenzymatic glycosylation of proteins or the interaction of glucose with protein (4) which can alter the function these proteins. These glycosylated proteins can undergo further chemical change to form AGEs (111). These compounds can link to amino acids on other similar proteins, forming crosslinks, which can cause further damage. In the diabetic myocardium AGEs form stable and irreversible crosslinks with adjacent collagen polymers resulting in fibrosis and reduced

myocardial compliance (112). DM also causes the formation of AGEs on SERCA2a resulting in slowed cardiac relaxation (113). Furthermore, it is possible for cytosolic proteins such as contractile proteins to be glycosylated (109). AGE formation can promote free radical formation, thereby aggravating oxidative stress, and conversely free radicals can promote AGE formation (4).

Hyperglycemia also alters PKC-regulated pathways. Increases in the activity of PKC can cause alterations in myocardial contractility through inhibition of myofibrillar ATPase by phosphorylation of troponin T and I and through inhibition of SR Ca^{2+} uptake by phosphorylation of PLB (106). In addition, increased PKC β isoform activity in myocardium of diabetic rats is associated with increased expression of connective tissue growth factor and transforming growth factor β (114), both of which are important mediators of tissue fibrosis (106).

In diabetic cardiomyopathy, there are abnormalities in the regulation of Ca^{2+} homeostasis which results in altered cardiac contractile function. This impairment may be due to alterations in the expression and function of proteins that regulate intracellular Ca^{2+} such as the SERCA2a and the sarcolemmal sodium-calcium exchanger (NCX). These changes may result from the accumulation of toxic molecules such as long-chain acylcarnitines, the formation of AGEs and reactive oxygen species, as well as alterations in membrane lipid composition (103). Diminished Ca^{2+} sensitivity of the regulatory proteins involved in the actin-myosin complex, along with shifts in cardiac myosin isoenzyme distribution from V_1 with high ATPase activity to V_3 with low ATPase activity may also contribute to impaired cardiac contractile function (103).

2.8.3.2 Small Vessel Disease

Whether small vessel disease contributes to diabetic cardiomyopathy remains controversial (103, 106). Chronic DM can lead to coronary microvascular changes including abnormal vascular sensitivity and reactivity to various ligands, depressed autonomic function, increased stiffness of the vascular wall, and abnormalities of various proteins that control ion movements, particularly intracellular Ca^{2+} (103). While it is still unclear how these abnormalities lead to diabetic cardiomyopathy, one potential mechanism involves myocardial ischemia which can result from both structural and functional abnormalities in small vessels during increased myocardial demand or from microvascular spasm due to changes in Ca^{2+} distribution. Accordingly, microvascular spasm and reperfusion injury could lead to focal cell loss with the subsequent development of focal fibrosis and reactive hypertrophy in response to myocardial necrosis (103).

2.8.3.3 Cardiac Autonomic Neuropathy

Sympathetic autonomic dysfunction may play a role in the development of diabetic cardiomyopathy. The change in sympathetic innervation may be associated with alterations in catecholamine levels and adrenergic receptors in the myocardium (103). Cardiac norepinephrine content and β-adrenergic receptor density have been reported to be increased during the early stage of DM (115). However, as the diabetic state developed cardiac norepinephrine content, β-adrenergic receptor density, and adenylyl cyclase activity returned to control levels. Conversely, some studies have shown plasma norepinephrine levels to be reduced in diabetic patients (103). Sympathetic autonomic

dysfunction can contribute to cardiac contractile dysfunction as catecholamines activate Ca^{2+} channels and pumps in myocardial cell membranes.

Calcium Handling

2.9 Calcium Handling in the Normal Heart

Ca^{2+} ions play a pivotal role in cardiac excitation-contraction coupling, which is the process from electrical excitation of the myocyte to contraction of the heart (12). It is therefore important to understand the mechanisms that regulate intracellular Ca^{2+} concentration in the heart. During the cardiac action potential, a small amount of (trigger) Ca^{2+} enters the cell through the sarcolemmal L-type Ca^{2+} channel, also termed the *dihydropyridine receptor (DHPR)*. This trigger Ca^{2+} initiates the release of a large amount of (activator) Ca^{2+} from the SR through the Ca^{2+} release channel or ryanodine receptor (RyR2), a phenomenon termed *calcium-induced calcium release* (116). The combination of Ca^{2+} influx and release raises the free intracellular Ca^{2+} concentration, allowing Ca^{2+} to bind to the myofilament protein troponin C enabling the interaction between actin and myosin, leading to force generation. The magnitude of force development is dependent upon the sensitivity of the myofilaments to Ca^{2+} as well as the amplitude or duration of the Ca^{2+} transient (12). For relaxation to occur the intracellular Ca^{2+} concentration must decline allowing Ca^{2+} to dissociate from troponin. This is primarily achieved by the resequestration of activator Ca^{2+} into the SR through the SERCA2a as well as by extrusion through the sarcolemmal NCX. In addition, the sarcolemmal Ca^{2+}-ATPase and mitochondrial Ca^{2+} uniporter remove negligible amounts of Ca^{2+}.

2.9.1 Calcium Handling in the Diabetic Heart

Alterations in Ca^{2+} handling play an important role in the pathogenesis of diabetic cardiomyopathy. Studies in murine models of type 1 DM have demonstrated unaltered (16) or reduced (117) L-type Ca^{2+} channel activity, decreased NCX expression and function (16, 118), as well as depressed sarcolemmal Ca^{2+}-ATPase activity (119). In addition, numerous studies have reported defects in the function and regulation of the SR in type 1 diabetic murine models (15-26). In human cardiomyocytes of patients with type 2 DM L-type Ca^{2+} channel activity was unaltered (120), while studies in murine models of type 2 DM have shown unaltered (121) or decreased (122) NCX activity as well as depressed sarcolemmal ATP-dependent Ca^{2+} transport (123). There is scanty and conflicting information available on the function of the SR in murine models of type 2 DM as three studies have reported decreased SR function (36, 121, 124) while one study has reported augmented SR function (125). There are also contradictory results on the effect of type 1 DM on myofilament sensitivity to Ca^{2+} with studies reporting increased (126), unaltered (127), or decreased (128) sensitivity. There is a relative lack of data on myofilament sensitivity in type 2 DM.

2.10 The Sarcoplasmic Reticulum

The SR regulates intracellular Ca^{2+} concentration by delivering trigger Ca^{2+} to initiate cardiac contraction and by taking up and storing Ca^{2+} during cardiac relaxation (116). Anatomically, the SR is a fine network (in Latin *reticulum* means small network) spreading throughout the myocytes, defined by its lipid bilayer (129). The SR can be divided into two regions the subsarcolemmal cisternae, that contain the Ca^{2+} release channels through which Ca^{2+} flows to initiate contraction, and an extensive sarcotubular

network that contains a densely packed array of Ca^{2+} pump ATPase proteins (116). In the myocardium, the subsarcolemmal cisternae are located both beneath the plasma membrane and alongside the transverse tubular system, while the sarcotubular network surrounds the contractile proteins (116).

2.10.1 The Sarcoplasmic Reticulum Calcium Release Channels

Ca^{2+} release from the SR is facilitated by RyR2, the main RyR isoform in cardiac muscle. The RyR2 is a very large protein with a molecular weight of 565 kilodaltons (kDa) and it exists as a tetramer (130). The RyR2 also serves as a scaffolding protein for numerous regulatory proteins which can modulate RyR2 function, making it a huge macromolecular complex (130). These RyR2 complexes are arranged in arrays of up to 200 RyR2s at the junction between the SR and the sarcolemmal DHPRs, where corners of neighbouring RyR2 tetramers may interact physically and even functionally in coupled gating (130). The proteins that interact with the cytoplasmic part of the RyR2 include calmodulin; FK-506 binding protein-12.6 (FKBP-12.6); PKA, which is bound to RyR2 via its targeting protein muscle A kinase anchoring protein (mAKAP); CaMK II; PP1 and protein phosphatase 2A (PP2A) that are bound to RyR2 via their targeting proteins spinophilin and PR130 respectively; sorcin; triadin; junctin; calsequestrin; and Homer (130).

2.10.2 The Sarcoplasmic Reticulum Calcium Pump

The transport of Ca^{2+} from the cytosol into the SR is facilitated by SERCA2a, the cardiac isoform of 110 KDa molecular weight. SERCA2a physically interacts with its regulatory protein PLB and this interaction can be disrupted by an elevated Ca^{2+} concentration or by phosphorylation of PLB (131). The amount of activator Ca^{2+}

resequestered by SERCA2a varies between species as it removes 70% of activator Ca^{2+} (NCX extrudes 28%) in rabbit ventricle versus 92% (NCX extrudes 7%) in rat ventricle (12). The activity of SERCA2a in mouse is quantitatively like the rat (132), whereas activity in ferret, dog, cat, guinea-pig, and human is more like the rabbit (133).

2.10.3 Regulation of Sarcoplasmic Reticulum Function

The mechanisms involved in regulating SR function include the complementary reactions of protein phosphorylation by protein kinases and protein dephosphorylation by protein phosphatases (PP). Phosphorylation of the SR Ca^{2+} cycling proteins is mediated by the protein kinases PKA and CaMK II, while dephosphorylation is mediated by PP1 and PP2A, the major PPs in the heart (134). PP2B also plays a role in protein dephosphorylation in heart muscle (135).

SR Ca^{2+} release is augmented by phosphorylation of RyR2 by PKA at amino acid residue serine-2809 in rabbit or the corresponding amino acid residue serine-2808 in human (136). Marx *et al.* (135) proposed that PKA-mediated RyR2 phosphorylation dissociates FKBP12.6 from RyR2, which relieves inhibition of the channel and consequently increases RyR2 open probability and sensitivity to Ca^{2+}-induced activation; however, this mechanism is not supported by others (137, 138). CaMK II also phosphorylates RyR2 increasing RyR2 Ca^{2+} sensitivity and open probability (139). Previous studies have suggested that CaMK II phosphorylates RyR2 at serine-2809 (136, 140), while a recent study by Wehrens *et al.* (139) showed that serine-2815 is the principal CaMK II phosphorylation site on RyR2. Contradictory results have also been reported regarding the functional effects of CaMK II phosphorylation of RyR2 (141).

PP1, PP2A (130), and PP2B (142) dephosphorylate RyR2 thereby decreasing SR Ca^{2+} release.

SR Ca^{2+} uptake by SERCA2a may be modulated through its accessory protein PLB or by direct phosphorylation. In its unphosphorylated state PLB inhibits SERCA2a activity, while PKA-mediated phosphorylation of the serine-16 residue or CaMK II-mediated phosphorylation of the threonine-17 residue relieves this inhibition and thereby increases the affinity of SERCA2a for Ca^{2+} (143, 144). Some studies have also reported an increase in the maximal velocity (V_{max}) of SR Ca^{2+} transport upon PLB phosphorylation (145, 146). Similarly, the direct phosphorylation of the serine-38 residue of SERCA2a by CaMK II increases the V_{max} of SR Ca^{2+} transport (147). However, a few studies have found SERCA2a phosphorylation to have no effect on SR Ca^{2+} uptake (148, 149). The stimulatory effects of the protein kinases on Ca^{2+} uptake can be reversed primarily by PP1 which can dephosphorylate PLB at both the PKA and CaMK II phosphorylation sites (134). PP2B dephosphorylation of PLB at threonine-17 has also been shown to decrease SR Ca^{2+} transport (14).

It should be acknowledged that cGMP-dependent protein kinase phosphorylates PLB at serine-16 *in vitro*, but not in the intact cardiac muscle (150). Furthermore, PKC phosphorylates PLB at serine-10, however this mechanism has been shown to increase (151, 152) or decrease (153, 154) SR Ca^{2+} uptake.

2.10.4 Alterations in the Sarcoplasmic Reticulum in Diabetes Mellitus

Alterations in the function and regulation of the SR have been well documented in murine models of type 1 DM (15-26). However in comparison there are only four studies on SR function in murine models of type 2 DM (36, 121, 124, 125), one of which was

conducted in an insulin resistant model (36). Furthermore, no study to date has examined SR regulation in insulin resistance or type 2 DM.

2.10.4.1 The Sarcoplasmic Reticulum in Type 1 Diabetes Mellitus

A number of studies have shown that SR dysfunction in type 1 DM is associated with decreased SR Ca^{2+} uptake activity (17, 19-21, 23, 25), and this decreased activity has been further differentiated as decreases in the maximum Ca^{2+} uptake rate and affinity of SERCA2a for Ca^{2+} (18, 26). The depression in the resequestration of Ca^{2+} into the SR occurs primarily through a reduction in SERCA2a protein content (16, 18, 20, 24-26) as well as through an increase in PLB protein content (16, 18, 26). However, protein content of PLB has also been reported to be decreased, though the ratio of PLB to SERCA2a was increased (25). The decrease in the reuptake of Ca^{2+} into the SR may also be attributed to a reduction in the phosphorylation of PLB by PKA and CaMK II (25, 26). The depression in SR function in type 1 DM is also associated with decreased SR Ca^{2+} release (16, 20). The depression in SR Ca^{2+} release results from a decrease in the protein expression of RyR2 (15, 16, 20) as well as from a loss of RyR2 function (15).

In addition to the impairments in SR function, there are alterations in the SR regulatory mechanisms, phosphorylation and dephosphorylation, in the type 1 diabetic heart. The activities of the SR-associated PKA and CaMK II have been reported to be increased (20, 25). Similar augmentation in the activities of the SR-associated PPs have been reported (20, 22, 25), which suggests that enhanced dephosphorylation of the SR Ca^{2+} cycling proteins may also contribute to SR dysfunction. The SR Ca^{2+} store has also been reported to be decreased in type 1 diabetic hearts (16).

2.10.4.2 The Sarcoplasmic Reticulum in Type 2 Diabetes Mellitus

To date three studies have examined SR function in murine models of type 2 DM. One such study conducted in the streptozotocin (STZ)-induced type 2 diabetic rat model reported decreased SR Ca^{2+} uptake and SR Ca^{2+}-Mg^{2+}-ATPase activity (124). Conversely, a study conducted in the genetic JCR:LA corpulent (cp/cp) rat model reported increased SR Ca^{2+} uptake and unaltered SR Ca^{2+}-ATPase activity and [^{3}H]ryanodine binding (125). As [^{3}H]ryanodine binds only to RyR2, [^{3}H]ryanodine binding is dependent on the amount of RyR2 in each SR vesicle and the functional integrity of RyR2 (15). A third study conducted in the genetic diabetic (db/db) mouse model reported decreased Ca^{2+} handling by the SR based on decreases in caffeine-induced Ca^{2+} release and decay rate of the Ca^{2+} transient in myocytes (121). The decrease in SR Ca^{2+} handling in the db/db mice was also attributed to alterations in SR protein content, specifically small non-significant decreases in SERCA2a and serine-16 PLB and a large increase in PLB; RyR2 protein content was unaltered (121).

Furthermore, only one study has assessed SR function in an insulin resistant (i.e. prediabetic) murine model. This study reported slowed SR Ca^{2+} uptake based on slowed cytosolic Ca^{2+} removal and myocyte relaxation in myocytes from sucrose-induced insulin resistant rats (36). No change in SERCA2a protein content as well as PLB protein content and extent of phosphorylation were also reported (36).

To date no study has examined the SR regulatory mechanisms of protein phosphorylation and dephosphorylation by the SR-associated protein kinases and protein phosphatases respectively in insulin resistant or type 2 diabetic murine models.

2.11 Models of Diabetes Mellitus

There are many different experimental models with which to study the pathophysiology of type 1 and type 2 DM. Each particular model has advantages and disadvantages regarding its use and applicability to the clinical situation. However, no model of animal diabetes precisely replicates all the clinical features of the human diabetic condition (155).

2.11.1 Murine Models of Type 1 Diabetes Mellitus

Three different models of type 1 DM have been used to study the function and regulation of the SR: STZ-treated young adult rats or mice (15-18, 20-22, 24-26), alloxan-treated rats (19), and spontaneously diabetic BB rats (23). These murine models exhibit β-cell damage and necrosis and the resultant hypoinsulinemic condition that resembles the type 1 diabetic state in humans (156).

2.11.2 Murine Models of Type 2 Diabetes Mellitus

Murine models of type 2 DM can be broadly divided into three categories: chemically-treated, diet-induced, and genetic. The chemically-treated model is produced by injection of neonatal rats with STZ (157); the diet-induced models involve sucrose or fructose feeding; and the genetic models include the JCR:LA cp/cp rats, db/db mice, Otsuka Long-Evans Tokushima fatty (OLETF) rats, Zucker diabetic fatty (ZDF) rats, and Goto Kakizaki rats. Unlike the murine models of type 1 DM which exhibit the primary cause of the disease, the murine models of type 2 DM are heterogeneous.

2.11.2.1 The High Sucrose Model of Insulin Resistance

Sucrose is a disaccharide composed of one molecule of glucose and one molecule of fructose. During absorption, sucrose is hydrolyzed into equal amounts of

glucose and fructose, of which fructose has been found to be the primary nutrient mediator of sucrose-induced insulin resistance (158). The high sucrose model can be achieved by the administration of sucrose in solid or liquid form. High sucrose diets administered in solid form commonly compose 68% of total calories (28-31, 33, 35, 36, 158, 159), while liquid diets are commonly composed of 30% to 35% sucrose in drinking water (27, 160, 161). No difference in results between the two forms of sucrose feeding (35% sucrose solid diet vs. 35% sucrose solution) has been reported (160).

Diets rich in sucrose have been shown to produce whole body insulin resistance in male rats (27-36). In contrast to male rats, female rats do not develop sucrose-induced insulin resistance (159). Characterization of the development of sucrose-induced insulin resistance in male rats has shown that hepatic insulin resistance precedes muscle insulin resistance (33). The metabolic abnormalities associated with high sucrose feeding include hyperinsulinemia, hypertriglyceridemia, and hyperglycemia (34, 162, 163), as well as significant elevations in plasma cholesterol (27, 162) and body weight. Therefore, high sucrose feeding is an excellent model of multifactorial diseases–such as the metabolic syndrome, obesity, and type 2 DM in humans–and is superior to monogenic murine models in which a single gene mutation is the predominant cause of disease development. Furthermore, the insulin resistance caused by high sucrose feeding results in cardiac contractile dysfunction, specifically reduced myocyte contractility (28-32, 36) and decreased myofibrillar protein ATPase activities (34). As prediabetic metabolic derangements such as insulin resistance may produce abnormalities in cardiac structure and function before the development of overt DM (10) and given that high sucrose feeding has been shown to lead to the development of cardiac contractile

dysfunction (28-32, 34, 36), the high sucrose model is highly relevant for the study of diabetic cardiomyopathy. Additional benefits of this model are that it is economical and highly reproducible.

III. STATEMENT OF HYPOTHESIS

Diabetes mellitus causes a cardiomyopathy characterized by reduced cardiac contractile function that is primarily the result of changes in Ca^{2+} handling within the myocyte. Numerous studies have suggested that a dysfunctional SR, leading to alterations in Ca^{2+} handling, is responsible for decreased contractile function in type 1 DM. Conversely, there is a relative lack of data on the affect of type 2 DM on cardiac contractile function and specifically SR function and its regulation. Insulin resistance is commonly the first lesion in the development of type 2 DM and can have detrimental effects on the heart. Accordingly, this study provides a serial echocardiographic characterization of heart structure and function during the progression of insulin resistance. Furthermore, this study determines the role of SR Ca^{2+} cycling and its regulatory mechanisms in modulating myocardial Ca^{2+} handling and contractile function in the insulin resistant state. This aspect has not been investigated before and therefore this study will provide invaluable information on the phenotypic and intracellular changes that occur in the hearts of insulin resistant individuals.

We hypothesize that high sucrose feeding will induce whole body insulin resistance in association with derangements in glucose and lipid metabolism. These prediabetic metabolic derangements will cause abnormalities in cardiac structure and function and therefore a diabetic-like cardiomyopathy before the development of overt type 2 DM. Furthermore, the alterations in cardiac contractile function will be associated with alterations in the function and regulation of the SR.

IV. MATERIALS AND METHODS

The following experimental protocol was approved by the Animal Care Committee of the University of Manitoba and conforms to the guidelines of the *Canadian Council on Animal Care Concerning the Care and Use of Experimental Animals* (Vol. 1, 2nd Edition, 1993).

4.1 Animal model

Four week old male Sprague-Dawley rats (Central Animal Care, University of Manitoba) weighing 75 to 100 grams at the time of arrival were housed two to three per cage under controlled temperature (20°C ± 2°C), humidity (30% to 70%), and lighting (12h:12h light-dark cycle). On arrival, all rats were given standard chow and water *ad libitum*. After one week, rats were randomly assigned to one of two groups. The control group continued to receive water and rat chow while the experimental group received 32% (935 mM) sucrose (Sigma, St. Louis, MO) in their drinking water and rat chow.

4.2 Experimental Protocol

The control and sucrose-fed rats underwent echocardiographic and metabolic assessments at 1, 2.5, 5, and 10 weeks post-diet initiation. At the 2.5, 5, and 10-week time points a random subset of control and sucrose-fed rats were sacrificed. Prior to sacrifice, rats were weighed and anaesthetized using a cocktail of ketamine (90mg/kg) and xylazine (10mg/kg). Hearts were excised, washed in 0.9% (154 mM) NaCl and total heart weights were measured. The atria were then removed and the remaining heart tissue was quick frozen in liquid nitrogen. The heart tissue was subsequently stored at -85°C until experimentation.

4.3 Echocardiographic assessment of cardiac structure and function

Rats were weighed and anaesthetized with 5% isoflurane and maintained under anaesthetic with 2% isoflurane (Ohio Medical Products, Madison, WI) carried by oxygen at a flow rate of 2 l/min. Two-dimensional transthoracic echocardiography was performed using a SONOS 5500 ultrasound system (Aligent Technologies, Andover, MA). A 12-MHz probe coated with ultrasonic transmission gel (Parker Laboratories, Inc., Fairfield, NJ) was placed in the parasternal short-axis orientation to obtain M-mode and pulsed wave Doppler images. LV M-mode tracings were taken at the level of the papillary muscles at a sweep speed of 150 mm/s and a depth setting of 3 cm. M-mode images were used to measure the following parameters according to the leading-edge method of the American Society of Echocardiography (164): interventricular septal dimensions (IVS), left ventricular posterior wall dimensions (LVPW), and left ventricular internal dimensions (LVID) at both end diastole (IVSd, LVPWd, and LVIDd respectively) and end systole (IVSs, LVPWs, and LVIDs respectively). These dimensions were used to calculate the systolic parameters: LV fractional shortening (FS), ejection fraction (EF), and cardiac output (CO) as follows. FS = [(LVIDd-LVIDs)/LVIDd] X 100, EF = [(end diastolic volume (EDV) – end systolic volume (ESV)/EDV], CO = [(EDV – ESV)/1000] X heart rate (165). EDV and ESV were calculated by the cubic formulas $LVIDd^3$ and $LVIDs^3$ respectively (165).

Doppler waveforms of mitral inflow and aortic outflow were obtained from the apical 4-chamber and 5-chamber views respectively. Diastolic variables determined from the apical 4-chamber view included the peak early diastolic filling velocity (E wave), peak late diastolic filling velocity (A wave), E/A ratio, and E wave deceleration time (E

decel time), which is the time interval of peak E wave velocity to zero. IVRT, the time from the end of systolic ventricular outflow to mitral valve opening, and aortic ejection time (ET) were obtained from the apical 5-chamber view. All measurements were based on the average of three selected cardiac cycles.

4.4 Metabolic measurements

Immediately following echocardiographic assessment 10% of the total circulating blood volume was collected through puncture of the jugular vein of the anaesthetized rats. Blood samples were centrifuged at 1500 rev/min for 10 min and the plasma was stored at -20°C for later analysis. Plasma glucose, triglyceride, and cholesterol levels were measured using the respective diagnostic kits (Roche Diagnostics Corporation, Indianapolis, IN). Plasma insulin levels were determined with an enzyme-linked assay kit (Alpco Diagnostics, Windham, NH).

4.5 Isolation of SR vesicles

SR vesicles were obtained using a method described previously (20, 25, 166-168). Heart tissue placed on ice was pulverized and homogenized using a Polytron homogenizer (Brinkman, Westbury, NY) for a period of 20 sec and 25 sec, separated by a 1 min interval, at a speed of 12,000 rpm. The homogenization buffer contained (in mM) 10 $NaHCO_3$, 5 NaN_3, 15 Tris·HCl (pH 6.8), and the protease inhibitors (in μM), 1 leupeptin, 1 pepstatin, and 100 phenylmethylsulphonyl fluoride. The homogenate was then centrifuged for 20 min at 10,919 g and the supernatant obtained was further centrifuged for 45 min at 43,666 g (JA 20.0 rotor; Beckman Instruments, Inc. Palo Alto, CA). The resultant pellet was suspended in a buffer containing 0.6 M KCl and 20 mM Tris·HCl (pH 6.8) and recentrifuged at the same speed and duration as in the previous

step. The final pellet containing the SR fraction was suspended in a buffer containing 250 mM sucrose and 10 mM histidine (pH 7.0). All steps were performed in the cold room (4°C).

4.6 Measurement of SR Ca^{2+} uptake

Ca^{2+} uptake of the SR vesicles was measured using a procedure described previously (20, 25, 166-168). The reaction mixture contained (in mM) 50 Tris-maleate (pH 6.8), 5 NaN_3, 5 ATP, 5 $MgCl_2$, 120 KCl, 5 K-oxalate, 0.1 EGTA, 0.1 $^{45}CaCl_2$, and 25 μM ruthenium red. The reaction was initiated with the addition of SR vesicles (20 μg protein) to the reaction mixture at 37°C and was terminated after 1 min by filtering 200 μl of the reaction mixture through 0.45 μm membrane filters (Fisher Scientific Ltd., Whitby, ON, Canada). The filters were then washed, dried at 60°C for 1 h, and counted in a β–liquid scintillation counter.

4.7 Measurement of SR-associated PKA and CaMK II activities

Determination of PKA and CaMK II activities of the SR vesicles was based on a technique previously established (20, 25, 166, 167). PKA and CaMK II activities were measured using assay kits from Upstate Biotechnology (Lake Placid, NY). The assay kit for PKA activity is based on the phosphorylation of a specific substrate, kemptide, by the transfer of the γ-phosphate of [γ-^{32}P]ATP by PKA. The assay kit for CaMK II activity is based on the phosphorylation of a specific substrate, autocamtide, by the transfer of the γ-phosphate of [γ-^{32}P]ATP by CaMK II. The reactions for PKA and CaMK II were initiated by adding [γ-^{32}P]ATP to the reaction mixture containing the respective assay dilution buffers, substrates, and inhibitor cocktails and incubating for 10 min at 30°C. The reaction was stopped by spotting the reaction mixture on phosphocellulose filter papers.

The filters were then washed three times with phosphoric acid and once with acetone and counted in a β–liquid scintillation counter. The activities of PKA and CaMK II were calculated as the difference between the values obtained in the presence and absence of the exogenous substrate.

4.8 Measurement of SR-associated PP activity

PP activity of the SR vesicles was measured using a method described previously (20, 25, 166, 167). PP activity was determined using the serine/threonine phosphatase assay kit (Upstate Biotechnology, Lake Placid, NY), which is based on the dephosphorylation of the phosphopeptide KRpTIRR. The reaction was initiated with the addition of SR vesicles (30 μg) to microtiter wells in the presence or absence of the phosphopeptide (200 μM) and incubated for 30 min. The reaction was terminated by adding malachite green, and the absorbance was read after 15 min at a wavelength of 650 nm to determine the amount of inorganic phosphate released.

4.9 Western blot analysis

The protein content of the SR proteins SERCA2a, PLB, and its phosphorylated forms, serine-16 PLB and threonine-17 PLB, were determined by Western blot analysis according to a procedure described previously (20, 25, 167, 168). The protein concentration of SR samples was normalized to 2.0 mg/ml after which SR samples (20 μg) were separated on either 10% (for SERCA2a) or 20% (for PLB, serine-16 PLB, and threonine-17 PLB) gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were probed with mouse monoclonal SERCA2 ATPase (Affinity Bioreagents, Golden, CO), mouse monoclonal PLB (Upstate Biotechnology, Lake Placid, NY), rabbit polyclonal serine-16

PLB, and rabbit polyclonal threonine-17 PLB (Badrilla Ltd, Leeds, UK). Appropriate secondary antibodies were used and the antibody-antigen complexes of all probed membranes were detected using an enhanced chemiluminescence kit (Amersham, Buckinghamshire, U.K.). An imaging densitometer (model GS-800; Bio-Rad Laboratories, Inc., Hercules, CA) was used to scan the protein bands, and the data were quantified using Quality One 4.5.0 software (Bio-Rad Laboratories, Inc., Hercules, CA).

4.10 Statistical analysis

Data were expressed as mean ± standard error (SE) in the figures and tables. Student's t-test was used to compare data between control and sucrose-fed groups. A difference between control and sucrose-fed groups was considered significant for $P<0.05$.

V. RESULTS

The general characteristics of the control and sucrose-fed rats are provided in Table 1. The body weights of the control and sucrose-fed rats were comparable at 1 week after which the sucrose-fed rats gained more weight than the control rats. In contrast, there were no changes in heart weights between the control and sucrose-fed groups at any time points. Due to the substantial difference in body weight at the 10-week time point, the heart weight to body weight (HW/BW) ratio was significantly lower in the sucrose-fed rats. Plasma insulin, glucose, triglyceride, and cholesterol levels were significantly elevated in sucrose-fed than control rats at all time points.

To determine whether changes in cardiac structure and function occurred in the sucrose-fed rats, *in vivo* echocardiography was performed and compared with control rats. Parameters of cardiac structure and function obtained by M-mode and Doppler echocardiography in the control and sucrose-fed rats are provided in Tables 2 and 3 respectively. With regard to cardiac structure, the dimensions of the IVS at diastole and systole did not differ between the control and sucrose-fed rats until 10 weeks when significant thinning of the IVS was observed at both diastole and systole in the sucrose-fed rats. Similarly, the internal dimensions of the LV did not change until the 10-week time point when significant increases in the LVID were evident at both diastole and systole in the sucrose-fed rats. The dimensions of the LVPW at diastole and systole were similar among the two groups at all time points. M-mode echocardiograms of representative left ventricles from the control and sucrose-fed rats at the 10-week time point are displayed in Figure 1.

Table 1. General characteristics of the control and sucrose-fed rats.

	1 week		2.5 weeks		5 weeks		10 weeks	
Parameter	Control (n=8-17)	Sucrose (n=10-19)	Control (n=9-15)	Sucrose (n=9-17)	Control (n=5-16)	Sucrose (n=5-16)	Control (n=8-38)	Sucrose (n=6-40)
Body Weight (g)	199.4 ± 5.0	202.6 ± 4.5	277.7 ± 7.7	302.2 ± 6.5*	421.4 ± 5.6	438.3 ± 6.6	544.9 ± 6.6	617.2 ± 12.1*
Heart Weight (g)	-	-	0.94 ± 0.02	1.00 ± 0.02	1.19 ± 0.02	1.25 ± 0.03	1.41 ± 0.02	1.45 ± 0.02
HW/BW (mg/g)	-	-	3.39 ± 0.05	3.30 ± 0.03	2.81 ± 0.02	2.84 ± 0.04	2.59 ± 0.02	2.35 ± 0.03*
Glucose (mmol/l)	10.7 ± 0.27	12.5 ± 0.31*	8.56 ± 0.50	12.0 ± 0.57*	11.8 ± 0.60	14.9 ± 0.96*	10.3 ± 0.25	11.6 ± 0.52*
Insulin (pmol/l)	15.7 ± 3.1	78.6 ± 21.0*	34.1 ± 8.5	81.6 ± 12.5*	66.1 ± 16.7	147 ± 15.7*	60.4 ± 7.5	200.1 ± 18.3*
Triglycerides (mmol/l)	1.17 ± 0.09	1.99 ± 0.16*	1.81 ± 0.12	2.63 ± 0.18*	1.80 ± 0.16	2.63 ± 0.28*	2.42 ± 0.12	3.74 ± 0.22*
Cholesterol (mmol/l)	2.05 ± 0.04	2.26 ± 0.09*	1.89 ± 0.05	2.06 ± 0.06*	1.70 ± 0.03	2.04 ± 0.12*	1.78 ± 0.04	2.18 ± 0.06*

Data are means ± SE. HW/BW, ratio of heart weight to body weight. *P<0.05 vs. control rats.

Table 2. Parameters of cardiac function and structure obtained by M-mode echocardiography in the control and sucrose-fed rats.

Parameter	1 week		2.5 weeks		5 weeks		10 weeks	
	Control (n=16-17)	Sucrose (n=17-18)	Control (n=69-72)	Sucrose (n=70-74)	Control (n=63-69)	Sucrose (n=65-69)	Control (n=35-39)	Sucrose (n=37-39)
FS (%)	40.8 ± 1.2	41.1 ± 1.1	44.4 ± 0.67	43.2 ± 0.66	41.9 ± 0.67	42.5 ± 0.65	43.2 ± 0.90	39.3 ± 1.4*
EF	0.77 ± 0.01	0.77 ± 0.01	0.80 ± 0.01	0.79 ± 0.01	0.78 ± 0.01	0.78 ± 0.01	0.79 ± 0.01	0.73 ± 0.02*
CO (ml/min)	266.9 ± 10.1	281.6 ± 14.4	272.1 ± 8.7	265.5 ± 7.8	324.6 ± 10.5	343.5 ± 9.7	392.5 ± 11.1	427.1 ± 15.3
HR (beats/min)	391 ± 4.5	398 ± 5.5	382 ± 2.7	399 ± 2.7*	358 ± 2.9	376 ± 2.9*	337 ± 4.1	354 ± 3.6*
IVSd (mm)	1.49 ± 0.06	1.39 ± 0.06	1.69 ± 0.03	1.74 ± 0.04	1.80 ± 0.03	1.84 ± 0.03	1.90 ± 0.06	1.74 ± 0.05*
IVSs (mm)	2.50 ± 0.07	2.41 ± 0.09	2.88 ± 0.04	2.88 ± 0.05	3.06 ± 0.04	3.10 ± 0.05	3.17 ± 0.05	2.91 ± 0.07*
LVIDd (mm)	7.32 ± 0.10	7.41 ± 0.12	7.29 ± 0.08	7.17 ± 0.09	8.08 ± 0.08	8.10 ± 0.08	8.81 ± 0.09	9.26 ± 0.18*
LVIDs (mm)	4.34 ± 0.11	4.37 ± 0.12	4.05 ± 0.07	4.08 ± 0.08	4.68 ± 0.06	4.66 ± 0.08	5.02 ± 0.10	5.68 ± 0.25*
LVPWd (mm)	1.22 ± 0.03	1.30 ± 0.04	1.72 ± 0.05	1.77 ± 0.06	1.87 ± 0.04	1.79 ± 0.04	1.80 ± 0.04	1.77 ± 0.06
LVPWs (mm)	2.15 ± 0.07	2.21 ± 0.07	2.70 ± 0.05	2.76 ± 0.05	2.89 ± 0.04	2.85 ± 0.04	3.03 ± 0.05	2.98 ± 0.08

Data are means ± SE. FS, fractional shortening; EF, ejection fraction; CO, cardiac output; HR, heart rate; IVS, interventricular septal dimensions; LVID, left ventricular internal dimensions; LVPW, left ventricular posterior wall dimensions; s, systole; d, diastole. *P<0.05 vs. control rats.

Table 3. Parameters of cardiac function obtained by Doppler echocardiography in the control and sucrose-fed rats.

	1 week		2.5 weeks		5 weeks		10 weeks	
Parameter	Control (n=6-18)	Sucrose (n=6-19)	Control (n=28-37)	Sucrose (n=27-38)	Control (n=26-36)	Sucrose (n=31-36)	Control (n=8-17)	Sucrose (n=8-15)
E wave (cm/s)	116 ± 4.2	120 ± 4.9	110 ± 3.2	110 ± 2.5	116 ± 2.3	119 ± 2.1	109 ± 3.5	119 ± 5.9
A wave (cm/s)	77.4 ± 6.7	75.9 ± 5.3	72.9 ± 2.6	79.2 ± 1.2*	76.1 ± 2.4	87.8 ± 2.2*	69.8 ± 4.0	81.6 ± 3.0
E/A	1.55 ± 0.10	1.60 ± 0.06	1.55 ± 0.06	1.40 ± 0.03*	1.56 ± 0.05	1.36 ± 0.02*	1.61 ± 0.13	1.47 ± 0.09
E decel (ms)	40.7 ± 1.6	41.5 ± 4.9	42.1 ± 1.8	40.1 ± 1.6	47.5 ± 1.6	46.2 ± 1.5	50.9 ± 1.6	52.4 ± 3.8
IVRT (ms)	19.3 ± 0.74	18.8 ± 0.56	19.2 ± 0.48	19.0 ± 0.58	20.3 ± 0.42	19.7 ± 0.39	22.3 ± 0.72	20.1 ± 0.43
ET (ms)	68.9 ± 0.85	66.8 ± 0.86	69.2 ± 0.80	67.8 ± 0.61	69.5 ± 0.73	67.5 ± 0.85	70.6 ± 0.92	70.0 ± 0.99

Data are means ± SE. E wave, peak early diastolic filling velocity; A wave, peak late diastolic filling velocity; E/A, ratio of the peak early to late diastolic filling velocity; E decel, E wave deceleration time; IVRT, isovolumic relaxation time; ET, aortic ejection time. *P<0.05 vs. control rats.

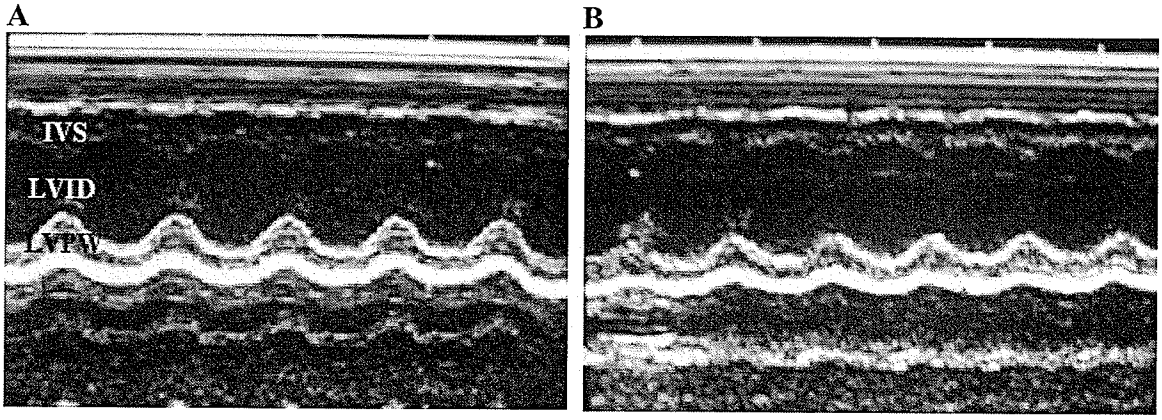

Figure 1. M-mode echocardiograms of representative left ventricles from the control (A) and sucrose-fed (B) rats at 10 weeks.

Subtle yet significant impairments in LV systolic function expressed as FS and EF were observed in the sucrose-fed rats at the final time point and were not different at any of the other time points. There was no change in HR 1 week post-diet initiation, however, after this time point significant increases HR were evident in the sucrose-fed rats. CO was similar among the two groups at all time points.

E wave, A wave, and E/A ratios were measured as parameters of LV diastolic function. The control and sucrose-fed rats exhibited similar E waves throughout the 10-week time course. The two groups showed equivalent A waves at 1 week, after which the sucrose-fed rats exhibited significant increases in the A wave. As a result, the sucrose-fed rats had reduced E/A ratios after 2.5 weeks comparative to control rats. An example of a higher A wave observed in the sucrose-fed rats in comparison to the control rats is displayed in Figure 2. There were no changes in the parameters concerning time, E decel time, IVRT, and ET, among the two groups.

Given the role of the SR in regulating cardiac contraction and relaxation, its function was examined commencing at the 2.5-week time point when a difference in LV diastolic function first became apparent. SR Ca^{2+} uptake was greater in the 2.5-week sucrose hearts relative to control hearts, which was followed by a decrease in SR Ca^{2+} uptake to control values 2.5 weeks later (Figure 3). The decline in SR Ca^{2+} uptake continued as the hearts of the 10-week sucrose-fed rats showed a significant 31% reduction in SR Ca^{2+} uptake compared to control hearts. Given the substantial depression in SR Ca^{2+} uptake at 10 weeks, we proceeded to examine the status of the SR Ca^{2+} cycling and regulatory mechanisms at this time point.

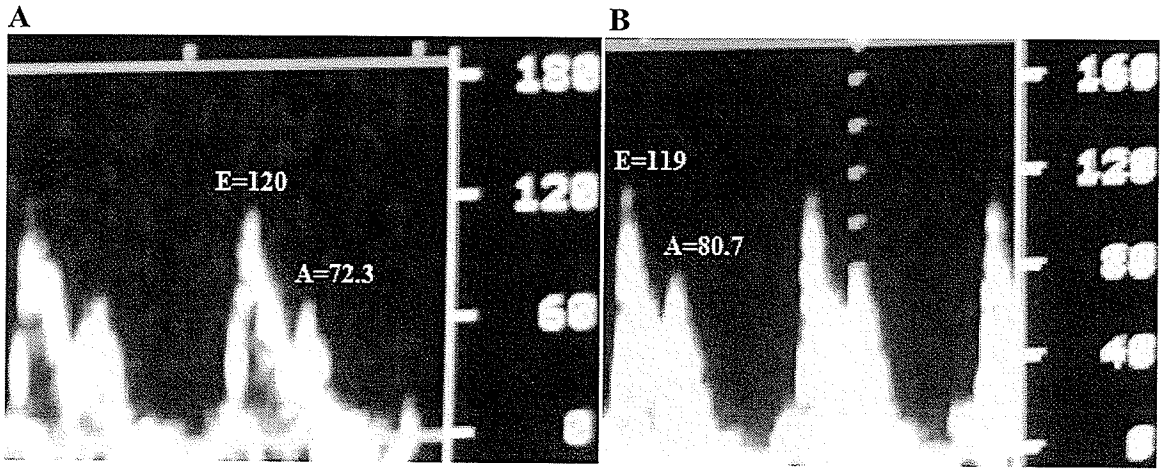

Figure 2. Representative Doppler waveforms from the control (A) and sucrose-fed (B) rats illustrate the increase in peak late diastolic filling velocity (A wave) in the sucrose-fed rats at 5 weeks.

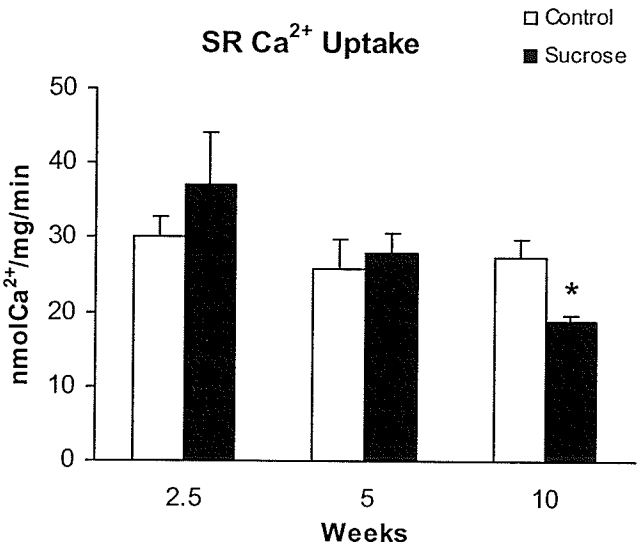

Figure 3. SR Ca^{2+} uptake in the hearts of the control and sucrose-fed rats. The data are mean ± SE. The n values are given for each group at 2.5, 5, and 10 weeks in sequential order: Control (6, 6, 18); Sucrose (6, 6, 15). *$P < 0.05$ vs. control group.

To determine whether the significant reduction in SR Ca^{2+} uptake in the 10-week sucrose hearts was due to alterations in the expression of SR Ca^{2+} cycling proteins, the content of SERCA2a and PLB were measured. Figure 4 shows a marginal decrease in the expression of SERCA2a and concomitant slight increase in the expression of its inhibitory protein PLB in the sucrose hearts comparative to control hearts. Accordingly, the ratio of PLB to SERCA2a (PLB/SERCA2a) protein content was relatively higher in the hearts of the 10-week sucrose-fed rats.

As SR Ca^{2+} uptake is regulated by the PKA and CaMK II-mediated phosphorylation of PLB, the reduction in SR Ca^{2+} uptake in the hearts of the 10-week sucrose-fed rats may be attributed to abnormalities in the phosphorylation of PLB. Examination of the phosphorylation status of PLB in the sucrose hearts yielded a significant 71% decrease in the PKA-mediated phosphorylation of PLB at serine-16 and further, a significant 68% reduction in the CaMK II-mediated phosphorylation of PLB at threonine-17 (Figure 5). To find the underlying mechanisms for the significant reduction in PLB phosphorylation at the serine and threonine residues, the SR-associated PKA and CaMK II activities were assessed. There were significant increases in the activities of both SR-associated PKA (67%) and CaMK II (41%) (Figure 6), indicating that the decline in PLB phosphorylation at the serine-16 and threonine-17 residues respectively was not due to decreased activities of these two kinases. Subsequently, in order to determine if the SR-associated PP contributed to the dephosphorylation of PLB, the SR-associated PP activity was examined in the hearts of the 10-week control and sucrose-fed rats. Figure 7 shows a minor increase in the SR-associated PP activity in the 10-week sucrose hearts comparative to control hearts.

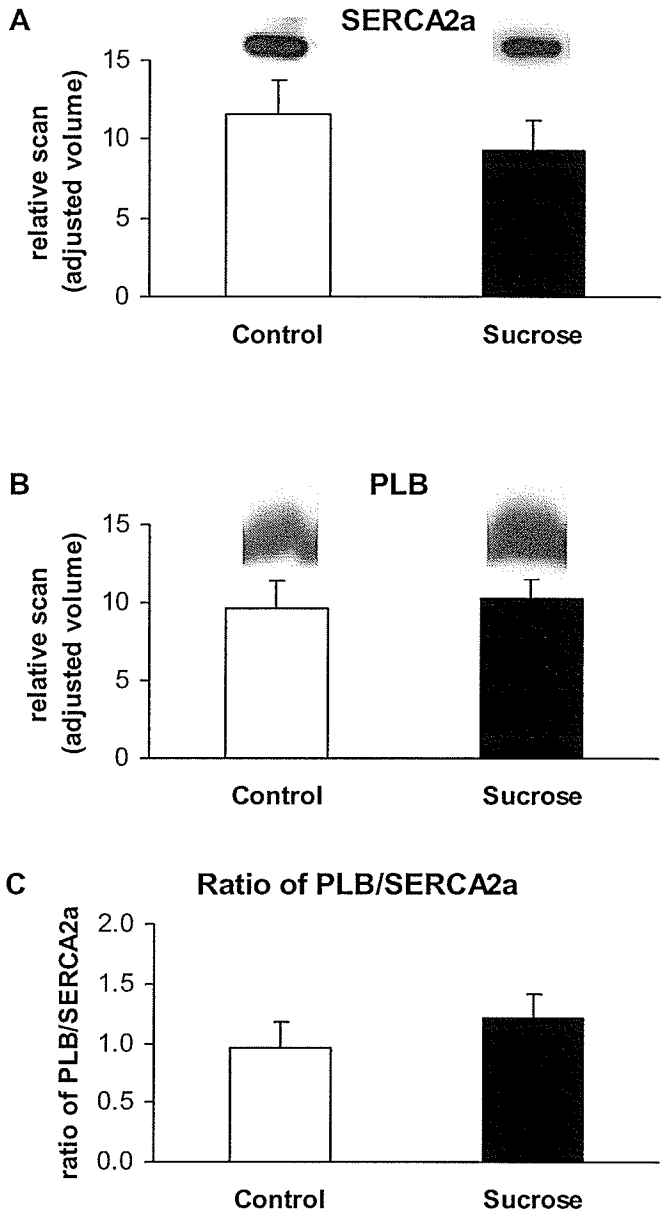

Figure 4. Western blot analysis of sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) (A) and phospholamban (PLB) (B), and the ratio of PLB to SERCA2a (C) in the hearts of the 10-week control and sucrose-fed rats. The data are mean ± SE; n=5.

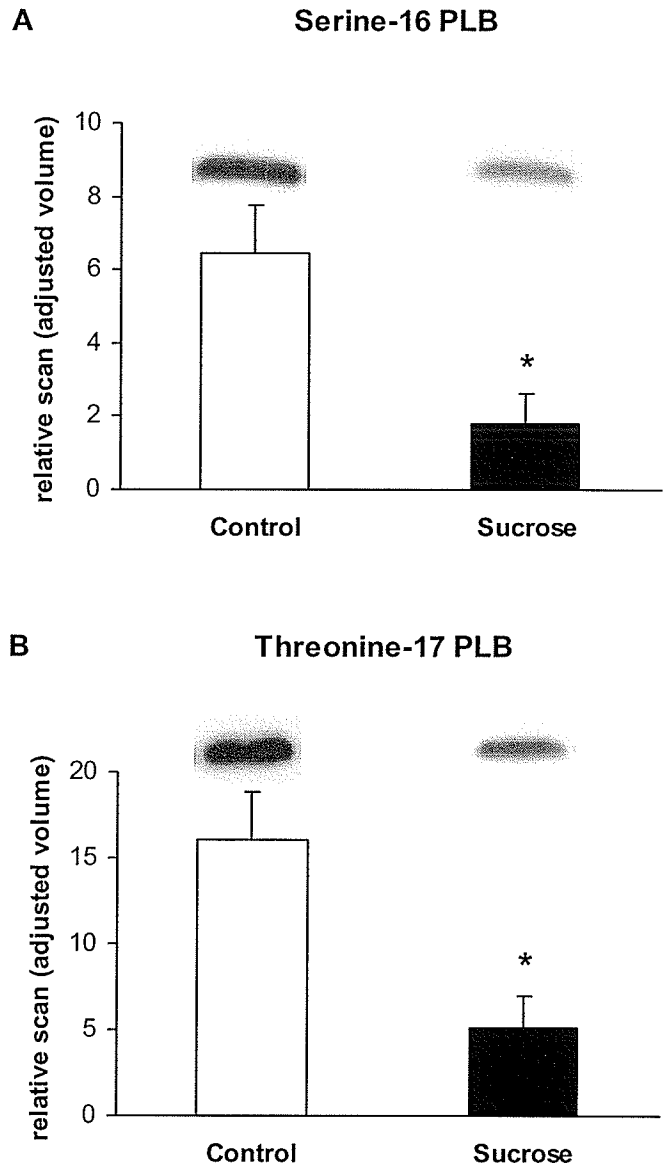

Figure 5. Western blot analysis of the phosphorylation status of phospholamban (PLB) at the serine-16 residue (A) and the threonine-17 residue (B) in the hearts of the 10-week control and sucrose-fed rats. The data are mean ± SE; n=4. *$P < 0.05$ vs. control group.

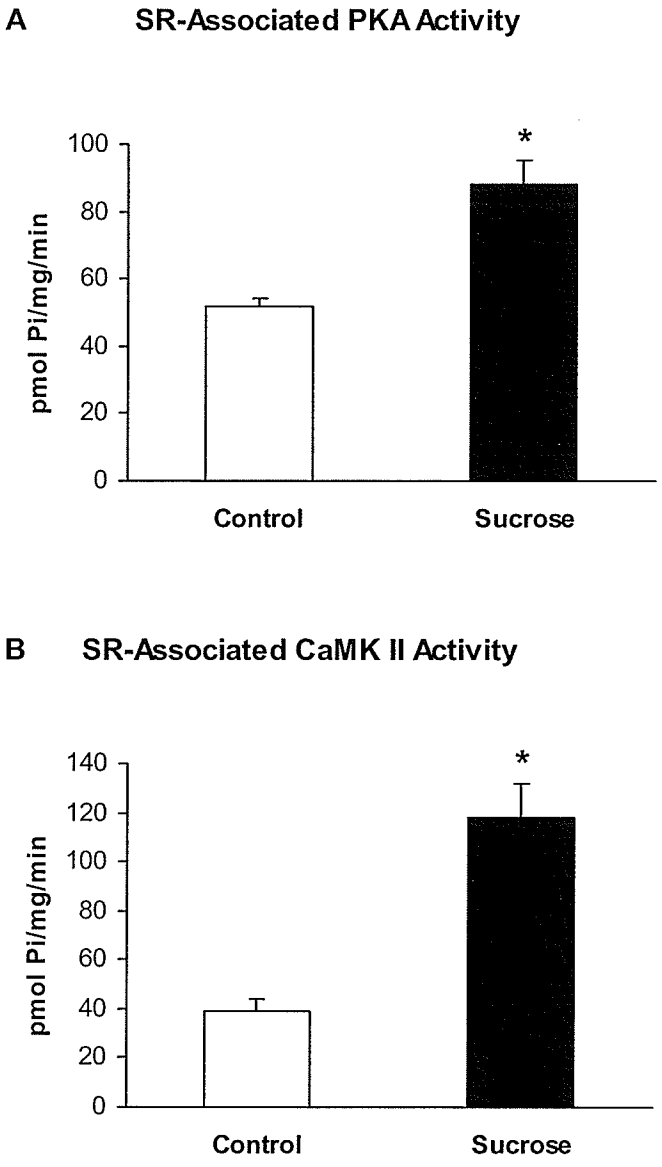

Figure 6. SR-associated cAMP-dependent protein kinase (PKA) (A) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II) (B) activities in the hearts of the 10-week control and sucrose-fed rats. The n values are given for each group for PKA and CaMK II activities in sequential order: Control (5, 5); Sucrose (6, 7). The data are mean ± SE. *$P < 0.05$ vs. control group.

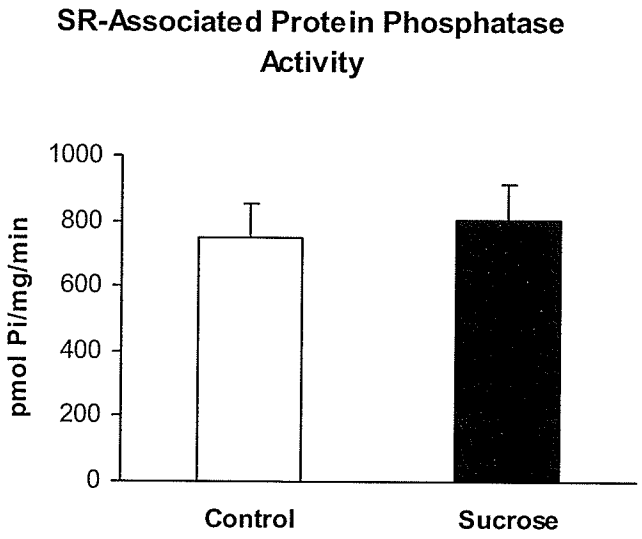

Figure 7. SR-associated protein phosphatase activity in the hearts of the 10-week control and sucrose-fed rats. The data are mean ± SE; n=9.

VI. DISCUSSION

This study examined serial alterations in cardiac structure and function and determined the status of cardiac SR Ca^{2+} cycling and its regulatory mechanisms in insulin resistant rats.

High sucrose feeding has been shown to produce whole body insulin resistance in male rats (27-36), however, the metabolic changes observed in these studies varied due to the different strains of rat used, as well as the mode, amount, and duration of sucrose feeding. In this study, the sucrose rats exhibited higher plasma insulin and glucose levels than control rats within 1 week of feeding suggesting that these rats had developed whole body insulin resistance and had further progressed into a glucose intolerant state. Concurrently, these rats developed moderate hypertriglyceridemia and hypercholesterolemia with no change in body weight. After 2.5 weeks of feeding the sucrose rats were relatively heavier than control rats which resulted in a lower HW/BW ratio at the final time point. Our findings are consistent with previous studies which have demonstrated that high sucrose feeding in rats leads to hyperinsulinemia, hypertriglyceridemia, and hyperglycemia (34, 162, 163). Sucrose rich diets have also been shown to cause significant elevations in plasma cholesterol (27, 162) and body weight (27, 32, 34). Insulin resistance, glucose intolerance, dyslipidemia, and obesity are all components of the metabolic syndrome, a major risk factor for type 2 DM (70), as well as metabolic characteristics of the early stages of type 2 DM (54). Hence, the altered metabolic status of our sucrose-fed rats closely resembles that of the insulin resistant stage of type 2 DM in humans (10). As prediabetic metabolic derangements such as insulin resistance may produce abnormalities in cardiac structure and function before the

development of overt DM our model is particularly relevant in understanding the mechanisms underlying the cardiomyopathy caused by type 2 DM.

Previous studies have shown that the development of insulin resistance in male rats caused by high sucrose feeding results in cardiac contractile dysfunction, specifically depressed myocyte contractility (28-32, 36) and reduced myofibrillar protein ATPase activities (34). However, no study to date has examined cardiac structure and function by *in vivo* echocardiography in a sucrose-induced model of insulin resistance. Hence, we performed serialized echocardiographic assessments which revealed alterations in myocardial structure and impairments in diastolic and systolic performance in the sucrose-fed rats compared to control rats.

With regard to diastolic function, the sucrose-fed rats exhibited early abnormalities in LV filling as demonstrated by higher A waves and reduced E/A ratios from the 2.5-week time point onwards. These changes are consistent with echocardiographic findings in other murine models of type 2 diabetes (169, 170, 171). Moreover, our results correspond with human studies which have shown early diastolic dysfunction marked by a significant reduction in the E/A ratio in patients with impaired glucose tolerance or overt type 2 DM (172, 173). The early impairment in diastolic function was followed by moderate yet rapid reductions in FS and EF between 5 and 10 weeks, indicating decreased cardiac contractility in the sucrose-fed rats. The late development of systolic dysfunction has been previously observed in 12-week old db/db mice (171) and in 20-week old ZDF rats (174). Alterations in myocardial structure also occurred between 5 and 10 weeks as the sucrose-fed rats exhibited a thinner

interventricular septal wall concomitant with a slightly enlarged left ventricular internal cavity, suggesting minor left ventricular dilatation.

Numerous studies have demonstrated that the cardiac contractile dysfunction in type 1 diabetic murine models is closely associated with defects in the function and regulation of the SR (15-26). In contrast, there is scanty and conflicting information available on the status of the SR in insulin resistant (i.e. prediabetic) and type 2 diabetic murine models (36, 121, 124, 125). Schaffer *et al.* (124) reported a decrease in SR Ca^{2+} uptake in 12-month old diabetic rats which had been injected with STZ as neonates to produce a type 2 diabetic-like condition. However, the experimental model used in their study had certain limitations that included no elevation in basal insulin levels and normal body weight, which differs from the clinical state seen in the majority of type 2 diabetic patients. In contrast, Misra *et al.* (125) observed an increase in SR Ca^{2+} uptake in 3-month old JCR:LA cp/cp rats. Despite this observation, Misra *et al.* (125) found no changes in active cell shortening and intracellular Ca^{2+} concentration under basal conditions in cardiomyocytes from the cp/cp rats. Belke *et al.* (121) reported a decreased rate of relaxation in isolated working hearts and a reduced rate of decay of the Ca^{2+} transient in isolated myocytes from 12-week old db/db mice. They attributed these changes to diminished SR activity as the sarcolemmal NCX activity was unchanged. Similarly, Wold *et al.* (36) reported that cytosolic Ca^{2+} removal and myocyte relaxation (with normal NCX function) involved impaired SERCA2a activity in sucrose-induced insulin resistant rats. However, the limitation with these studies was that functional parameters were not assessed in isolated SR vesicles, which restricts the conclusions of these studies.

Given the critical role of the SR in regulating cardiac contraction and relaxation, defective SR function may explain the alterations in *in vivo* cardiac contractile function observed in our sucrose-induced insulin resistant rats. We examined Ca^{2+} uptake in isolated SR vesicles commencing at 2.5 weeks a difference in LV diastolic function first became apparent. We observed no differences in SR Ca^{2+} uptake in the hearts of the 2.5 and 5-week control and sucrose-fed rats. These results suggest that other cellular mechanisms such as altered Ca^{2+} affinity of the contractile proteins or incomplete Ca^{2+} removal across the sarcolemmal membrane may also play a role in diastolic dysfunction. In support of this, Schaffer *et al.* (122) reported the activity of the NCX to be depressed in a murine model of type 2 DM. Furthermore, we observed a significant decrease in SR Ca^{2+} uptake in the 10-week sucrose hearts, a finding that was not reflected in the diastolic parameters of these rats. As a decline in SR Ca^{2+} uptake would decrease SR Ca^{2+} loading thereby making less Ca^{2+} available for the next wave of contraction, it is not surprising that we observed significant reductions in FS and EF in the 10-week sucrose-fed rats.

Given that studies examining SR function in insulin resistant and type 2 diabetic murine models are rare, the mechanisms underlying defective SR function are not completely understood. Since considerable SR dysfunction was apparent in the 10-week sucrose-fed rats, we proceeded to examine the expression of the SR Ca^{2+} cycling proteins as well as the status of the SR regulatory mechanisms in the hearts of these rats.

With regard to the SR Ca^{2+} cycling proteins, we observed a relatively small decrease and increase in the expression of SERCA2a and its inhibitory protein PLB respectively, which resulted in an increased PLB/SERCA2a ratio in the sucrose hearts compared to control hearts. A similar observation has been made by Belke *et al.* (121) in

db/db mice, although they observed a much larger increase in the expression of PLB and therefore a greater increase in the ratio of PLB/SERCA2a. Our results suggest that augmented inhibition of SERCA2a by PLB may have played a minor role in decreasing SR Ca^{2+} uptake in the 10-week sucrose hearts. Furthermore, it is possible that changes in SERCA2a function precede changes in protein expression, which has been observed in type 1 diabetic rat hearts (26).

The mechanisms involved in regulating SR function include the complementary reactions of protein phosphorylation by protein kinases and protein dephosphorylation by protein phosphatases. Given that SR Ca^{2+} uptake is augmented by the phosphorylation of PLB by PKA or CaMK II (11, 131), the depression in SR Ca^{2+} uptake in the 10-week sucrose hearts may have been attributed to decreased PLB phosphorylation by these protein kinases. We observed a significant decrease in the PKA-mediated phosphorylation of PLB at serine-16 as well as a significant decrease in the CaMK II-mediated phosphorylation of PLB at threonine-17. A similar observation has been made by Belke *et al.* (121) in db/db mice, where the phosphorylation status of serine-16 was slightly decreased. Since PKA and CaMK II are endogenous to the SR (175), the observed decrease in PLB phosphorylation at the serine and threonine residues may be attributed to reductions in the SR-associated PKA and CaMK II activities respectively. However, our results show a significant upregulation in the activities of both the SR-associated PKA and CaMK II in the 10-week sucrose hearts. One other study (176) has assessed the activity of PKA *in vitro* using normal rat ventricular myocytes cultured in a high glucose (HG) medium. While their study found the total PKA activity to be depressed in the HG myocytes, the phosphorylation status of serine-16 was unaffected as

was the phosphorylation status of threonine-17. A great limitation of their study was that global PKA activity was measured, which may or may not have affected sarcoplasmic reticular PLB. This global measurement contrasts with our study which measured PKA activity localized to the SR. Additionally, it is possible that activation of PKC may have contributed to the decrease in SR Ca^{2+} uptake. In support of this, Davidoff *et al.* (177) reported that PKC activation contributed to slowed shortening and relengthening in myocytes cultured in a HG medium.

Given that phosphorylation and dephosphorylation are complementary regulatory mechanisms, increased SR-associated PP activity may have contributed to the decreased PLB phosphorylation observed in the 10-week sucrose hearts. PP1 has been reported to be endogenous to the SR (178) and dephosphorylates PLB at both the PKA and CaMK II phosphorylation sites (134), thereby inhibiting SR Ca^{2+} uptake. PP2B has also been shown to reduce SR Ca^{2+} transport through dephosphorylation of PLB at threonine-17 (14). Our results show a relatively small increase in SR-associated PP activity in the 10-week sucrose hearts compared to control hearts.

Considering our protein kinase and protein phosphatase results collectively we would expect augmented phosphorylation of PLB, however, this was not observed. This discrepancy indicates that additional factors may affect the regulation of SR function such as anchoring proteins. Anchoring proteins are thought to target protein kinases and protein phosphatases to intracellular locations positioning them close to their substrates, thereby facilitating the phosphorylation or dephosphorylation of specific targets (179). In this respect, A-kinase anchoring proteins (AKAPS) may modulate the compartmentalization of PKA to the cardiac SR (180, 181), while α-kinase anchoring

proteins may modulate the compartmentalization of CaMK II to the cardiac SR (182). AKAPS specific to PP1 have also been identified (179). Furthermore, though the activities of the SR-associated PKA and CaMK II were upregulated, their protein targets are not limited to PLB in that that PKA and CaMK II also phosphorylate RyR2 (12).

The macronutrient content of a diet can be an important factor in the development of insulin resistance. Recent research suggests that a high intake of refined carbohydrates, particularly refined sugars high in fructose, increase the risk of insulin resistance (183). We therefore decided to feed male rats 32% sucrose, a disaccharide composed of fructose and glucose, to determine the adverse effects of insulin resistance on heart function. Understanding the detrimental consequences of sucrose on heart function is especially pertinent for human disorders involving insulin resistance, such as the metabolic syndrome, type 2 DM, obesity, and hypertension. Moreover, the sole effects of dietary nutrients cannot be examined in genetically altered animals. The dosage of sucrose administered was based on previous studies which added 30% to 35% sucrose in the drinking water of male rats to induce insulin resistance and disorders of insulin resistance, specifically the metabolic syndrome and hypertension (27, 160, 161). Furthermore, we examined only male rats because Horton *et al.* (159) reported that female rats do not develop sucrose-induced insulin resistance. They postulated that the presence of estrogen and/or progesterone, or the absence of male androgens, may protect female rats against the impairment in insulin action. In addition, Busserolles *et al.* (184) reported that female rats were protected against the pro-oxidant effect of a high sucrose diet and that this may be attributed to the effect of estrogens on antioxidant capacity. In contrast, numerous studies have shown that high sucrose feeding in male rats induces

insulin resistance (27-36) and furthermore, a high sucrose diet has a pro-oxidant effect in male rats (184). However, high sucrose feeding is disadvantageous because the specific mechanisms by which the moieties of the sucrose disaccharide induce whole body insulin resistance are not completely understood. Another disadvantage of high sucrose feeding is that it causes subtle changes in cardiac structure and function as well as in SR Ca^{2+} cycling and its regulatory mechanisms rather than overt impairment. Additionally, the specific mechanisms by which a high sucrose diet contributes to these subtle changes are not completely understood.

There are several pathways by which a diet rich in sucrose could potentially alter cellular metabolism which in turn causes cardiomyopathy. The detrimental effect of a high sucrose diet may be attributed to its glucose content. As glucose is the key stimulator of insulin secretion, persistent hyperglycemia may result in hyperinsulinemia, leading to decreased insulin sensitivity as insulin downregulates the insulin receptor by increasing its rate of degradation and by suppressing its synthesis (185). Given that the human myocardium is insulin sensitive (109), reduced insulin sensitivity leads to decreased glucose uptake and glucose oxidation consequently inhibiting glycolysis. This affects Ca^{2+} reuptake into the SR through SERCA2a because this ion pump preferentially uses glycolytically derived ATP (106). Hyperglycemia may also cause myocardial damage by the glycation of myocardial cytosolic proteins such as contractile proteins with the formation of AGEs and associated free radical-mediated damage (109). The formation of AGEs on SERCA2a during diabetes has been demonstrated and correlated with a prolonged rate of cardiac relaxation (113).

The detrimental effect of a high sucrose diet may also be attributed to its fructose content. Excess fructose in the diet can perturb glucose metabolism and glucose uptake pathways as well as lead to enhanced lipogenesis and triglyceride accumulation thereby contributing to insulin resistance (186). In addition, fructose facilitates oxidative stress (187).

Sucrose-induced insulin resistance may further cause changes in cardiac structure and function and SR function through its induction of abnormalities in lipid metabolism. The rise in plasma triglycerides and cholesterol in the sucrose-fed rats indicates the defective removal and/or increased breakdown of lipids. Consequently, increased plasma FFAs can lead to changes in the fatty acid composition or fluidity of sarcolemmal and intracellular membranes (188). Hence the function of major Ca^{2+} regulating proteins attached to or inserted within these membranes such as the sarcolemmal NCX and the SR Ca^{2+} cycling proteins SERCA2a, PLB, and RyR2 may be affected. Furthermore, increased FFA oxidation in the myocardium results in the accumulation of toxic intermediates, long-chain acylcarnitines for example, that can result in free-radical mediated damage to both the sarcolemma and intracellular membranes (109). In turn this damage may result in impaired ion pumping and exchange mechanisms. Such abnormalities of Ca^{2+} pumping and exchange as well as of the sarcolemmal sodium-potassium adenosine phosphatase have been described in the myocardium in experimental diabetes models (109). Additional FFA-induced disturbances include the induction of insulin resistance in muscle and liver and the stimulation of glucose production and release by the liver (68).

The abnormalities in cardiac structure and function may also be attributed to alterations in nitrogen metabolism caused by the presence of insulin resistance, which can alter protein anabolism in heart muscle (189). Such alterations in the hearts of the sucrose-fed rats may have resulted in reduced production of myocardial structural and contractile proteins, which would account for the decrease in interventricular septal wall thickness and concurrent enlarged left ventricular cavity as well as the decline in systolic function.

The detrimental effects of obesity must also be considered in our model. The sucrose-fed rats were heavier relative to the control rats and the weight gain may be attributed to an increase in adipose tissue. Increased adiposity perturbs insulin action and insulin secretion leading to enhanced lipolysis with subsequent FFA release, which aggravates insulin resistance. Furthermore, dysfunctional adipocytes produce excessive amounts of insulin resistance-inducing, inflammatory, and atherosclerotic-provoking cytokines and fail to secrete normal amounts of insulin-sensitizing adipocytokines (68).

Our study is novel in that it is the first to examine serial alterations in cardiac structure and function by echocardiography while determining the status of cardiac SR Ca^{2+} cycling and its regulatory mechanisms in sucrose-induced insulin resistant rats. The early abnormalities in LV diastolic filling and late impairments in systolic function concomitant with alterations in myocardial structure in the sucrose-fed rats suggest the development of a diabetic-like cardiomyopathy at an insulin resistant state before the manifestation of overt type 2 DM. Furthermore, the cardiac contractile dysfunction in the sucrose-induced insulin resistant heart is associated with a decline in SR function as well as defective SR regulation. The changes in cardiac structure and function and SR function

may be the result of abnormalities in carbohydrate, lipid, and/or protein metabolism associated with myocardial insulin resistance.

In conclusion, our study provides a better understanding of the phenotypic changes that occur in the myocardium during the insulin resistant state and specifically identifies potential targets for improving SR function. This information is of paramount importance as it will enable the prevention or therapeutic treatment of cardiac contractile dysfunction in human disorders involving insulin resistance such as the metabolic syndrome, type 2 DM, obesity, and hypertension.

VII. FUTURE DIRECTIONS

Potential areas to explore include further examination of whole heart and SR structure and function in the hearts of the 10-week sucrose-fed rats as well as in the hearts of rats fed a high sucrose diet for a longer period of time. Additional prospective areas involve examining the influence of gender on the development of insulin resistance as well as on cardiac contractile performance and SR Ca^{2+} cycling and its regulation. Lastly, progress in gene therapy will provide therapeutic strategies aimed at attenuating SR dysfunction and subsequently improving cardiac contractile function in disorders of insulin resistance.

The hearts of the 10-week sucrose-fed rats showed decreased SERCA2a function with no change SERCA2a protein expression which suggests that other factors may affect the function of this SR membrane transport protein. The chronic elevation in the triglyceride and cholesterol level of the sucrose-fed rats may be one factor and accordingly one could determine whether changes in the fatty acid composition or fluidity of the SR membrane affect SERCA2a function. The chronic elevation in the glucose level of the sucrose-fed rats may be another factor and hence one could determine if there is glycation of the SR Ca^{2+} cycling proteins. The hearts of the 10-week sucrose-fed rats also showed significant decreases in the PKA and CaMK II-mediated phosphorylation of PLB at the serine-16 and threonine-17 amino acid residues respectively, paradoxically, the activities of PKA and CaMK II were significantly upregulated. One potential mechanism for this discrepancy may involve changes in the compartmentalization of PKA and CaMK II-mediated phosphorylation of PLB.

Accordingly, one could evaluate the function and expression of anchoring proteins specific for PKA and CaMK II.

In addition to Ca^{2+} uptake, the SR releases (activator) Ca^{2+} into the cytosol facilitating cardiac contraction. Accordingly, one could determine whether changes in SR Ca^{2+} release through RyR2 plays a role in the decline in systolic function in the 10-week sucrose-fed rats. If changes in SR Ca^{2+} release are apparent, then one could assess the protein expression and phosphorylation status of RyR2.

We and others have demonstrated that high sucrose feeding induces whole body insulin resistance in male rats. However, the mechanism by which sucrose affects insulin action in insulin-sensitive tissues is not clear. Accordingly, one could assess the status of the insulin signalling pathways in these tissues and more specifically, determine if there is a link between insulin signalling and SR function in the heart.

Gender plays a major role in the development of sucrose-induced insulin resistance. It is therefore important to determine whether high sucrose feeding leads to differential changes in cardiac structure and function as well as in SR Ca^{2+} cycling and its regulation in male versus female rats.

Our results show that impaired cardiac contractile function and depressed SR function occur in the prestage of type 2 DM. In order to determine whether cardiac contractile performance and SR function and its regulation further deteriorate or whether there are additional alterations in these parameters in the development of overt type 2 DM, it would be highly relevant to extend the duration of high sucrose feeding.

Lastly, examination of the function and regulation of the SR in the progression towards overt type 2 DM will enable the identification of SR Ca^{2+} cycling and SR Ca^{2+}

cycling regulatory proteins as potential targets for gene therapy. Gene therapeutics will improve SR function and subsequently cardiac contractile performance in disorders of insulin resistance and overt type 2 DM in humans. Furthermore, the targeted approach offered by gene medicines is much better than the systemic effects and toxicities associated with pharmacological agents in use today.

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