S100B EXPRESSION MODULATES DIABETIC MYOCARDIUM FOLLOWING MYOCARDIAL INFARCTION AND DEVELOPMENT OF DIABETES

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ABSTRACT

S100B regulates left ventricular (LV) remodelling following myocardial infarction (MI) by interaction with the receptor for advanced glycation end products (RAGE). Since S100B and RAGE are induced in diabetic vasculature, we hypothesized that i) hyperglycemia will augment expression or functional impact of S100B and RAGE post-MI and ii) abrogated or forced expression of S100B will alter post-MI structure and remodeling specifically in diabetes. Also, as S100B Knockout (BKO) mice appear resistant to streptozotocin (STZ)–induced diabetes, we postulated a role for S100B in development of diabetes.

In mouse, post-MI remodeling in diabetes exhibited an attenuation of LV dilation, myocyte hypertrophy, S100B expression and matrix metalloproteinase-2 (MMP-2) activity, increased apoptosis and fibrosis. Despite reduced LV dilation, impairment of cardiac function was similar to non-diabetic controls. Following MI, diabetic BKO demonstrated increased LV dilation compared to S100B transgenic (BTG) and WT with greater impairment of cardiac function, decreased glucose transporter type 4 (GLUT4) mRNA expression and increased AGE levels. These data suggest that S100B expression may serve to limit adverse diabetic post-MI remodeling and regulate associated metabolic changes in the heart.

In addition, we have defined a novel role of S100B in the development of diabetes. As BKO mice were resistant to STZ induced-diabetes with lower value for food and water intake and urine volume and increased body weight compared to WT mice, we hypothesized that S100B
played a role in islet cell damage and/or insulin sensitivity. BKO exhibited enhanced glucose tolerance and insulin sensitivity with increased insulin in serum. S100B deficiency prevented β-cell disruption and functional impairment in response to STZ. Expression of S100B was increased in the WT islet after induction of diabetes and S100B induced apoptosis in β cells. These findings indicate that S100B expression is involved in islet dysfunction and in peripheral insulin resistance, at least in an STZ model.

In conclusion, our findings support a differential, paradoxical effect of S100B signalling in post-MI diabetic hearts, serving to limit dilation and functional impairment. Moreover, S100B may play a role in diabetes itself contributing to β-cell loss in pancreas and peripheral insulin resistance.
We do not receive wisdom;
We must discover it for ourselves
After a journey
No one can take for us
Or spare us.

- Marcel Proust
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Dedicated to My Sons: Eilyad and Faraz
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• James N. Tsoporis, Forough Mohammadzadeh, Shehla Izhar, and Thomas G. Parker S100B Acts as an Intracellular Regulator and an Extracellular Ligand for the Receptor for Advanced Glycation End-Products (RAGE) and Toll-Like Receptor 4 (TLR4) in Cardiovascular Disease, International Journal of Medical and Biological Frontiers. 2012, Volume 18

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LIST OF ABBREVIATIONS

ACE: angiotensin-converting enzyme
AGE: advanced glyated end product
ANF: atrial natriuretic factor
ATP: adenosine triphosphate
BNF: brain natriuretic factor
BW: body weight
Ca2+: calcium
CAD: coronary artery disease
cGMP: cyclic guanosine monophosphate
CHF: congestive heart failure
CREB: cAMP response element binding
CVD: cardiovascular disease
DBP: diastolic blood pressure
DM: diabetes mellitus
DNA: deoxyribonucleic acid
FABP: fatty acid binding protein
ECM: extracellular matrix
EF: ejection fraction
ERK: extracellular signal-regulated kinase
eNOS: endothelial nitric oxide synthase
GAD: glutamic acid decarboxylase
GAPDH: Glyceraldehyde-3 Phosphate Dehydrogenase
GFAP: glial fibrillary acidic protein
HF: heart failure
IA-2: islet antigen-2
ICA: islet cell antigen
IR: insulin resistance
LAD: left anterior descending
LV: left ventricle/ventricular
LVDD: left ventricular diastolic dimension
LVEDP: left ventricular end-diastolic pressure
LVEF: left ventricular ejection fraction
LVSD: left ventricular systolic dimension
LVSP: left ventricular systolic pressure
MAPK: mitogen activated protein kinase
MI: myocardial infarction
mRNA: messenger ribonucleic acid
MMP: matrix metalloproteinase
NFκB: nuclear factor κB
NOD: non obese diabetes
PI3K: phosphatidylinositol-3 kinase
PKC: protein kinase C
PMN: neutrophilic granulocytes
pSC: peri-islet Schwann cells
RAGE: receptor for advanced glycation end product
RT-PCR: reverse transcriptase-polymerase chain reaction

TEF-1: Transcription enhancer factor -1

RTEF-1: Related TEF-1

TGF-β: transforming growth factor-beta

TUNNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

SBP: systolic blood pressure

SERCA2a: sarcoplasmic reticulum Ca$^{2+}$-ATPase (cardiac isoform)

SF: shortening fraction

SGOT: serum glutamic oxaloacetic transaminase

WT: wild type

$+dP/dt$: maximum rate of pressure rise

α-SMA: α-alpha-smooth muscle actin

β-MHC: β -myosin heavy chain
CHAPTER 1

BACKGROUND REVIEW OF LITERATURE
1.1- Cardiovascular Disease

Cardiovascular disease (CVD) is the leading cause of mortality and morbidity in the western world including Canada (193), particularly among older adults (255). In the United States over 64 million individuals suffer from one or more forms of cardiovascular disease, with over 25 million being over the age of 65 (193). In Canada, over 80% of the population has one of the following risk factors for cardiovascular disease: smoking, physical inactivity, obesity, self reported hypertension and diabetes. CVD is also the leading cause of hospitalization among men and women (except for child birth). CVD imparts a large economic burden on the health care system of Canada, requiring 18 billion dollars a year, which includes the price of direct medical costs and supplies as well as indirect medical costs like disability payments. This cost in the United States exceeds 300 billion dollars annually.

1.2 Development and Pathogenesis of Heart Disease

A variety of pre-existing conditions can cause heart disease and heart failure: hypertension, cardiomyopathy, and most importantly myocardial infarction (MI). However, there are many other factors that play into whether a person will develop heart disease. Different lifestyle choices affect a person’s chances of developing heart disease (for example, poor diet, inactivity and smoking). Other factors such as genetics, gender, age and diabetes may also contribute to developing the above conditions. Among the myocardial diseases, MI is the most overwhelming contributor to heart failure and death (255). Because of the high incidence of MI, it is important to investigate post-MI events that contribute to adverse outcomes, particularly heart failure, including remodeling of the extracellular matrix (ECM), myofibroblast function, collagen production, cellular hypertrophy and apoptosis, and the development of fibrosis and healing of the infarct scar. Of interest, among risk factors for MI, diabetes may independently contribute to heart failure through various mechanisms,
including hypertension, accelerating the development of coronary atherosclerosis, and existence of a specific diabetic cardiomyopathy.

1.2.1 Myocardial Infarction

1.2.1.1 Prevalence

MI is imprecisely referred to in the lay vernacular as a heart attack. Acute MI (AMI) alone is one of the leading causes of mortality and morbidity in the United States and other developing countries (255). In the United States, annually more than 1.3 million Americans suffer from an AMI and approximately one-third of them die (193). Furthermore, over 8 million individuals have suffered an AMI and its subsequent complications. Although the mortality rate from an AMI has been steadily declining over the last three decades (70), an AMI is still a major source of mortality in the United States and Canada.

1.2.1.2 Acute Myocardial Infarction

Myocardial infarction (MI) occurs when diminished blood supply to the heart (ischemia) exceeds a critical threshold that overwhelms myocardial cellular repair mechanisms required to maintain normal operating function and homeostasis. Ischemia at this critical threshold level for an extended period results in irreversible myocardial cell damage or death. Myocardial ischemia results from increased myocardial metabolic demand or more commonly reduced oxygen and nutrient supply to the myocardium through the coronary circulation. The reduction of oxygen and nutrient supply occurs when a thrombus is superimposed on an unstable atherosclerotic plaque and results in coronary arterial blockage (63). In addition to rupturing a plaque alternatively, a dynamic stenosis associated with coronary vasospasm can also limit the supply of oxygen and nutrients results in an MI. There are several conditions that increase myocardial metabolic demand including extreme physical activity, severe hypertension and aortic valve stenosis.
Based on the anatomical and morphological properties, MI is subcategorized in two types; transmural and nontransmural. A transmural MI is characterized by ischemic necrosis of the complete thickness of the affected area, from endocardium through to the epicardium. A nontransmural MI is defined as an area that ischemic necrosis does not extend through the full thickness of myocardium wall to the epicardium, often as a consequence of subtotal occlusion or reperfusion (258).

A main consequence of myocardial ischemia is the switch from aerobic to anaerobic glycolysis to produce energy. This switch results in an inadequate production of high–energy phosphates such as adenosine triphosphate (ATP) and creatine phosphate and the accumulation of the glycolysis end product, lactic acid. The high-energy phosphates are essential for myocardial contractility. Therefore, within minutes of initiation of ischemia, the reduction of available high-energy phosphates severely attenuates myocardial contractility. Moreover, myocardium undergoes several structural changes such as cell and mitochondria swelling, myofibrillar relaxation and electrolyte disturbances, which also reduce myocardial contractility. These early changes can be reversed by restoration of blood flow. However, once ischemia is sustained, more than 20-40 minutes, irreversible myocardial damage or infarction results. At this stage, cardiomyocyte loss happens through necrosis and subsequent apoptosis. Therefore, early detection of MI and early coronary reperfusion by using angioplasty or thrombolysis to prevent the loss of myocytes as well as expanding the infarct size (272).

1.2.1.3 Arrhythmias

Ionic disruption and ventricular arrhythmias are significant consequences of MI. A major cause of sudden death after ischemia or MI is usually due to ventricular fibrillation as a result of myocardial irritability. Normally, 50-70 percent of sudden death in patients with acute MI
is attributed to ventricular arrhythmias, especially ventricular fibrillation (VF). The other prominent form of ventricular arrhythmia is ventricular tachycardia (VT), which results from a rapid sequence of ventricular impulses. The incidence of ventricular arrhythmias during the acute phase of myocardial infarction is higher than for transient ischemic episodes (28). During the first 30 min after experimental, complete coronary artery occlusion, ventricular arrhythmias (ventricular premature depolarization, ventricular tachycardia, and ventricular fibrillation) occur.

Multiple agents have been identified as initiators of ventricular arrhythmias in the presence of myocardial ischemia and MI. Among these agents, potassium (K⁺) appears to play a pivotal role in the cellular electrophysiology of ischemic or infarcted tissue. In the presence of ischemia there is an acute increase in interstitial K⁺ levels (135). The result of these ionic imbalances is the abrupt depolarization of transmembrane resting potential, mostly due to the change in the equilibrium potential of K⁺.

These electrical changes do not happen uniformly within the myocardium and are differentially distributed in ischemic versus non-ischemic myocardium (135). The generation of premature impulses within this unstable environment will further augment the electrical instability and ultimately result in the onset of ventricular arrhythmias. The re-entrant process results in the initiation of VF after coronary artery occlusion (152).

The therapeutic goal in the early post-MI period, in addition to re-establishing blood flow to limit the infarct area, is to increase the threshold for arrhythmia induction. Pharmaceutical intervention such as β-blockers has been widely used (130) and twenty percent reduction has been shown in patients by using these agents. This reduction in mortality is believed to result from a combination of their anti-arrhythmic effects, prevention of re-infarction and a reduction of myocardial oxygen demand.
1.2.1.4 Post MI Events

Abnormalities in the function of the heart are typically seen within 24-48 hours after the MI but can be seen even earlier (102). Once blood flow has been restored, for example when the blood clot has been dissolved pharmacologically or mechanically disrupted by percutaneous coronary intervention (angioplasty) allowing an increase in blood flow, oxygen and nutrients are reperfused back into the tissue which causes a sudden production of free oxygen radicals (24). Free oxygen radicals can contribute to the formation of detrimental substances which can promote signaling in apoptosis pathways, provoking further cellular damage and cell death in the myocardium (24).

Irreversible loss of contracting myocardium due to MI results in a chronic increase in the workload of the remaining viable myocardium. The heart responds with an increase in muscle mass and this process of heart hypertrophy represents a critical compensatory mechanism that permits the ventricle to sustain normal stroke volume and cardiac output. However, if this increased load on the heart is allowed to continue for a prolonged period, cardiac pumping function may become ineffective and heart failure happens in the setting of progressive hypertrophy. As a result, research efforts with regards to the pathogenesis and management of post-MI heart failure have been increased. In this regard, an improved and expanded knowledge of the pathophysiological mechanisms that ultimately contribute to progressive cardiac dysfunction and failure have led to an approach for heart failure treatment following myocardial infarction.

1.3 Ventricular Remodeling

The term "ventricular remodeling" refers to global changes in ventricular chamber size, shape, mass and structure that develop in a complex and coordinated fashion in response to altered myocardial loading conditions. These changes occur at the cellular level in both
myocyte and the extracellular matrix compartments (ECM) (261). However, structural alterations after infarction are not limited to the infarcted area but also encompass the non-infarcted myocardium (60). Studies have shown that the extent of the remodeling that occurs in post-MI myocardium depends on the size of the infarct zone that is formed during the event (52). Left ventricular (LV) remodeling is regarded as an early adaptive and beneficial response to the reduced contractility and function that results from the MI. Despite a depressed ejection fraction, the increase in ventricular cavity size serves to restore stroke volume. However chronically, remodeling becomes maladaptive because of the progressive enlargement and dilation of the LV resulting in pump failure, and is associated with a reduction in survival. Post-infarction remodeling has been arbitrarily divided into an early phase (within 72 hours) and a late phase (beyond 72 hours; Fig.1.1) (309).

The process of remodeling involves reduction of myocardial wall thickness, dilation of ventricular cavity and hypertrophy of myocytes in the remote region. Early remodeling includes extracellular matrix remodeling and expansion of infarct zone leading to wall thinning (309). This LV dilation may result in early ventricular rupture or aneurysm formation. Events happening in the early phase of remodeling are infiltration of immune cells into the infarcted area, wound healing, scar formation, and collagen deposition. Associated inflammatory responses in this phase are divided into two main categories: acute and chronic. Activation of the inflammatory system lasts up to 6 weeks post-infarction. The hallmark later phase of remodeling are alterations in the non-infarcted area of LV and is associated with progressive dilatation, alteration of myocardial geometry and compensatory myocyte hypertrophy in response to increased workload (pre-load and after-load) and increased wall tension. Also this phase includes activation of neurohormones and growth factors. In
consequence, progressive LV dilatation and geometric distortion increase regional wall tension and result in a progressive deterioration in ventricular contractile function (309).

![Figure 1.1) Ventricular remodeling following a myocardial infarction](image)

Within hours to days after an infarction, the myocardium is affected as the infarction begins to expand and become thinner. Within days to months, global ventricular remodeling can occur, resulting in overall ventricular dilatation and decreased ventricular function. Reproduced with permission from Opie, et. al. Lancet. 2006 Jan 28;367(9507):356-67

1.3.1 Early Ventricular Remodeling

The early phase of remodeling and LV cavity dilatation is primarily due to the expansion of infarct region. In fact, cardiac rupture accounts for the 5–30% of in-hospital mortality after AMI (128). Infarct expansion occurs within hours of myocyte injury, and thereafter, a progressive dilation of noninfarcted LV occurs over weeks (241). These progressive changes in LV geometry contribute to the development of depressed cardiac function, clinical heart failure, and increased mortality. The earlier infarct expansion occurs prior to fibroblast proliferation and collagen deposition in the scar area, therefore, dynamic synthesis and breakdown of ECM proteins could play an important role in this deleterious event. In particular, degradation of the extracellular collagen struts by serine proteases and the
activation of matrix metalloproteinases (MMPs) within hours of infarction have been implicated in this process (59, 64). In parallel, thinning of the infarct, which occurs as a result of slippage between muscle bundles, could cause a reduction in the number of myocytes across the infarcted region. Expansion of the infarct area does not occur uniformly after all MIs and is more prevalent in large transmural infarction. Patients exhibiting infarct expansion are more likely to experience post-MI complications like congestive heart failure, myocardial aneurysm and rupture.

1.3.1.1 Wound Healing

Wound healing after MI mimics the same process observed in other tissues such as skin. However, cardiac wound healing has unique characteristics. First, the capacity of the heart to regenerate functional myocardium throughout life is limited (236). Therefore, the factors that are involved in myocyte regeneration or cell proliferation have less importance than the factors that control formation of granulation tissues. Secondly, a cyclic stretch caused by rhythmic contraction of non–infarcted area which is absent in other tissues could affect the wound healing process (291). In general, four overlapping phases can be distinguished during post-MI wound healing: phase 1: myocyte cell death, phase 2: acute inflammation, phase 3: granulation tissue and phase 4: remodeling and repair.

1.3.1.1.1 Phase 1: Cardiomyocyte Death

The first phase is the death of the cardiomyocytes which can occur by two pathways: apoptosis or necrosis. The peak of cardiomyocyte apoptosis occurs at 6-8 hours after a MI and the peak for necrosis occurs between 12 hours to 4 days after a MI (127). Myocardial necrosis is generally characterized by swelling of the cells, while apoptosis is characterized by cell shrinkage. Apoptosis is a major source of cell loss in post-MI in both rats and humans (41). Myocyte death begins in the sub-endocardial area and spreads to the epicardium making
the infarct area transmural. Early interventions could be directed to limit the degree of myocyte loss by inhibiting the apoptotic factors like caspases or their underlying pathways (359). The death of cardiomyocytes releases many different substances into the bloodstream like troponin-T, fatty acid binding protein (FABP), serum glutamic oxaloacetic transaminase (SGOT) and creatine kinase, skeletal-brain hybrid type (CK-MB) (58, 127), which act as markers for damage and are also responsible for initiating the second phase of wound healing. The second phase of healing is marked by an immune response.

1.3.1.1.2 Phase 2: Acute Inflammation

One of the earliest features of the inflammatory response is activation of the complement system and release of cytokines and interleukins like IL-6 and IL-8 within hours after the onset of the infarction in humans, and persisting until the fifth or seventh day post-MI (303). Within 6-8 hours post-MI, neutrophilic granulocytes (PMN) migrate into the infarcted area peaking within 24-48 hours after the infarct. The PMN infiltration is followed by the influx of other inflammatory cells, including lymphocytes and macrophages. The importance of this inflammatory period has been illustrated by studies with anti-inflammatory agents, which by impairing scar healing increase ventricular dilatation and post-MI complications (123). Infiltrating mononuclear cells which differentiate into macrophages and mast cells by a phenotypic switch appear to orchestrate the cardiac repair process through a complex cascade involving cytokines and growth factors. The production of these mediators is necessary to support fibroblast proliferation and neo-vessel formation and the regulation of extra-cellular matrix metabolism. On the other side, coronary reperfusion significantly enhances the post-MI inflammatory response, and this is thought to be one of the major mechanisms by which this intervention improves outcome post-MI. Two to three days after infarction extracellular matrix proteins begin to be deposited, first within the border zone, between infarcted and
non-infarcted tissues and later in the center of scar area. This marks the onset of phase 3 of
the process of cardiac wound healing, the formation of granulation tissue (58).

1.3.1.1.3 Phase 3: Granulation Tissue

The formation of granulation tissue during wound healing provides the tensile strength of the
infarcted area and prevents cardiac rupture. First, fibrin is deposited followed by deposition
of other extracellular matrix proteins, like fibronectin and tenascin (353). Then, within a few
days post-MI, the infarcted area is surrounded by myofibroblasts, which produce collagen.
Elevation of type-III collagen content in the beginning of phase 3 is followed by a lower and
slower rise in type-I collagen. The cross-linking of type-I collagen is responsible for the
development of the tensile strength. The crosslinking process may take up to several weeks
after the initiation of the wound healing process (31). Along with collagen synthesis, collagen
degradation is also activated. Increased collagenolytic activity by specific matrix
metalloproteinases that cleave interstitial collagens happens in the first week after infarction
(59). These enzymatic activities can result in loss of structural support, distortion of tissue
architecture, reduction of cardiac stiffness, wall thinning and even rupture of the myocardium
(58). Appearance of new blood vessels in the wound area is one of the features of granulation
tissue. These new vessels are derived from pre-existing vessels and/or from circulating
endothelial or progenitor cells that migrate from the peri-MI region into the wound. These
newly formed blood vessels attempt to normalize coronary blood flow. Previous studies in
rats have demonstrated that within a week after the infarction, basal coronary blood flow is
normalized and that by this time the center of infarcted area receives about 25% of the
maximal left ventricular flow (222). After 2-3 weeks granulation tissue in the infarcted zone
contains cross–linked interstitial collagens, macrophages, blood vessels, and myofibroblasts.
Subsequently cells begin to disappear from the scar except for myofibroblasts (303), which is the main characteristic of the fourth phase of cardiac wound healing, scar tissue formation.

1.3.1.4 Phase 4: Remodeling/Repair

During this period, disappearance of the various cell types due to apoptosis along with the cross-linking of collagen play important roles in this wound healing phase (32). Formation of permanent scar tissue occurs in the absence of cardiomyocyte regeneration. An increase in interstitial collagen could have a beneficial effect by reducing the dilation rate. However, increased interstitial matrix contents will also increase the stiffness of the heart and result in reduced cardiac function with impaired filling (38). Today, the transmural scar is regarded as a living tissue, as opposed to previous belief that the scar was an inert, acellular tissue with the primary role of restoring structural integrity. It is now well established that the scar is composed of a population of cells, myofibroblasts, which persist at the infarct site for years. There are some cells that may account for the production of myofibroblasts; interstitial fibroblasts, adventitial fibroblasts, pericytes, fibrocytes, or circulating monocytes or bone marrow-derived progenitor cells that transdifferentiate at the infarct site (305). Moreover, it has also been demonstrated that in addition to the production of types-I and III collagen; myofibroblasts and their α-smooth muscle actin microfilaments, are connected to one another through gap junctions and are attached to the scar’s extensive collagen network, creating a contractile assembly network. Scar tissue has also been reported to demonstrate contractile behaviour (32). Newly established vessels nourish these cells and enable them to be metabolically active to express the components needed for angiotensin II (Ang II) generation, including angiotensin-converting enzyme (ACE), angiotensinogen, renin and angiotensin II Type 1 (AT1) receptor as well as transforming growth factor-β1 (TGF-β1), an important regulatory peptide in fibrous tissue formation. Along with collagen production in the scar
area, Ang II and TGF-β1 formation contribute to ongoing scar tissue collagen turnover and to fibrous tissue formation in the non-infarcted area (47).

### 1.3.2 Late Ventricular Remodeling

Late ventricular remodeling includes time-dependent ventricular dilatation, increased apoptosis in remote myocardium, altered myocardial geometry, compensatory noninfarcted myocardial hypertrophy in response to workload, pre-and after-load tension and the chronic hyper-activation of neurohormones and growth factors. Late remodeling is closely related to “infarct expansion,” which is not explained by additional myocardial necrosis (4). Evidence from experimental and clinical studies have demonstrated a loss of intrinsic contractility in surviving myocardium after myocardial infarction (210). Increasing myocyte length with a disproportionate increase in cell diameter leads to a reduction in the wall thickness-to-chamber radius ratio (eccentric hypertrophy) (206). This structural rearrangement of myocytes contributes in increasing the chamber volume and results in an increased diastolic wall stress in myocardium remote from the area of infarction. Consequently, this increased stress can lead to myocyte slippage, resulting in a decreased number of myocytes across the wall (350) and concomitant ventricular wall thinning and LV dilation (206). Wherever the wall thinning occurs, it is exposed to the same tension per unit muscle thickness as the rest of the myocardium but increased mechanical stress. This phenomenon can further affect wall thinning.

Progressive increases in the number of apoptotic myocytes in the myocardium remote from the area of ischemic damage may play a role in late ventricular remodeling. Although specific factors stimulating apoptosis up to 6 months post-MI have not been determined, increased LV dimension post-MI by 5-10 fold suggest a contribution of mechanical stress in this event (265). Despite strong correlation between the frequency of apoptotic myocytes and
the decrement in maximal developed pressure, it is also possible that apoptosis is a result of worse LV function rather than the cause.

Ventricular dilation during the post-myocardial infarction period is accompanied by increased left ventricular end-systolic volume (124, 352) associated with depressed ventricular function and poor survival in both animals and patients (11, 243, 340), which in turn stimulates further ventricular dilation. Thus a vicious cycle can be created in which compensatory dilatation to maintain cardiac pump function results in further dilation and increased heart failure and this may serve as a basis for aneurysm formation (205) and subsequent cardiac rupture (273). Prevention of such infarct expansion attenuates a decrease in ejection fraction and preserves ventricular function (162).

1.3.2.1 Myocardial Hypertrophy

Myocyte hypertrophy of remaining viable myocardium is viewed as an initial compensatory response to the increased-ventricle wall stress. Hypertrophy works to stabilize contractile function and restore stroke volume and can offset the increased wall stress and reduce the stimulus for further enlargement. Increased wall stress is a powerful stimulus for hypertrophy, initiated by neurohormonal activation, myocardial stretch, and the activation of the local tissue renin-angiotensin-system (RAS), which initiates the increased synthesis of contractile assembly units (192) and paracrine/autocrine factors (309). Studies have demonstrated tremendous growth and increase in cell volume within the viable myocardium after experimental coronary artery ligation (192). Excessive ventricular hypertrophy can unfortunately become maladaptive and result in ventricular dysfunction. Moreover, the cellular hypertrophy exhibited in the remote area is often insufficient to reduce the loss of myocyte contractility within the infarct territory. The end result is progressive ventricular dilatation as described above.
Cardiac hypertrophy is associated with both morphological changes (increase in cell size and myofibrillar content/organization) as well as changes in gene expression. In the hypertrophied myocardium, there is an up-regulation of genes associated with cardiac development, collectively named as fetal genes. The re-expression of fetal sarcomeric proteins α-skeletal actin, β-myosin heavy chain, transcriptional factors such as c-fos, c-jun, c-myc, atrial natriuretic factor (ANF), and brain natriuretic factor (BNF) are just a few examples of the genetic changes associated with a hypertrophied myocardial phenotype. Although these changes improve energy efficiency, they may also contribute to the development of LV dysfunction. Various neurohormonal systems such as the RAS, endothelin-1 (ET-1) and the adrenergic signaling pathways have been identified as potent hypertrophic agents in both cultured myocytes and in vivo hearts. These pathways induce both the morphologic and transcriptional changes associated with the hypertrophic response. Directly pathways initiate their signaling in the heart by acting through G protein coupled receptors, principally activating the Gq subfamily of G proteins (95). This, in turn activates phospholipase C, leading to the hydrolysis of phosphatidylinositol 4, 5-bisphosphate, producing the dual second messengers inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG). DAG is the physiological activator of protein kinase C (PKC), while IP3 causes the release of calcium from intracellular stores. Together, PKC and the elevated intracellular calcium concentrations promote the expression of growth related transcription factors such as c-fos, c-myc and c-jun (196, 216).

The activation of G-protein coupled receptors also activates the mitogen-activated protein kinases (MAPKs), a superfamily of proline-directed Ser/Thr protein kinases, which are involved in transcriptional regulation and the regulation of hypertrophic gene expression (95). The three subfamilies of the MAPKs superfamily, including the extra-cellularly
regulated kinases (ERK), the stress activated protein kinases/c-Jun N-terminal kinases (SAPKs/JNKs) and p38 have been implicated in the induction of transcriptional changes and an increase in cell size. Additionally, various growth factors including fibroblast growth factor, epidermal growth factor, platelet-derived growth factor, insulin, insulin-like growth factor and TGF-β1 are also involved in the induction of myocyte hypertrophy by activating receptor tyrosine kinases, p21 ras and MAPKs.

While transcriptional regulation of protein synthesis appears to be a significant component of cardiac hypertrophy, post-translational modification and degradation of proteins may also play a role.

1.3.2.2 Myocardial Fibrosis

After transmural MI, fibrosis, the major component of adverse ventricular remodeling, occurs at the site of infarction as well as in the remote area (345). Although increased collagen content at the MI site may be beneficial to ensure the formation of a mature scar tissue to maintain ventricular structure and prevent the cardiac dilation and rupture, the interstitial fibrosis remote to the MI involving noninfarcted myocardium alters the tissue stiffness by crosslinking of extracellular matrix proteins resulting in diastolic ventricular dysfunction (242).

In response to MI, myofibroblasts, phenotypically transformed fibroblasts are the only cell types present in the mature infarct tissue that can play a major role in collagen formation (345). Myofibroblasts are not part of the normal cellular constituents of myocardium. They appear in pathological situation for example in infarcted area (311). Once present, they start
to proliferate and produce several matrix proteins, specifically collagen III and I, which are the main fibrillar collagens involved in tissue repair (237).

There are several factors involved in regulation of tissue fibrogenesis. One of these regulators is TGF-β1, that in addition to stimulation of myofibroblast proliferation, plays an important role in transforming of fibroblast to myofibroblast (308), promoting collagen synthesis and inhibiting collagen degradation (228). Inflammatory macrophages, present in the infarcted area within hours of the initial injury, are the major source of the TGF-β1 production (308). A number of histological studies have highlighted the role of Ang II in fibrosis of non-infarcted myocardium following MI (156).

1.3.2.2.1 Extracellular Matrix

Alterations in the extracellular matrix (ECM) have also been suggested to contribute to the development of heart failure subsequent to myocardial infarction (237) and cardiomyopathy. After myocardial infarction, abnormal collagen (155) deposition occurs in the scar region as well as in the remaining viable myocardium remote from the site of infarction (156). The cardiac ECM acts to direct the contractile force produced by myocytes and in addition, contributes to the passive stretch characteristics of the ventricular chamber (237). It has been suggested that structural abnormalities within the cardiac interstitium serve as the primary mechanism for myocardial failure.

The cardiac ECM is predominantly composed of collagen, types I and III being the most abundant forms within the heart (19). Type I collagen is usually present in the form of thick fibers and provides the great tensile strength to the myocardium (45), while type III collagen forms a fine reticular network. Other types of collagen proteins including type IV and type V collagens present in small amounts within the myocardium are associated with cell membranes. Concentration of collagen in the cardiac interstitium is dependent on the relative
balance between synthetic pathways and degradation of collagen mediated by proteases, the matrix metalloproteinases (MMP's). Results from experimental studies (237) and those from clinical investigations (337) have provided incremental evidence for increased deposition of collagen proteins in regions of the left ventricle remote from the infarction zone. This abnormal increase in myocardial collagen concentration results in myocardial fibrosis (346) which is defined as a reactive interstitial and perivascular fibrosis (in the surviving myocardium) and replacement fibrosis for dead cardiac muscle (346). Development of cardiac fibrosis may be a result of increased collagen synthesis, reduced collagen degradation, or both (79). Excessive collagen deposition may lead to diastolic dysfunction by increasing cardiac muscle stiffness and reducing ventricular chamber compliance (191). Presence of excessive cardiac collagen has also been reported in human dilated cardiomyopathy (268), in the Syrian hamster with genetic cardiomyopathy (79), and pressure-induced hypertrophy (74).

Recent studies suggest that the RAS system plays an important role in the stimulation of myocardial fibrosis (38). Upregulation of angiotensin type 1 receptors (AT1) has been show to occur in cardiomyopathic hamsters (170). Administration of losartan, an AT1 blocker, resulted in a significant decrease in cardiac fibrosis in post-MI rat hearts and this reduction was associated with an improved left ventricular function in these experimental animals (156). These studies provide further evidence linking cardiac ECM remodeling in the development of heart failure.

1.3.2.2.2 Matrix Metalloproteinases

The matrix metalloproteinase family consists of at least 16 members that together target all basement membrane and interstitial matrix components. Originally, these enzymes were classified into several groups; collagenases, gelatinases, stromelysins, and matrilysin, based
on their substrate specificity. However, currently MMPs are classified according to their structure. Most MMPs possess a signal peptide for secretion, an N-terminal propeptide domain that is removed when the enzyme is proteolytically activated, a zinc-coordinated catalytic domain, a hemopexin-like domain (shares structural homology-with a blood plasma protein that binds heme groups and prevents their elimination in urine), and a C-terminal domain (89). Gelatinases (MMP-2 and MMP-9) also contain a fibronectin domain that is inserted in the catalytic domain (30). MMPs are soluble with the exception of Membrane-type MMP (MT-MMPs) (351). Cleavage of 10 kDa prodomain activates latent MMPs. This can be observed by the appearance of two distinct bands using protein electrophoresis.

Before and during the period of myocytes necrosis there is extensive deposition of collagen and an increase in the tensile strength allowing the infarcted region to thin and elongate (242). This involves degradation of the extracellular scaffolding, allowing side-by-side slippage of muscle bundles to occur, resulting in a reduction of the number of myocytes across the infarcted region (347). Central to expansion is the degradation of the inter-myocyte collagen struts by serine proteases and the activation of MMPs released from neutrophils (58). Tissue inhibitors of MMPs (TIMPs) confine collagenolytic activity to the region of injury and control matrix degradation. Synthesis of MMPs and TIMPs is regulated by locally generated growth factors including TGF-β1 (307). Infarct expansion reflects an imbalance in production between MMPs and TIMPs (58).

Studies have demonstrated that MMPs are involved not only in LV remodeling and heart failure (189) but also in cardiac rupture (133). Among the various known MMPs, MMP-9 has been shown to play an important role in post-MI remodeling (86), and it is mainly expressed in such infiltrating inflammatory cells as neutrophils and macrophages (188). Conversely, MMP-2 is widely distributed in cardiac myocytes and fibroblasts (55), and it has been shown
to be persistently upregulated after MI (245). MMP-2 KO mice exerted less LV cavity
dilatation and improved fractional shortening after MI (128). Therefore, MMP-2 may play an
important role in early myocardial healing and the late post-infarct remodeling process.

1.3.2.3 Heart Failure

Heart failure (HF) affects nearly 5 million Americans and approximately 400,000 new cases
are diagnosed annually in the US. HF is the leading cause of hospitalization in adults over the
age of 65, with an incidence rate approaching 1 per 100 individuals (153). In fact over the
past decade the rate of hospitalization for HF has increased by over 159%. Increased survival
of acute MI, as the result of improved surgical and pharmaceutical interventions, has played
an important role in the increased prevalence of HF.

Heart failure is a progressive, pathophysiological state characterized by ventricular dilatation,
thinning of the LV wall and a reduction in LV function where the heart is unable to pump
blood at a rate to meet the metabolic demands of the various tissues, at normal cardiac filling
pressures or volumes. Post MI HF represents the ultimate clinical consequence of the
constellation of mechanisms that contribute to adverse ventricular remodeling. An additional
hallmark of post-MI heart failure includes elevated systemic vascular resistance and the
activation of compensatory neurohormonal systems. Indeed, current clinical management
focuses on these latter features and includes beta-adrenergic blockade, angiotensin converting
enzyme inhibition or angiotensin receptor blockade, and aldosterone antagonism.

1.4 Overview of Diabetes Mellitus

Diabetes mellitus (DM) is defined as a state of abnormal chronic fat, carbohydrate and
protein metabolism characterized by hyperglycemia resulting from defects in insulin
secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with
long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels (96).

Diabetes has become a major public health problem and social burden in North America and around the world (165). According to the World Health Organization (WHO) in 2000, more than 176 million individuals were affected by diabetes around the world and this number is predicted to increase to more than 300 million by the year 2025. In Canada, over 6% of the population suffers from diabetes, with the prevalence of glucose intolerance for individuals over 65 years of age between 25%-35%. It has been estimated that the number of people diagnosed with diabetes in Canada will be nearly double in the next 25 years, due to a number of factors such as continued increase in the prevalence of obesity and a sedentary lifestyle (WHO, 2003).

Diabetes has become a worldwide concern, and its associated morbidity and mortality will continue to cast an enormous burden upon personal, public and economic costs.

1.4.1 Classification of Diabetes Mellitus

Uniform diagnostic criteria for diabetes were first recommended by the American Diabetes Association and WHO in 1979 and 1980. Originally, DM was mainly classified as two types: insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM). The classification and diagnostic criteria were later updated and revised in the late 1990s (6). The Canadian Diabetes Association adopted the updated diagnostic criteria in 1998.

According to the current revised criteria, diabetes mellitus is classified into the following four categories: 1) Type 1 diabetes (called type 1, IDDM or juvenile diabetes), 2) Type 2 diabetes (called type 2, NIDDM or adult-onset diabetes), 3) Other specific types of diabetes include those of known etiologies, such as diabetes caused by genetic defects of beta-cell
function or insulin action (this group also includes drug- or chemical-induced pancreatic dysfunction causing diabetes), 4) The last category, gestational diabetes refers to diabetes that occurs during pregnancy.

1.4.1.1 Type 1 Diabetes

Type 1 diabetes is a chronic metabolic disorder characterized by a loss of pancreatic islet β cell mass, decreased serum insulin, and hyperglycemia. It occurs in childhood or early adolescence and accounts for approximately 10% of all cases of diabetes. It manifests itself when nearly 90% of the pancreatic β cells are destroyed (229). Although the pathogenetic mechanisms of this disease have not been fully understood, genetic, environmental, and autoimmune factors have been postulated (179). Primarily, Type 1 diabetes is known as a T cell-mediated autoimmune disease directed against one or more β cell autoantigens. Failing immunoregulatory mechanisms activate T cells specific for islet cell autoantigens leading to the development of type 1 diabetes. Later these T cells start to expand clonally and initiate a cascade of immune and inflammatory processes in the islets, resulting in β cell destruction. There could be three strategies to prevent the development of immune -mediated type 1 diabetes: β cell protection, cell regeneration, or β cell replacement. β cell destruction starts with the appearance of antigen presenting cells, such as macrophages and dendritic cells, followed by CD4 and CD8 T cells and B cells. In the beginning this non-destructive insulitis is usually located around the islets. Later, these cells become destructive. T cell-mediated cellular destruction is regulated through the release of cytotoxic molecules or perforin, or through Fas activated cell-death signaling pathways. β cell apoptosis occurs by activation of caspase pathway via numerous mechanisms including, Fas interaction with Fas ligand, action of nitric oxide and oxygen-derived free radicals and finally membrane disruption by perforin and granzyme B produced by cytotoxic T cell (161).
Although the focus in type 1 diabetes is the pathogenetic infiltration of T cells in the islet, many islet autoantigens are constituents of the central and peripheral nervous system, such as glutamic acid decarboxylases (GAD65, GAD67), islet antigen (IA)-2 and islet cell antigen (ICA) (354). Diabetic autoimmunity in humans and non-obese diabetic (NOD) mice contain development of T-cell autoreactivities that target nervous system autoantigens. The best example of this is the development of diabetes in multiple sclerosis patients (199). The best evidence is the early presence of T lymphocytes surrounding peri-islet Schwann cells (pSC) in NOD mice. The accumulation of these T cells around pSC takes a few weeks before penetrating into the islets and casusing destruction of them, which results to the destruction of beta cells (354). Therefore, autoimmune diabetes is not β cell-exclusive and can develop through two distinct pathways, with or without pancreatic Schwann cell failure.

1.4.1.2 Type 2 Diabetes

Type 2 diabetes is a complex disorder accounting for approximately 90% of all diabetic cases. It is generally characterized by three pathophysiologic abnormalities: peripheral insulin resistance (IR), impaired insulin secretion and excessive hepatic glucose production. While the diagnosis of DM is generally predetermined by levels of hyperglycemia, abnormal glucose metabolism in type-II diabetes develops over time. It is not well understood whether the primary cause of diabetes is β-cell dysfunction or IR; because once the disease has developed it is impossible to determine the initial event. It is widely believed that resistance to the action of insulin occurs first (114). This is supported by studies reporting that individuals with type 2 diabetes often present as hyperinsulinemic, even though these levels are regarded as low compared to blood glucose concentrations (209). In the beginning, many type 2 diabetic patients have a sufficient number of functional β - cells to maintain glycemic control with proper dietary restrictions. However in order to compensate for the increased
blood glucose level in response to chronic peripheral IR, pancreatic β-cells become hyperactive. Initially, the compensatory hyperactivity of the pancreatic β-cells allows for a degree of glycemic control in these patients. However, as time passes, the insulin secretory response declines and the β-cells can no longer compensate, failing to respond appropriately to glucose and hyperglycemia. At this time, hepatic glucose production and plasma concentration of glucose increase in parallel with the decline in plasma insulin concentration (185). Ultimately this leads to the deterioration of glucose homeostasis and the development of glucose intolerance. Studies have shown that approximately 5 to 10% of glucose-intolerant patients progress to frank diabetes, which continues to worsen as IR increases. Over time, the unregulated production of fatty acids and glucose by adipose tissues and the liver, respectively in diabetic patients, leads to a complete β-cell failure as well as other complications such as ketoacidosis (264).

1.5 Cardiovascular Complications in Diabetes

Consistent hyperglycemia in chronic diabetes can cause metabolic abnormalities, which affect functions of various organs and eventually lead to complications. Both type 1 and type 2 diabetes are associated with long-term complications that threaten life and the quality of life. Specific long-term complications of diabetes include: (1) Retinopathy with potential loss of vision; (2) Nephropathy leading to end-stage renal disease and failure; (3) Neuropathy with risk of foot ulcers, amputation, sexual dysfunction, and potentially disabling dysfunction of the stomach, bowel, and bladder; (4) Cardiovascular complications such as arteriosclerosis, congestive heart failure, and diabetic cardiomyopathy.

Diabetic patients are very susceptible to cardiovascular complications. It has been shown that the incidence of cardiovascular diseases is 2-3 fold higher in diabetic patients than nondiabetic patients (159). The death rate from cardiovascular diseases was also significantly
higher in diabetic patients and up to 80% of mortality in diabetic patients is related to cardiovascular complications. Diabetes can cause both macrovascular and microvascular pathological changes. Macrovascular pathological changes may contribute to the development of atherosclerosis, coronary heart disease, medial calcification, stroke, peripheral vascular disease, hypertension and myocardial infarction. Microvascular pathological changes of diabetes can result in diabetic retinopathy, nephropathy, cardiomyopathy and neuropathy (194, 317, 327). Abnormalities in endothelial function and vascular smooth muscle cell function as well as a prothrombotic state, also contribute to atherosclerosis and its complications in patients with diabetes (141). Hyperglycemia has also been identified as an independent risk factor for the development of coronary artery disease (CAD) in three important clinical trials (46, 92, 233). Other cardiovascular risk factors such as hypertension, dyslipidemia, increased vascular LDL cholesterol and collagen deposition, increased platelet aggregation and increased oxidative stress, only serve to compound the development of micro and macrovascular complications in these patients (121). Also, elevated levels of oxygen free radicals, likely play a key role in initiating and maintaining endothelial dysfunction, reducing bioactivity of NO thus shifting the balance toward the vasoconstrictor agents.

Diabetic patients also have an increased risk of congestive heart failure (CHF) (158). In spite of the very well established adverse impact of diabetes on survival post-MI, the mechanism behind the higher risk for the development of HF in people with diabetes after an initial injury such as an acute MI is not fully understood. Jaffe and colleagues have previously reported a 4-fold increased rate of LV failure in diabetic patients, particularly in patients with moderate sized infarcts which generally have a good prognosis in non-diabetic patients (151). This study suggests that MI size may not be an important predictor of HF in individuals with
diabetes. Similarly, LV remodeling and dilatation are also presently being questioned as factors in the onset of HF in people with diabetes.

Given the fact that diabetic patients develop HF at a higher rate than non-diabetic people post-MI and the fact that post-MI LV remodeling is the best predictor of HF and post-MI prognosis in the non-diabetic population, it had been assumed that diabetic hearts would demonstrate more adverse remodeling and dilation post-MI. One small study of 49 post-MI patients suggested a worsening of LV function and increased LV dilatation in diabetic hearts compared with non-diabetic hearts. Another study of 100 patients by Dini et al. reported that non-diabetic patients with total or subtotal coronary occlusion demonstrated significantly greater end systolic and diastolic volume indices as compared to controls without any coronary occlusion; while no significant differences were observed between diabetic and non-diabetic post-MI patients (78). By contrast, in a sub study of the SAVE trial, Soloman et al. reported that diabetic patients exhibited significantly less post-MI dilation and remodeling as compared to the non-diabetic patients regardless of treatment with the ACE inhibitor, Captopril, despite the higher prevalence of HF in the diabetic patients (286). This group further concluded that LV dilation was not a predictor of HF in diabetic patients even though HF was observed more frequently in the diabetic group. A small study by Marfella et al. has suggested an alternative mechanism which may result in diabetic HF (198). In this study, Marfella et al. reported that worsening diabetes, as measured by fasting blood glucose levels and increased HbA1c, was associated with ventricular dyssynchrony. Dyssynchrony between right and left ventricular contraction and relaxation has been previously identified as an independent predictor of HF and cardiac mortality amongst HF patients (1). Similar to clinical data, there is some inconsistency in the impact of diabetes on cardiac structure and function in animal models. The altered cardiac phenotypes associated with
diabetic cardiomyopathy have been investigated in a wide array of experimental animal models. Table 1.1 summarizes the biochemical, structural, and functional abnormalities reported in these models (246).

Table 1.1. Metabolic and cardiac phenotypes in experimental models of diabetic cardiomyopathy. Adapted from Poornima et al. Circ. Res. 2006; 98; 596-605

From a mechanistic perspective, diabetic cardiomyopathy should be predictable based on the duration and the severity of the abnormalities in non-esterified fatty acids, insulin, and glucose homeostasis. However, the experimental conditions under which the models are studied add significant complexity to the findings. The paradoxical findings might be due to differences in the species examined (mice vs. rats), dose of STZ injected, lack of requirement for insulin therapy for animal survival, the time-point examined or the sensitivity of these hemodynamic measures to detect subtle alterations in LV relaxation and compliance.

1.5.1 Diabetic Cardiomyopathy

Diabetic cardiomyopathy is a known heart complication that affects heart function in patients with diabetes. The pathological condition of diabetic cardiomyopathy was originally described by Rubler et al. in 1972 on the basis of observations in four diabetic patients who presented with heart failure without evidence of hypertension, coronary arterial disease,
valvular or congenital heart disease (259). Later, diabetic cardiomyopathy was defined as cardiac dysfunction caused by diabetes independent of dominant pathophysiological changes such as coronary atherosclerosis, macroangiopathy and autonomic neuropathy (21) (Fig 1.2). Cardiac dysfunction in diabetic cardiomyopathy may be caused by defects in myocardial cellular organelles such as myofibrils, mitochondria, sarcoplasmic reticulum and sarcolemma.

Impairment of calcium handling and lipid accumulation in cardiomyocytes may also play a role in depressing heart function in patients with diabetic cardiomyopathy (169). In regard to the cardiovascular system, diabetes mellitus is associated with multiple pathophysiological changes. Among them, endothelial dysfunction and hemostatic disorders are the most important factors for the higher risk of CAD. However microangiopathy, myocardial fibrosis, and abnormal myocardial metabolism have been implicated in the pathogenesis of a diabetic cardiomyopathy. In most cases HF in diabetic patients is a consequence of CAD; other possible causes include the co-morbidities frequently encountered in diabetic patients such as hypertension or other causes of nonischemic cardiomyopathy. The existence of a diabetic cardiomyopathy may increase the risk of HF in response to these insults; however, whether diabetic cardiomyopathy alone may be responsible for HF remains unknown. Cardiac failure in diabetic cardiomyopathy despite preserved ventricular systolic function indicates that there is a decreased diastolic compliance in these patients (280). Both active and passive diastole are thought to contribute in this abnormal function (364). It has been shown that there is a correlation between blood glucose control and diastolic dysfunction (284). One reason for this type of impaired ventricular filling in the latter could be due to alteration in calcium handling mechanisms in the early stage of relaxation such as defects of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) and concomitant regulatory proteins such as
phospholamban (75). The second reason could be because of alteration of extracellular matrix properties of the heart as a consequence of myocardium fibrosis, which manifests LV stiffness (333).

**Figure 1.2. Potential mechanisms linking diabetes mellitus to heart failure.** Adapted from Bauters, et. al. Cardiovascular Diabetology 2003 Jan 8;2:1

**1.5.2 Myocardial Fibrosis in Diabetes**

The excessive diastolic LV stiffness due to accumulation of fibrosis, which modifies ischemic LV dysfunction, could be a reason for the reduced LV remodeling and increased HF after acute MI (333). Although the mechanisms underlying these changes are not well understood, few mechanisms have been shown to contribute. One of them is TGF- β, a growth factor with pro-fibrotic and hypertrophic effects (200). TGF- β is stimulated by increased aldosterone and angiotensin II (ANGII) (306). The other mechanism is related to circulating advanced glycation end products (AGE), which are able to crosslink with some proteins of the matrix. This concept will be discussed in detail later. Next to nonenzymatic
glycation of collagen, dysregulation of collagen degrading proteins, MMPs and their tissue inhibitors, TIMPs, are supposed to be a hallmark in diabetic myocardium fibrosis.

1.5.2.1 Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) regulation is accompanied with maladaptive changes in the composition of extracellular cardiac matrix leading to the increase of total collagen content in human diabetic cardiomyopathy, as well as in animal models (336). MMP-2 and MMP-9 have shown to have an effect on cardiac extracellular matrix. Little is known about cardiac MMP levels in diabetes (314). While MMP-9 activity is enhanced with hyperglycemia or DM as observed in diabetic rats, hyperglycemic bovine aortic endothelial cell and coronary collateral development MMP-2 activity did not change (328). Notably, MMP-9 in spite of having the same structural protein substrates as MMP-2 expresses much lower proteolytic activity and therefore might not be important for altering collagen content. Although it has been shown that MMP-9 upregulates biological activity of TGF-β (335), there is a recent study demonstrating significant decreased protein content of MMP-2, but not MMP-9, regulated by Angiotensin II type 1 receptor (AT1) in STZ-induced diabetes which was consistent with increased cardiac fibrosis (350). MMPs are synthesized by cardiac fibroblasts (CF) and treatment of these cell types with high glucose concentration promoted fibrosis by increasing CF protein and collagen synthesis and decreasing MMP-2 activity via the upregulation of AT1 receptors, which can be blocked by losartan (16).

1.5.3 Diabetes and Cardiac Metabolism

Diabetes impacts the metabolism of the heart by numerous biochemical changes, which alter a diabetic heart’s energy metabolism and are associated with contractile dysfunction present in individuals with diabetes. Similar to the non-diabetic failing heart, the diabetic heart
undergoes changes in myocardial metabolism, which can overtime significantly decrease cardiac function. Viewed most simply, glucose uptake decreases, and fatty acid metabolism predominates (369).

Under normal aerobic conditions, the main sources of ATP synthesis (>98%) comes from oxidative phosphorylation in the mitochondria, while glycolysis counts for less than 2% of ATP. In a normal heart 60-90% of ATP re-synthesis in the mitochondria comes from the β-oxidation of fatty acids, while only 10-40% comes from pyruvate, which is formed from lactate and glycolysis (220).

The healthy heart easily extracts free fatty acids from the plasma and either oxidizes them instantly or stores them as triglycerides. The rate of uptake of free acids mainly depends on the plasma levels of fatty acids as well as the concentration of the specific fatty acid transport proteins in the sarcolemmal membrane (35). β-oxidation of fatty acid happens in the mitochondria, through a series of repetitive oxidative steps. Each step produces one molecule of acetyl CoA, one NADH and one FADH$_2$ with each successive turn, which continues until the entire fatty acid chain has been completely oxidized. Then, resulted acetyl CoAs are oxidized within the Krebs cycle to produce NADH and FADH$_2$, which in turn initiate phosphorylation and produces ATP.

As mentioned above a lower percentage of ATP is generated from converting glucose and lactate into pyruvate in the cytosol, which is ultimately oxidized, to CO$_2$ in the mitochondria. The uptake of extracellular glucose happens in two ways: 1- Diffusion from blood into the cell via transmembrane proteins, which is regulated by a glucose concentration gradient; 2- Transporting by plasma membrane glucose transporters, GLUT1 and GLUT4 (369), which is regulated by the concentration and activity of these transporters within the sarcolemmal
membrane. The concentration of these glucose transporters is influenced by several factors such as insulin and ischemia (260). Upon entering the cell, free glucose is phosphorylated into glucose-6-phosphate. At this stage the glucose can either be stored as glycogen or enter glycolytic pathways and undergo glycolysis (Fig. 1.3).

There is metabolic dysregulation in the failing heart, which has been shown in both animal models and humans. At the present time contradictory data are available on the rate of myocardial glucose, lactate and fatty acid metabolism and oxidation in ischemic hearts. These studies highlight the concept that ischemic heart disease is associated with profound changes in the expression of key regulatory proteins in both glucose and fatty acid metabolism within the viable myocardium, which could play a role in contractile dysfunction observed in failing hearts. However, it is assumed that glucose plays a central role for energy production in the ischemic heart, when lack of oxygen induces a shift to anaerobic metabolism with rapid increases of glucose uptake (29, 212). In theory, the oxidation of fatty acids requires a higher rate of oxygen consumption for every mole of ATP produced as compared to glucose and lactate; suggesting that the oxidation of glucose and lactate is slightly more oxygen efficient than fatty acids (294). This theory is supported by a study indicating that further enhancing the heart’s glucose and lactate metabolism instead of fatty acid oxidation improves cardiac function under pathological conditions (296).

The relative contribution of glucose to energy production is highly dependent on the severity of ischemia. In moderate ischemia (reduction of coronary flow by 75%), glucose uptake remains unchanged, while glucose extraction increases and metabolism of glucose is regulated from oxidation to lactate production (34). In severe ischemia, myocardial glucose uptake is inversely related to coronary flow (295), until the degree of ischemia becomes so
severe that glycolysis is prevented by increases in its products (219). At the time glycolysis is inhibited, glucose extraction gradually starts to decrease. This reduction is attenuated by several factors protecting the heart against prolonged ischemia injury, like increases of extracellular glucose or insulin (12, 71, 87, 334). There is controversy about how the beneficial or deleterious effects of glucose during ischemia depend on the models to be used and parameters to be measured.

In contrast, diabetic hearts differentially modify their fuel use, in which carbohydrate metabolism is also drastically reduced so that fatty acid oxidation can provide between 90 to 100% of a diabetic heart’s ATP requirements, without appropriate treatment. In one study of diabetic subjects fatty acid uptake was three-fold higher than normal, which coincided with a two-fold decrease in glucose uptake (131). A number of factors are involved in the observed diabetic switch to fatty acid oxidation as the sole source of metabolic energy, some of which

**Figure 1.3. Insulin controls glucose uptake in the heart.** GSV, GLUT4-containing small vesicles; IR, Insulin receptor; IRS, Insulin receptor substrate, Adapted from Allard, et. al. Heart and Metabolism Number 7, 2000
are very similar to those observed in ischemic hearts. Insulin deficiency, insulin resistance and subsequent reduction in GLUT-1 and GLUT-4 expression/translocation can only partly explain the metabolic changes. High circulating levels of fatty acids in individuals with diabetes, in addition to alterations in the regulation of fatty acid oxidation, also appear to play a significant role in the switch. The whole body shift to reduced glucose uptake and increased fatty acid availability occurs in diabetes because of reduced insulin action in most tissues. Increased release of fatty acids by the adipocyte and liver results in elevated circulation of fatty acids and triglycerides (360). To adapt to the requirement for increased fatty acid oxidation, mitochondrial proteins involved in fat metabolism show dramatic up-regulation (326) such as PPARβ and PPARα (145).

The preference for fatty acid oxidation at the expense of carbohydrate metabolism in diabetic hearts suggests that the hearts of diabetic individuals is already metabolically disadvantaged in the absence of cardiovascular complications and that with the onset of an acute event such as MI, these metabolic changes are further augmented which can in turn be responsible for the higher rates of myocardial failure and mortality in this patient population.

1.6 S100 Protein Signaling

The S100 proteins are one of the most extensive EF-hand (helix E-loop-helix F) protein families. These small acidic proteins with molecular weight of 10-12 kDa are called S100 because of their solubility in a 100% -saturated solution with ammonium sulphate at neutral pH. They contain two distinct EF-hands, 4 α-helical segments, and a central hinge region of variable length. The C terminal EF hand is referred to as the canonical Ca^{2+} binding loop including 12 amino acids and the N terminal loop is formed of 14 amino acids with the lower affinity for Ca^{2+}. To date, 25 members of this family have been identified. Of these, 21
family members (S100A1-S100A18, trichohyalin, filaggrin and repetin) have genes clustered
on a 1.6-Mbp segment of human chromosome 1 (1q21) while other members are found at
chromosome loci 4q16 (S100P), 5q14 (S100Z), 21q22 (S100B), and Xp22 (S100G). S100
proteins exhibit a tissue and cell type specific expression with diverse functional properties.
For example, S100A1 and S100A2 are found in the cytoplasm and nucleus respectively of
smooth-muscle cells of skeletal muscle tissue (83), S100P is located in the cytoplasm of
placental tissue (91) and S100B in cytoplasm of astrocytes of nervous system (166).
However their expression might be repressed in other cell types by negative regulatory
factors, which are controlled by environmental conditions. For instance induction of S100B
in rat myocardium post infarction (320) and inhibition of adipose S100 protein release by
insulin (310) imply that transcription/translation/post translation regulation of these proteins
is strongly controlled by negative and positive elements (80).
S100 proteins do not express intrinsic catalytic activity. However, they are calcium sensor
proteins and through interaction with several intracellular effector proteins they contribute in
the regulation of a broad range of functions such as regulation of myocardial and skeletal
muscle contractility, hypertrophy, apoptosis, the regulation of metabolic enzymes,
proliferation, migration, cell growth and differentiation, motility, cell cycle transcription,
organization of membrane-associated cytoskeleton elements and protein phosphorylation
(267). In order to modulate these types of activities S100 proteins might undergo
conformational changes (148). Upon Ca$^{2+}$ binding, the helices of S100 protein rearrange,
revealing a cleft, which forms the target protein binding site (88). In addition, some members
of S100 are able to bind to Zn$^{2+}$ and or Cu$^{2+}$ rather than Ca$^{2+}$ (129). The best example for
Ca$^{2+}$ - independent but Zn$^{2+}$ - dependent target protein recognition is the S100B (283).
Nitrosoglutathione (GSNO) related S-nitrosylation of the conserved C-terminal cystein is
strongly activated by the binding of Ca and Zn to S100B which results in a protein structure alteration (363). Some studies have shown that Cu$^{2+}$ binding to S100B might have a neuroprotective function (224). Although most target binding by S100 proteins are calcium dependent, several calcium independent interactions have been reported. Enzymes are the most common calcium independent targets binding for the S100 proteins. For instance, S100B and S100A1 bind with glycogen phosphorylase (367). The interaction of several S100 proteins with the same target can result in differential affects. For example, whereas S100A1 stimulates phosphoglucomutase activity, S100B inhibits the enzyme (171). The most significant calcium-independent interactions of S100 proteins are their ability to bind to each other. Typically, they are homodimers, but heterodimerization adds considerably to the complexity of this multiprotein family. Association of homodimeric S100 proteins provides an indication of stability of these proteins. In most of the cases dimerization could be enhanced in the presence of calcium (98). Ostendorp et al. have purified and characterized recombinant tetrameric, hexameric and octameric S100B from Escherichia coli. S100B tetramer caused stronger activation of cell growth than S100B dimer and promoted cell survival (231). Growing evidence indicates that in addition to intracellular activities, some S100 proteins such as S100B, and S100A1-13 have extracellular function as cytokines and chemokines. However, secretion has been proven only for S100B, S100A8, and S100A9 by an as yet obscure mechanism (81). It is suggested that S100A8/A9 heterodimer is secreted by novel secretion pathways that depends on an intact microtubule network and acts as a chemotactic molecule in inflammation (248). The extracellular effects of some S100 proteins are dependent on receptor activation, predominantly the receptor for advanced glycation end products (RAGE). RAGE is a member of the immunoglobulin superfamily of cell-surface molecules. In addition to its well known ligands, advanced glycation end products (AGE),
RAGE interacts with divergent members of S100 family such as S100A6 and S100B (231) as well as S100A12 (106). An interesting feature of the receptor is its apparent colocalization to sites where its ligands tend to accumulate. Thus, where AGEs and S100B accumulate, higher levels of RAGE are also identified in cellular elements (44). Although structurally S100 members are similar, they can interact with different RAGE extracellular domains and differentially stimulate cell survival. For example, at micromolar concentration while RAGE interaction with S100B increases cellular proliferation, S100A6 triggers apoptosis (175).

1.6.1 S100B Signaling

S100B is predominantly expressed in astrocytes, oligodendrocytes, and Schwann cells in the central and peripheral neuronal system. In addition to playing a major role in brain physiology (65), S100B is considered a biochemical marker for brain injuries after bypass graft surgery (10). Also, abnormal levels of S100B have been detected in Alzheimer’s, Down syndrome, schizophrenia and epilepsy (110, 119, 281). S100B has intracellular and extracellular effects (Fig.1.3). Intracellularly, S100B can regulate cytoskeletal dynamics through disassembly of tubulin filaments, type III intermediate filaments (81) and binding to fibrillar proteins such as CapZ (164, 292, 332) or inhibiting glial fibrillary acidic protein (GFAP) phosphorylation when stimulated by cAMP or Ca $^{2+}$/calmodulin (104). Moreover, S100B interacts in a Ca $^{2+}$-dependent manner with the cytoplasmic domain of myelin-associated glycoprotein and inhibits its phosphorylation by protein kinase A (168). It is implicated in the phosphorylation of tau protein and modulation of kinase activity by NDR kinase (nuclear Dbf2-related protein kinase) (298), GTPase Rac1, the Cdc effectors (24) and IQ motif containing GTPase activating protein (IQGAP) (204). IQGAP1 plays a role during the process of dynamic rearrangement of cell membrane morphology of astrocytes (204).
Interaction of S100B with the C terminus of p53, disrupting its tetramer structure, has inhibitory effects on phosphorylation of this tumor suppressor (187) and of protein kinase C (20).

After secretion by astrocytes, S100B has autocrine effects such as activation of extracellular signal-regulated kinase (117), and paracrine effect on neurons by promoting their survival during development and after injury through the NF-KB pathway, as well as through neurite outgrowth (8, 166). It has been well documented that extracellular concentrations of S100B play an important role in the physiological response. At nanomolar quantities it has trophic effects on neurite outgrowth by formation of covalent disulphide bonds (274), however, at micromolar concentration it has been implicated in glial activation (a prominent feature in Down syndrome and Alzheimer’s disease), up-regulation of nitric-oxide synthase, and apoptosis (142, 146). Moreover, higher concentrations of S100B with formation of disulphide bonds can induce an inflammatory response by stimulating microglial nitric oxide or cyclooxygenase-2 (3, 25). The mechanisms regulating secretion of S100B still remain unclear. Under basal conditions, S100 proteins are localized in specific cellular compartments from which they can move in response to Ca\(^{2+}\) activation (69, 197). This implies that translocation of S100B might be involved in its interactions with different partner proteins. Therefore most of the S100B signal transactions, whether induced through interaction with intracellular partners or binding to extracellular receptor domains, are most likely preceded by translocation and/or secretion of S100B. Astrocytes (glial cells), in order to secrete S100B, have developed a complex system regulating the ratio of intracellular Ca\(^{2+}\)/Zn\(^{2+}\) concentration. This function is not affected by the endoplasmic reticulum–Golgi classical secretion pathway inhibitor, brefeldin A (68). Secretion happens in response to external signals, produced by neurons and other astrocytes, induced Ca\(^{2+}\) signaling (68).
Secretion can be reversed by activation of glutamate receptor 3 regulated by neural and synaptic activity (263). Davey et al. showed that GFP-tagged S100B translocates from perinuclear areas toward the periphery of the cell in the form of vesicle-like structures in response to Ca\(^{2+}\) increase and Zn\(^{2+}\) decrease (68). Also they showed that S100B secretion is not likely dependent on the ER-Golgi pathway because the classical signal sequence is not used. Translocation of S100B in endothelial cells has been shown to be induced by its own extracellular presence via the RAGE receptor (140). In line with this observation, Perrone et al. have demonstrated that the Src kinase orchestrates a vesicular pathway that leads to RAGE recycling and S100B secretion (238), which in turn activates Src–caveolin-1 in a RAGE dependent manner (251). It has been shown that the actin cytoskeleton is not required for S100B secretion (68). After secretion or leakage from damaged cells, S100B accumulates in the extracellular space and/or enters the blood stream and cerebrospinal fluid and can be detrimental, causing pathological glial activation and neuroinflammation in response to injury (239).

In addition to astrocytes and neuronal cells adipocytes contain high levels of S100B. Hidaka et al. obtained the first evidence of S100B in adipose tissue and Michetti et al. found that S100B protein in adipose tissue was comparable to that measured in the brain tissue (134, 207), in agreement with later investigations indicating high level expression of S100B in human adipose and this amount varies depending on the anatomical area of adiposity (100, 116). There is clear evidence that adipocytes release S100B with the similar mechanisms to astrocytes (112). A positive regulation of S100B secretion in this tissue was observed in response to glucagon and catecholamines (244). Comparing the ratio of adipose/body weight to brain, the secretion of S100B from adipose tissue could be an important source of serum
S100B that is elevated during physical exercise (126) and correlates with body mass index (299). The biological significance of extracellular S100B from adipose tissue has not been investigated.

RAGE is a well-characterized cell surface receptor for S100B. It was first known as a receptor for glucose modified proteins, associated with the development of chronic inflammation in diabetes (221). The innate immune response can be induced by S100B, recruiting monocytes (276) and activating macrophages (94), both in a manner that depends on RAGE. Moreover, S100B via RAGE is potentially able to increase endothelial adherence to leucocytes (99), to reduce vasodilation induced by acetylcholine (107), and to increase neovascular proliferation (253). Therefore, the proinflammatory effect of S100B is regulated by RAGE. S100B/RAGE interactions are strictly Ca^{2+} dependent and can be altered with S100B concentration and in a cell specific manner (146). RAGE regulates both trophic and toxic effects of extracellular S100B in the brain (136). The S100B/RAGE binding has been mapped to the VC1 domain of RAGE, with the V domain as a key contributor compared to the C domain (67). Multiple RAGE binding sites in tetrameric or octameric S100B have higher binding affinity and result in the multimerization of the receptor and induction of signal transduction (231) leading to the production of reactive oxygen species, phosphatidylinositol 3-kinase/AKT and NF-KB, resulting in cellular proliferation(175). However, signal transduction by RAGE does not always involve S100B/RAGE interaction. For example, NO stimulation in microglia is dependent on the extracellular part of RAGE not the cytoplasmic domain and mostly serves to aggregate S100B on the BV-2 cell surface (3). An endogenously produced soluble RAGE (sRAGE) may act as a decoy for increased
pathological levels of S100B. Reduction of S100B and sRAGE levels in serum was associated with clinical improvement of acute schizophrenia (301).

Alternatively, S100B may interact with an unidentified receptor, as has been shown in myoblast cell apoptosis (293). One candidate for a non-RAGE receptor S100B interaction is Dopamine receptor, widely distributed in the central nervous system. Interaction of S100B with Dopamine receptor activates ERK and inhibits adenylate cyclase (297).

Figure 1.4. Schematic representation of proposed intracellular regulatory effects of S100B. Reproduced with permission from Donato, et. al. Biochemica et Biophysica Acta, 2009 June 1793(6):1008-22

1.6.1.1 S100B and Cardiovascular Disease

The first evidence that the myocardium could be an organ or tissue expressing S100B was the detection of the protein in the hearts of deceased patients with chronic lung disease (157). This led to a confirmatory study by our laboratory demonstrating that S100B was induced in hearts of rats 7-14 days following coronary artery ligation (321). Subsequently, induction of S100B in post- infarct myocardium of patients at the terminal stage of heart failure further confirmed these observations (235). In mechanistic experiments in vitro in cultured neonatal
rat cardiac myocytes, an expression vector encoding the human S100B protein inhibits $\alpha_1$ – adrenergic induction of the fetal genes $\beta$-myosin heavy chain (MHC), skeletal isoform of $\alpha$-actin (skACT)(321). The inhibition of S100B is selective, as it does not inhibit the capacity of thyroid hormone to induce $\alpha$-MHC. Importantly, S100B gene expression in myocardium is transcriptionally controlled dependent on both positive (-782/-162 and -6,689/-4,463) and negative (-4,463/-782) elements upstream of the transcription initiation site, selectively activated by $\alpha_1$ –adrenergic signaling regulated by $\beta$-protein kinase C (PKC$\beta$) and by the transcription factors, transcription enhancer factor (TEF)-1 and related TEF-1 (RTEF-1) as an inhibitor or stimulator, respectively (322).

Dilated cardiomyopathy (DCM) is a cardiac disease characterized by progressive dilation and impaired contractility of the left or both ventricles, leading to HF. Identifying reliable biochemical markers of ventricular dysfunction could improve diagnostic accuracy. S100B is known as an appropriate biochemical marker for central nervous system injury. Also, Bordignon and his colleagues by using an experimental model of isolated rat heart perfusion have demonstrated that rat hearts release low levels of S100B which is augmented by ischemia, indicating that in addition to brain, heart also is a source of serum S100B (221). Furthermore, this group showed there was a positive correlation between S100B and N-terminal pro B-type natriuretic peptide (NT-pro BNP, cardiac natriuretic biomarkers for cardiac disease) in DCM patients, which indicates that S100B in conjunction with other markers could be used for LV systolic dysfunction diagnosis (203).

Apoptosis in the infarct border zones is a hallmark of pathological ventricular remodeling after MI. Our laboratory has shown that S100B induction plays a role in the regulation of apoptosis in a RAGE dependent manner in post-MI myocardium (318). Intracellular S100B may modulate the apoptotic responses of post-infarct myocytes by activating a
transcriptionally inducible form of nitric oxide synthase (iNOS) and production of nitric oxide (NO) (210, 250) as has been shown for astrocytes (142). Thus, NO could be an intermediate pathway in the induction of apoptosis by intracellular S100B. In vitro administration of S100B to cultured cardiomyocytes induced apoptosis in a dose dependent manner beginning at 0.05 µmol/L, a local or regional concentration that is achieved in the peri-infarct myocardium (319).

Hypertrophy and its associated program of fetal gene re-expression such as β-MHC, skACT, and atrial natriuretic factor (ANF) can be induced in cardiac myocytes by treatment with trophic effectors, norepinephrine (NE) and phenylephrine (PE) (56, 341). As our group has shown, treatment of S100B transgenic mice with norepinephrine stimulates neither hypertrophy nor the fetal genes skACT or ANF expression indicating that S100B has an inhibitory effect on cardiac hypertrophy (235). In vivo studies performing descending aortic banding to produce pressure overload on S100B transgenic mice confirmed the previous results and the generalized anti-hypertrophic affect of S100B (321). To establish that S100B blocked α1-adrenergic induction of β-MHC and skACT by interrupting the PKC signaling pathway, the interaction between forced S100B expression and activated mutant PKCβ referred to as δPKCβ was tested in vitro (160). δPKCβ transactivated β-MHC and skACT genes suggest the regulation of α1-adrenergic induction of these genes by activation of the class-I PKC isoform β-PKC (160, 321). Forced S100B expression could only block δPKCβ-induced transaction of β-MHC and skACT treatment with a α1- adrenergic agonist or augmented extracellular calcium demonstrates the calcium depend capacity of S100B to modulate the hypertrophic phenotype (321). In contrast, in S100B knockout mice norepinephrine infusion as a α1–adrenergic agonist provoked a potentiated myocyte hypertrophic response and augmented arterial smooth muscle cell proliferation. In keeping
with an additional vascular role, in knockout mice, both the acute and chronic increase in blood pressure in response to $\alpha_1$–adrenergic agonist infusion was attenuated compared with wild-type mice (325).

To directly assess a role for S100B in post-MI remodeling, transgenic and knockout mice were subjected to coronary artery ligation. S100B transgenic mice demonstrated no hypertrophy, greater ventricular dilation, impaired function and higher mortality. In contrast knockout mice exhibited augmented hypertrophy, less dilation and improved survival compared to wild type mice (319). These results confirmed that S100B could be a potential therapeutic target to modify post-MI remodeling because deleting S100B was associated with greater hypertrophy, less LV dilation, less apoptosis, and improved hemodynamics in the early post-MI period (319).

1.6.1.2 S100B and Diabetes

One of the fundamental intracellular defects in animal models and patients with type 1 diabetes are abnormalities of calcium metabolism (141). However, the mechanism by which these changes in calcium metabolism could be transduced into cellular dysfunction has not clearly been established. Calmodulin/troponin C/S100 family of calcium regulatory proteins are involved in the intracellular calcium homeostasis responses and, therefore, might be proper candidates in diabetic cellular dysfunction. For example, alteration of the troponin complex has been associated with changes in contractility of type 1 diabetic hearts (141). On the other hand, elevated levels of calmodulin in pancreatic $\beta$-cells of mice resulted in secretion of insulin (202, 226, 252) and treatment of diabetic animals with insulin restores calmodulin transcripts to normal levels in insulin target tissues such as liver, heart, adipose tissue, kidney, and skeletal muscle (139, 213, 232, 287, 288). Despite the importance of these
calcium receptor proteins in linking calcium homeostasis and cell function, very little information is available regarding the effects of diabetes on their function. While some studies demonstrated diabetes and lack of insulin selectively decreased S100 expression in adipose tissue probably by increased release as observed in vitro studies, Zimmer et al. extended this study to various organs and determined the exact isoform of S100 by quantifying the effects of streptozotocin (STZ)-induced diabetes on S100A1 and S100B expression. They showed that these two members of S100 family had differential and non-coordinated alteration of mRNA and protein levels in various organs in response to diabetes (365). This regulation of S100A1 and S100B mRNA and protein levels indicate that transcriptional/ posttranscriptional and translational/ posttranslational modification of these genes is insulin-dependent. However, few insulin response elements are present in the S100A1 and S100B genes (9, 150, 227, 290). Moreover posttranslational regulation of some other genes such as S14 by insulin suggest that transcriptional effects may not be entirely responsible for altered S100A1 and S100B expression in diabetes (338).

As reviewed previously, alteration of adipose tissue containing high levels of S100B results in insulin resistance. It has been shown that an increased release of S100B in schizophrenia from brain and adipose tissue correlates with insulin resistance indicated by impaired insulin sensitivity and C-peptide/glucose ratios (300).

Damage to peri-islet Schwann cells (pSC) in islets, a lineage marked by the expression of S100B and GFAP (315, 354), could play an important role in development of type 1 diabetes. In STZ treated rats, pSC became reactivated following islet injury and began to express increased levels of nerve growth factor and GFAP (315). It has been shown that in NOD mice, immunotherapy with S100B significantly delayed diabetes development. Protected animals, despite showing strong TH1-biased peri-insulitis with removal of the pSC mantle,
were resistant to the islet invasion and β cell apoptosis (354). Since there is some S100B expression in β cells, this protection following S100B immunization could be due to the blocking of the S100B expression as an antigen in the β cells or alternatively in pSC. There is not enough evidence about the deleterious effect of S100B on β cells. One possible mechanism is the induction of Cyclooxygenase (PTGS2), which has been shown to occur in response to S100B treatment or diabetes in the pancreatic islet. PTGS2 modulates autoimmunity and thereby causes islet dysfunction (277). Thus, there is a possible linkage of S100B and progression to overt type 1 diabetes. One possible candidate for blocking the effect of S100B is Gabapentin (GBP) which attenuated the hyperglycemia-increased production of glial and neuronal markers, GFAP and S100B, mostly because of the reduction of the hippocampal and cortical neurodegeneration observed during diabetes mellitus (22).

1.7 Advanced Glycation End Products

During the past 20 years, one emerging area of interest and study has been advanced glycation end products (AGE) - the end products of amino-sugar reactions. The nonenzymatic reaction of the amino groups of amino acids, peptides, and proteins with reducing sugars, ultimately resulting in the formation of complex brown pigments and protein-protein crosslinks, was first studied by L.C. Maillard in the early 1900s. The Maillard reaction starts with the reaction of the carbonyl (aldehyde or ketone) of the reducing sugar with the amino group of proteins or lipids to form a reversible and unstable Schiff base. The Schiff base can undergo an intramolecular rearrangement to form the Amadori type products, which later can undergo further complex rearrangements, dehydration, and condensation to form irreversible and stable end products. Some of these products may be fluorescent (329). The most studied and first example of AGE in human is A1c (HbA1c), a naturally occurring minor human hemoglobin that is elevated in diabetic patients and serves as a marker of
glycemic control (51). The advanced glycation end products include N-(carboxymethyl)lysine (CML), known to be involved in diabetic nephropathy (218), pentosidine, which is seen in patients with diabetes and chronic inflammatory disorders (211) and the imidazolium salt cross-link, glyoxal-lysine dimer (GOLD), which is elevated in diabetes (53). In addition to proteins and lipids, DNA may also undergo AGE modification. It is assumed that the modification of DNA is involved in both regulatory and perhaps epigenetic components of the aging process (23).

AGE accumulation in diabetes is associated with development of cardiac dysfunction (15). Diastolic dysfunction is the first manifestation, which later progresses to systolic dysfunction. In the presence of other complications such as hypertension and CAD, this can accelerate the progression of heart failure. Enhanced AGE accumulation is not just implicated in diabetes and it can be involved in the development of heart failure in non-diabetic patients (125). AGES may contribute to the development of heart failure via two distinct pathways. Firstly, AGES affect the physiological properties of proteins in the extracellular matrix by creating cross-links. Secondly, AGES cause multiple vascular and myocardial changes via the interaction with AGE receptors.

Cross-linking of extracellular matrix proteins is necessary to strengthen the structure of tissues ensuring its integrity, without compromising flexibility. However, AGES, can covalently bind other AGES, and create additional cross-links between matrix proteins like collagen, laminin, and elastin (285) resulting in alteration of their biochemical properties by reducing enzymatic activity (97), altering biophysical properties (18), and changing protein interactions with other enzymes. AGE links form throughout the molecule, limiting the terminal positions for normal crosslinking, and this increases collagen AGE’s tensile stiffness. Moreover, AGE cross-links causes collagen to be less digestible by
metalloproteinases (215), which, in turn, favors its chronic accumulation in tissue. This excessive cross-linking attenuates the flexibility of matrix proteins. As a result it might affect the contractility of heart and lead to diastolic dysfunction. Also it has been shown that exposure to AGE caused a significant delay in calcium reuptake. As a consequence, the duration of the re-polarization phase of the cardiac contraction may increase, subsequently causing diastolic dysfunction.

Another pathway by which AGEs may contribute to the development of diastolic dysfunction is via activation of AGE receptors, which have been identified on several cell-types (37). AGEs act as ligands to various receptors; AGE-R1, AGE-R2, AGE-R3 and RAGE. RAGE is the most important receptor for AGEs, which induces intracellular signaling in respond to AGE and other ligands including S100 proteins as discussed previously. It mediates the induction of fibrosis via the upregulation of transforming TGF-β (302). RAGE affects calcium metabolism in cardiac myocytes as overexpression of RAGE in mice reduced the systolic and diastolic calcium concentration (237).

AGEs may affect the development of systolic dysfunction by accelerating the progression of CAD via direct vascular effects. Interaction of AGE/RAGE may induce atherosclerosis, thrombosis, and vasoconstriction by negatively influencing LDL-metabolism (230). Small soluble AGE peptides can form cross-links with low-density lipoprotein (LDL), rendering LDL particles more atherogenic and less susceptible to be recognized by LDL receptor which impairs clearance mechanisms resulting in the elevation of LDL levels in patients with diabetes (41).

AGEs also can affect intracellular protein properties, which results in changes to cellular functions that are critical in vascular homeostasis. One of these changes occurs in basic
fibroblast growth factor which once glycated, reduces the mitogenic activity of endothelial cells (113).

There are a variety of approaches that can block the pathophysiologic effects of AGE. First, AGE and AGE crosslink formation, can be inhibited by compounds such as aminoguanidine (AG), pyridoxamine, or OPB-9195 (2-iso-propylidenehydrazono-4-oxo-thiazolidin-5-ylacetanidide) (195). A second method for inhibiting AGE effects is to target RAGE by using a soluble extramembrane portion of the receptor (sRAGE) to act as a ligand scavenger or using RAGE antibodies. sRAGE administration has been demonstrated to attenuate early development of atherosclerosis as well as stabilize established atherosclerosis in diabetic apolipoprotein E–null mice (44). Another approach to inhibiting AGE pathophysiology is the use of cross-link breaker compounds. These agents contain a thiazolium structure that can break α-carbonyl compounds by cleaving the carbon-carbon bond between carbonyls. The best-known breaker is 4,5, -dimethyl-3-phenacylthiozolium chloride (ALT-711) that has been most widely studied (48).

1.8 Receptor for Advanced Glycated End Products

RAGE was originally isolated as a receptor for AGE (270) but has been found to bind diverse ligands (269). It has been identified that RAGE can bind to amphoterin, amyloid- β- peptide and β-fibrils as well as some members of the S100 protein family such as S100A12, S100A6 and S100B (82). Also S100A1 seems to be capable of binding and activating RAGE (138). RAGE belongs to the immunoglobulin superfamly of cell surface molecules (221) and is a 50 kDa protein consisting of 403 amino acids with an extracellular region containing one variable (V-type) Ig domain and two constant c (C-type) Ig domains with disulfide bridges, a hydrophobic transmembrane-spanning domain and a highly charged, short cytoplasmic domain (221). The V domain is critical in ligand binding and the cytosolic tail is important
for RAGE-induced intracellular signaling. Dominant-negative (DN) RAGE with a truncated cytosolic domain is unable to transduce a signal while embedded in the membrane even though it binds ligands (271). For example, overexpression of DN RAGE in vivo in SMC, cells of MP lineage, neuronal cells or CD4 lymphocytes, suppressed RAGE-mediated signaling cascades, thereby strikingly modulated injury-triggered outcomes in diverse settings (14, 49, 136, 262, 312).

The RAGE gene is composed of 11 exons and 10 introns. Additional RAGE isoforms lacking the transmembrane and cytosolic regions; soluble RAGE (sRAGE) which works as a ligand scavenger or lacking “V” immunoglobulin domain, and N-truncated RAGE (NtRAGE) were identified in human brain suggesting isoform-specific function for the receptor (76). The RAGE promoter contains binding sites for transcription factors NF-κB and Sp1 that have been shown to play important roles in the regulation of RAGE expression (181). Binding of NF-κB in the RAGE promoter creates a positive feedback loop in the regulation of RAGE expression because RAGE signaling in turn could activate NF-κB (358).

It has been clearly shown that RAGE is involved in various diseases and its expression is upregulated at pathological sites, such as in diabetes, atherosclerosis and Alzheimer (262, 269). Therefore, RAGE provides a potential therapeutic target for clinical studies. However, RAGE upregulation in response to some of its ligands is necessary in nervous system development, indicating that there are some non-pathophysiological roles for RAGE in homeostasis conditions.

One of the interesting features of this receptor is that its expression is characteristically low in the basal homeostatic condition but in situations characterized by enhanced cellular activation or stress, the expression of RAGE is significantly augmented (221). RAGE is
expressed in a variety of tissues and cell types such as endothelium, vascular smooth muscle, mononuclear phagocytes, cardiac myocytes and neural tissue (37).

**1.8.1 Signal Transduction of Ligand-RAGE Interaction**

RAGE modulation of gene expression and cellular properties are dependent on downstream signal transduction. RAGE is expressed in multiple, distinct cell types; thus, diverse signal transduction pathways may be impacted by RAGE. Also, in one cell type multiple parallel signaling pathways can be activated depending on the type of ligands and acuteness/chronicity of ligand stimulation.

Among the ligands such as AGEs, S100A12, S100B, amphoterin, amyloid- β-peptide and β-fibrils there is a cross-competition and binding to the V-type Ig domain of RAGE (122) with quite similar binding affinities in nanomolar concentrations (~ 50 nM)(136). The only known exception is modulation of cell survival by interaction of S100A6 with C1 and C2 RAGE Ig domains (175).

Upon ligand-RAGE interaction, reactive oxygen species (ROS) are generated, at least in part via activation of NADPH oxidase (344). ROS are important key intermediate factors of regulating intracellular signaling cascades such as NF-KB(66). Upstream signaling of NF-KB in response to ligand–RAGE interaction includes pathways such as p21ras, MAP kinases; Erk1/2 (p44/p42), p38 and SAPK/JNK MAP kinases, Rho-family GTPases (Cdc42-Rac1), phosphoinositol- 3 kinase (PI3-kinase), and JAK/STAT.

Pro-inflammatory effects of RAGE are mediated by increasing the expression of cytokines such as IL-1β, IL-2 (122), IL-6 (248) and TNF-α (136). Because NF-KB is specifically known to be involved in the regulation of genes in cellular defenses, immunological responses, expression of cytokines and cell adhesion molecules (66), its activation by RAGE is likely responsible for enhanced expression of most of the cytokines. Regarding neuronal
development, it is unlikely that neurotrophic effects of RAGE can be mediated through cytokines. Recently cAMP response element-binding (CREB) which is an important factor in neuronal development and plasticity has been identified as a downstream target of RAGE (279).

In the cytoplasmic region of RAGE, critical for signal transduction, there is neither a conserved Ser nor Thr residue to recognize cytoplasmic signaling molecules nor a phosphorylation site motif. Therefore, other non-receptor cell surface proteins are utilized to transduce intracellular signaling cascades. It has been shown that binding of S100B to RAGE induced vascular smooth muscle cell migration and inflammatory gene expression, NF-KB, via activation of non-receptor Src kinase and tyrosine phosphorylation of caveolin-1 (251). The other possibility is that the highly charged cytosolic tail of RAGE may induce signal transduction in respond to ligand stimulation. The C-terminally truncated RAGE, as discussed before, serves as a dominant negative receptor blocking the effects of RAGE-dependent cellular activation. The receptor clustering induced by ligation is important for any receptor activation resulting in signal transmission into the nuclei of cells. Whether conformational changes and/or clustering of RAGE in response to ligation results in the initiation of signaling cascades, is not clear (120).

It appears that all of the ligands for RAGE are multivalent. For instance, AGEs can modify more than one group on a single macromolecule. The formation of intermolecular crosslinks by the same process (39) further increases the number of potential modification sites on a particular protein. Likewise, tetrameric S100B binds two RAGE molecules with higher affinity than dimeric S100B. Subsequently their signalling activity is greater after aggregation (231). It seems that aggregation of all RAGE ligands, in the form of oligomers, in response to sustained and chronic tissue perturbation and injury may activate RAGE.
constantly and by reaching threshold levels lose their ability to recognize RAGE selectively. This process overwhelms the natural and rapid dissociation of monomeric ligands, which happens in response to acute stresses results in repair events. The best example is accelerating wound healing by administration of sRAGE to diabetic mice subjected to full-thickness excisional wounds because of suppression of inflammatory mechanisms and blunting of excessive MMP activity. In contrast, administration of sRAGE to nondiabetic mice had no beneficial or deleterious effect on wound healing (118).

1.8.2 RAGE and Cardiovascular Disease

Although the role of RAGE in the myocardium has not been broadly studied, experimental evidence supports the premise that RAGE mediates at least in part, cardiac dysfunction in the heart in response to clinically-relevant stressors. Interaction of AGEs with RAGE affects myocardial energy metabolism and function during ischemia/reperfusion (I/R). Furthermore it was associated with generation of iNOS, nitrite and nitrate, and cGMP in the heart. RAGE knockout mice were strikingly protected from the adverse effect of I/R as indicated by decreased lactate dehydrogenase (LDH) level and increased adenosine triphosphate (ATP) (43). A second study from same group showed that RAGE knockout mice demonstrated higher fractional shortening, reduced plasma levels of creatine kinase, and diminished myocardium apoptosis following left anterior descending coronary artery/reperfusion. The effect of RAGE on heart injury was mediated through JNK and STAT5 phosphorylation (7). Finally the results from a more recent study revealed that LDH release and glycoxidation products carboxymethyl-Lysin (CML) were reduced in isolated perfused diabetic hearts subjected to I/R in transgenic mice overexpressing cytoplasmic domain-deleted RAGE (DN RAGE). In this study the authors showed, at least in part, that these effects contribute to RAGE-expression in endothelial cells and mononuclear phagocytes (42). This finding
underscores the premise that AGEs and RAGE-dependent pathways likely synergize in the diabetic heart to drive exaggerated inflammation and metabolic dysfunction in the response to injury as a consequence of ischemia. The relevance of these findings to chronic coronary occlusion resulting in myocardial infarction in both non-diabetic and diabetic hearts is unknown.

1.8.3 RAGE and Diabetes

Many organ systems are strikingly susceptible to injury and dysfunction in chronic hyperglycemia and RAGE may play a functional role in this response. Human diabetic kidneys display increased AGEs and expression of RAGE. Hence, animal model (db/db mice) studies suggest that blockade of RAGE is beneficial in nephropathy (349). While the administration of streptozotocin (STZ) triggered albuminuria and mesangial expansion in wild type mice, these changes do not occur in RAGE knockout mice. In other studies, the role of RAGE blockade in diabetic neuropathy has been tested in transgenic mice in which β globin was under NF-κB control. In the sciatic nerve, diabetes was associated with a 20-fold increase in the induction of β-globin transcripts and expression of IL-6 which was inhibited fully by pretreatment of the diabetic mice with sRAGE or using RAGE knockout mice (27). Beyond microvascular complications, blockade of RAGE has also been shown to attenuate diabetic complications in distinct AGE-enriched environments. In peripheral wounds, blockade of RAGE limits the intense inflammatory response, thereby accelerating wound closure and triggering angiogenesis (118). These types of studies suggested that even in an environment with highly inflammatory factors, RAGE delays the repair process.

An interesting study that shows the direct effect of RAGE in Type 1 diabetes as an autoimmune disease has been demonstrated in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. RAGE and S100B ligands were not detected in the
islets from NOD/SCID mice. However, transferring the spleen cells from diabetic NOD mice into non-diabetic NOD/SCID caused an induction of RAGE and S100B expression on the islet cells as well as in a population of T cells (CD4+ and CD8+) and B cells. This was associated with an inflammatory infiltration of the sections of pancreas that had received a transfer of spleen cells and induction of the disease. Treatment with sRAGE significantly prevented transfer of diabetes and delayed recurrence of the disease in these mice. In parallel, the expression of cytokines, IL-1β and TNF-α was strictly reduced by sRAGE treatment compared to vehicle-treated animals (54). This observation raises the question as to whether RAGE has potential roles in the adaptive immune response and/or amplification of immune injury in islets in diabetes as an autoimmune disease. Treatment of normal human donor pancreatic islets with S100B resulted in a significant increase of Cyclooxygenase-2 (PTGS2), an important mediator of acute and chronic inflammation, through the RAGE receptor. Therefore, blocking RAGE may present a feasible approach to treatment, particularly in diabetic cases where RAGE ligands such as AGEs and S100B are increased.

1.8.4 RAGE and S100B

The first identified signal transduction S100 ligand for RAGE was S100A12 (extracellular newly-identified RAGE binding protein, ENRAGE), which binds RAGE in a dose-dependent manner on cultured endothelial, smooth muscle cells, macrophages and lymphocytes (136). Distinct mechanisms were identified by which these molecules may be released by inflammatory cells (105, 231), thereby permitting them to bind to the RAGE. S100A12-RAGE interaction plays a proximal role in the inflammatory cascade. For example, prevention of NF-KB activation and the generation of IL-2 and TNF-α due to inhibition of S100A12-RAGE ligation are critical early events leading to delayed-type hypersensitivity (136).
S100B regulates apoptosis in post-MI myocardium by interaction with RAGE after cellular release from damaged myocytes (318). Our laboratory has demonstrated in neonatal rat cultures, that exogenously administered S100B induced either apoptosis or necrosis in a dose dependent manner. In this S100B study, transfection of a full–length RAGE cDNA or a dominant negative (DN) RAGE caused an increase or decrease in myocyte apoptosis by S100B, respectively. Also, inhibition of MEK signaling or overexpression of a DN p53 inhibited S100B-induced myocyte apoptosis (318).

In the context of the neuronal system, binding of lower concentrations of S100B with RAGE induces neurite growth by induction of NF-KB and Bcl-2 (146). On the other hand, binding of high concentrations of S100B with RAGE results in the neuronal oxidant-dependent apoptosis. Both scenarios require expression of the full-length receptor. In fact, the deletion mutant of RAGE was unable to transduce trophic or toxic stimuli of S100B. This indicates that extracellular S100B can induce cell survival or apoptosis only when the functional receptor is expressed on the cell surface and these paradoxical effects are dose-dependent.

In all these studies, it has been shown that binding of S100B to RAGE induces intracellular signaling. However, a recent report indicates that S100B-induced nitric oxide production in microglia does not require RAGE transducing activity but depends on RAGE extracellular domains (3). Also there is the possibility that S100B has RAGE-independent functions as it has been shown in myotubes, although the specific distinct receptors were not identified (293).

1.9 Extracellular Regulated Kinase

Mitogen activated protein kinases (MAPK) are important mediators of signal transduction which convert different extracellular stimuli (cytokines, hormones, cell-cell and cell-matrix
interaction) into the appropriate responses inside the cells. MAP kinases are a family of serine/threonine kinases that are activated by dual phosphorylation at conserved threonine and tyrosine (Thr-X-Tyr) residues (109). These molecules are involved in the pathways of cell proliferation and growth, cell differentiation, cell migration and cell death, through affecting several types of substrates such as transcription factors, enzymes, and other kinases. There are three major mammalian MAP kinase subfamilies; Jun NH2-terminal Kinase (JNK), p38, and extracellular regulated kinase (ERK). The ERK family includes ERK1/2, ERK3/4, and ERK5/6. The most widely studied cascade is ERK1/2 which is activated by MEK1 and MEK2 in response to growth factors (180).

MAP kinases are involved in several signaling pathways. The most relevant to this thesis is the p21(ras)/MAP kinase pathways culminating in NF-KB activation which results from AGEs binding to RAGE in smooth muscle cells (172). Also, MAP kinases regulate expression of numerous MMPs through IL-1 in fibroblasts and vascular endothelial cells (235). It has been reported that ERK1/2 regulates both MT1-MMP and MMP-2 gene expression in endothelium (36).

With respect to the RAGE signaling pathways, the cytoplasmic domain of RAGE is considered to be responsible for inducing intracellular signaling as noted previously. ERK1/2 has been shown as the only protein that directly binds RAGE with a D-domain-like docking site that is independent of the phosphorylation status of ERK2. ERK1 can bind RAGE directly or through ERK-2. Since phosphorylation of ERK was not observed, it was assumed that RAGE stabilizes ERK under the proximal region of the plasma membrane and results in increases of ERK activation and its interaction with substrates (149).
Although ERK is mostly known as a survival factor during some steps of preconditioning stress such as cardiac ischemia (362), it has been demonstrated that stimulation of RAGE upregulated ERK1/2 activation, p53 phosphorylation at serine 15, with resultant cardiac myocyte apoptosis (318). The other example for toxic effects of ERK is attenuation of neurite cell viability in an ERK1/2 dependent manner in response to micromolar concentrations of S100B (146).

Similarly, this paradoxical dual mechanism of ERK in diabetes has also been demonstrated in several studies. There is an assumption that metabolic changes occurring during the early phase of diabetes may cause resistance to the I/R injury by chemically preconditioning (PC) the heart (249). By induction of signaling pathways ERK is involved in ischemia PC, which is an effective endogenous cardio-protective mechanism. In addition, ERK could be elevated by short-term hyperglycemia in the heart and in cells cultured under high glucose conditions (143, 217). Injury due to ischemia reperfusion (I/R) did not alter the hyperglycemia-elevated ERK. In this condition, attenuated infarct size could be explained by enhanced resistance to ischemia though already activated ERK1/2, which may cover the effect, indicating myocardial protection in response to short-term hyperglycemia. However, reduction of ERK phosphorylation after long term hyperglycemia and I/R revealed that glycation of ERK by AGEs alters its structure and function. In consequence, at this time having a larger infarct size indicates that PC has lost its protective effect on myocardium (217, 356).
Figure 1.5. Schematic representation of proposed interaction of RAGE and its ligands, S100B and AGE and their underlying signaling pathways. Presence of high levels of glucose and oxidant stress result in the production of AGE, which interact with their cell surface receptor, RAGE. RAGE as a multi ligand receptor is able to bind to some members of S100 protein family such as S100B. S100B via calcium binding interacts with several intracellular effectors proteins and contributes to the regulation of a broad range of functions. S100B after secretion may bind to non-RAGE receptor such as TLR4. Upon ligand-RAGE interaction, ROS are generated. ROS are key intermediate factors of regulating intracellular signaling cascades such as NF-ΚB. Upstream signaling of NF-ΚB in response to ligand–RAGE interaction includes pathways such as AKT, p21ras, MAP kinases, Rho-family GTPases, PI3-kinase, and JAK/STAT. Binding of NF-ΚB in the RAGE promoter creates a positive feedback loop in the regulation of RAGE expression.
CHAPTER 2

HYPOTHESES AND OBJECTIVES
**Hypotheses and Objectives**

The experiments outlined in this thesis tested three distinct hypotheses and relevant objectives:

**Hypothesis 1:** Hyperglycemia will augment the expression or functional impact of S100B and RAGE post-MI.

**Objective 1:** Characterize the impact of diabetes on cardiac structure and function following MI in mice.

The effect of diabetes on post-MI LV remodeling, specifically the impact on ventricular dimensions, remains controversial, although in total, existing data suggest an adverse impact of diabetes on cardiac function and clinical outcomes (282, 286). Therefore, we characterized the impact of diabetes on cardiac function and structure by inducing hyperglycemia in clinically relevant animal models, primarily coronary artery ligation in low dose STZ-induced diabetic mice complemented by studies in Akita mice as a non-pharmacological type 1 diabetic model. The impact of diabetes on post-MI myocardial fibrosis and MMP-2 activity was also assessed.

**Objective 2:** Examine patterns of expression of S100B, RAGE and associated signaling pathways in diabetic versus non-diabetic myocardium post-MI.

Diabetes mellitus (DM) is a major risk factor for development of coronary artery disease and is associated with greater mortality from cardiovascular causes. Although the adverse impact of diabetes on post MI survival is recognized (208), the underlying cellular and molecular mechanisms are not fully elucidated. As presented in the preceding section, S100B is anomalously expressed in myocardium following myocardial infarction and regulates remodeling. As diabetes also upregulates S100B (22) and its receptor, RAGE, expression
(136), we hypothesized that expression of S100B/RAGE signaling pathways would be amplified following myocardial infarction in the setting of experimental diabetes mellitus in mice. We examined patterns of expression of S100B and RAGE in diabetic versus non-diabetic myocardium post-MI. Our lab previously has shown that S100B induces apoptosis by an extracellular mechanism via interaction with RAGE and activation of ERK1/2 signaling (324) so we also determined whether any comcomitant alteration occurs in ERK, AKT, and NFκb activities as well as AGE levels in diabetes post-MI.

**Objective 3:** Define the contribution of AGE to the expression of the S100B/RAGE axis in cultured cardiac myocytes.

To define the specific effect on cardiac myocytes, we recapitulated the effect of diabetes alone on S100B and RAGE in vitro by treatment of mice or rat cardiomyocytes with AGE or glucose.

**Hypothesis 2:** Abrogated or forced expression of S100B will produce alterations in post-MI cardiac structure and remodelling that is specific to diabetes.

**Objective:** Assess the impact of S100B on diabetic post-MI remodeling in knockout (BKO) and S100B transgenic (BTG) mice.

We have previously demonstrated in non-diabetic mice, that absence of S100B confers less ventricular dilation and apoptosis and improved cardiac function. While diabetes may be associated with further amplification of S100B/RAGE signaling, the complex metabolic derangements associated with diabetes and the presence of high levels of AGEs may result in differential effects of abrogating or forcing expression of S100B alone in diabetes post-MI. Thus, we determined the specific effect of S100B on cardiac structure and function in diabetic post-MI hearts by using S100B knockout (BKO) and S100B transgenic (BTG) mice.
We also investigated the regulation of AGE levels in these genetically modified mice. Since S100B is shown to be involved in energy metabolism regulation (171, 366, 368) therefore, we further tested the impact on myocardial GLUT4 expression in these models.

**Hypothesis 3:** S100B plays a role in limiting islet cell damage in response to STZ and/or enhancing insulin sensitivity.

**Objective:** Investigate the impact of S100B deficiency in mice on STZ-induced diabetes and the associated regulation of insulin sensitivity and pancreatic islet cell structure and function.

In the process of establishing a diabetic model in S100B deficient mice, our preliminary data suggested that S100B may play a role in the development and progression of STZ-induced diabetes itself. We defined the impact of absence of S100B in the development of STZ-induced diabetes in knockout mice and studied the contribution of S100B in the functional and structural impairment of pancreatic β cells in this model. As S100B expression is known to reside in pancreatic Schwann cells, we also examined the expression of S100B in diabetic and non-diabetic pancreatic islets. There are several lines of evidence that show the involvement of S100B in dysfunction of adipose tissue by close correlation with the body mass index (BMI) as well as levels of leptin and adipocyte-type fatty acid binding protein (299). Interestingly, insulin resistance in schizophrenic patients may be closely linked to serum S100B changes (301). Therefore, we studied the effect of S100B on insulin sensitivity and resistance in STZ-induced diabetic mice. S100B in micromolar concentration behaves as a pro-apoptotic factor acting on glial cells and the neuronal system (166, 274). Moreover, ligation of S100B and RAGE resulted in β-cells apoptosis via oxidative stress (176). Thus, we investigated whether S100B treatment may induce β-cells apoptosis in cultured cells.
CONTRIBUTION

I would like to acknowledge the following contributions from members of the Terrence Donnelly Heart Centre at St. Michael’s Hospital and Pathology Core, Centre for Modeling Human Disease, Toronto:

i) Dr. Howard Leong Poi – Echocardiography analysis

ii) Jean-Francois Desjardins – Hemodynamics analysis

iii) Pathology Core, Centre for Modeling Human Disease, Toronto- Histopathology and Immunohistochemistry analysis

IV) Dr. Gerald Proteau – ELISA assays

V) Dr. Effat Rezaie – Flow Cytometry
CHAPTER 3

GENERAL METHODS
3.1 Animals

All animal procedures were approved by the Animal Care Committee of St. Michael’s Hospital. Upon weaning, ear notch samples from the pups were obtained for genotyping. Genomic DNA was extracted and polymerase chain reaction (PCR) performed using the buffers and PCR reaction mix from the REDExtract-N-Amp™ Tissue PCR Kit, following manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO).

3.1.1 S100B Knockout Mice

BKO mice with a null mutation of S100B were derived on a CD1 background as described previously (355). The following primers were used to detect endogenous S100B (~200bp) and the inserted neo-expression cassette (~500bp): (Endogenous S100B Forward) 5’-CCTGGTAGGCTCTCTACCC- 3’, and (Endogenous S100B Reverse) 5’–TTCAGCCTTGCTTGTGCACC– 3’. (Neo Forward) 5’-CCGACCTGTCCCGGTGCCTGAATGA– 3’, (Neo Reverse) 5’-CGAGGAAGCGGTAGCCATTCGCC, The amplification conditions were as follows: 1 cycle at 94°C for 3 min, followed by 35 cycles consisting of denaturing at 94°C for 40 sec, annealing at 58°C for 40 sec, and extension at 72°C for 1 min. WT littermates were used as controls in the experiments.

3.2 Induction of Diabetes

At six weeks of age, animals were randomized to receive either 40/50 (mg/kg body weight/day) of streptozotocin (STZ; Sigma, St Louis, MO, USA) diluted in 0.1 M citrate buffer pH 4.5 (BDH Chemicals, VIC, Australia) or citrate buffer alone (non-diabetic) by Intraperitoneal injection without fasting for 5 days.

3.2.1 Blood Glucose
Each week, mice were weighed and blood glucose was determined using tail amputation method by a Glucometer Elite testing system (Ascensia: Bayer). Animals with blood glucose greater than 15mmol/L were considered diabetic.

3.3 Tissues Harvesting and Preparation

Animals were given a further dose of anesthetic (pentobarbitone sodium 60mg/kg IP), and then underwent excision of the heart to use in chapter 4 and pancreas to use in chapter 5.

3.3.1 Heart

After a brief rinse in a saline solution and blotted dry, they were weighted. The heart was then dissected into right ventricle and left ventricle. Each was weighed separately and the weight recorded. The LV was divided into two parts by a cross-sectional cut perpendicular to the long axis apical to the insertion of papillary muscles; the base of the heart was snap frozen in liquid nitrogen for subsequent protein and RNA analysis and the apex was fixed in 10% formalin phosphate buffer for 24 hours on a shaker in room temperature then rinsed in phosphate-buffered saline (PBS), and stored in 70% ethanol until the day of paraffin-embedding.

3.3.2 Pancreas

After cardiac puncture under anesthesia, a midline laparotomy was performed and the pancreas was immediately dissected from surrounding tissues, cleared of fat and lymph nodes, blotted, weighed, and fixed as above mentioned. The procedure from completion of cardiac puncture to placement of tissue in fixative was completed within 5 min. After overnight fixation, the pancreas was washed in cold running water, cut into 8–10 segments and placed in 10% buffered formalin until processing for paraffin embedding. All of the segments from a single pancreas were embedded into one block, thus permitting analysis of the entire pancreas in a single section.

3.4 Blood Collection
Blood samples was collected by puncturing the heart and centrifuged at 10000g, 5 min to separate the plasma. For pancreas study, blood samples were collected by puncturing the left lateral saphenous vein with a 25-gauge needle after shaving the tarsal area of the left leg and mixed with two protease inhibitors; Aprotinin (5000 KIU, Sigma, St. Louis, USA) and Diprotin A (0.1 mM, Sigma, St. Louis, USA).

3.5 Histopathology and Immunohistochemistry

3.5.1 Hematoxylin and Eosin (H& E) Staining
To analyze infarct size, cardiac sections were stained with H & E. After deparaffinizing in xylene heart and pancreas slides were rehydrated with three changes of Absolute alcohol and 10 dips of 95% alcohol (ethanol). Then they were washed in running tap water and stained in Harris’ Hematoxylin (dissolved in alcohol) for 5 minutes. After that slides were washed in water, differentiated in acid alcohol 3 to 6 dips and washed again in water, blue in Scott's Tap water substitute 1 minute. They were washed well in running water and rinsed in 95% alcohol. Following that the slides were stained for 1 minute in eosin and washed well in running water. Finally slides were dehydrated in 95% alcohol and 3 changes of absolute alcohol and then were fixed in 3 changes of xylene and mounted.

3.6 RNA Extraction and cDNA Synthesis
Frozen cardiac tissue, stored at -80°C was homogenized and total RNA was isolated using Trizol reagent (Sigma, St. Louis, USA) according to the manufacturer’s protocol. The purified RNA was dissolved in sterile water and quantified spectrophotometrically (OD260). RNA concentration and purity were determined spectrophotometrically by measuring the absorbance at 260 and 280nm (corrected for background at 320 nm). Extracted RNA that has an A260/280 value ≥ 2.0 is considered relatively free of protein. RNA integrity was assessed by running the sample on a denaturing agarose (1%) gel and visualization of 18S
and 28S rRNA bands. cDNA was synthesized from DNase treated total RNA samples by reverse transcription kit (QuantiTect, Qiagen, CA) according to the manufacturer’s protocol.

3.7 Quantitative Real Time RT-PCR

Mouse S100B, S100A1 and RAGE gene expressions were quantified by real-time RT-PCR using sequence specific primers. PCR was performed using SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA). The fluorescence detection was performed by ABI Prism sequence Detection System (Applied Biosystems, Foster City, CA). RPL32 was used as an internal control for inequalities of loading. There was minimal variation in RPL32 mRNA expressions between experimental groups (mean Ct value of RPL32 mRNA, 21.8 ± 0.47). Cycle parameters were 94°C × 3 min, and then 40 cycle of 94°C × 60s, 58°C × 60s, 72°C × 60s followed by 95°C ×10 min. The nucleotide sequences of primers were: Mouse RAGE (forward); 5’- gtgtcgggaactacaggg-3’ (reverse); 5’- ctggctccaggaatctg-3’, Mouse S100B (forward); 5’- aacaacgagctctcactcc-3’ (reverse); 5’- ccttcactttgtgcaacca, Mouse RPL32 (forward); 5’- tttagcagatgtggtggtc-3’ (reverse); 5’- ctgcctcttttacgg, Mouse S100A1 (forward); 5’- tgtggacaaggtgaagcct-3’ (reverse); 5’- gatctggactgccactctg-3’, Mouse GLUT4 (forward); 5’-ttttaa aag aatg ccc ttc-3’ (reverse); 5’- cag tgt ttc aag agt cca ttg ct-3’. Mouse NFκb1 (p50), Bax and β-Actin specific sequences of oligonucleotide primers (Qiagen, Alameda, CA) based on DNA sequences in the National Centre for Biotechnology database were purchased from Qiagen (Alameda, CA). Real-time RT PCR data were calculated according to Pfaffl method (240).

\[
\text{Expression ratio} = \frac{(\text{Efficiency target})^{ΔCt \text{ target} (\text{calibrator} - \text{target})}}{(\text{Efficiency reference})^{ΔCt \text{ ref} (\text{calibrator} - \text{ref})}}
\]

3.8 Protein extraction
Cells were washed 3 times with 1X PBS and thereafter, 100µl of Cell Extraction Buffer (Invitrogen, Canada) containing protease inhibitor cocktail (150 µl/5ml, Sigma, St. Louis, USA) and PMSF (phenylmethanesulfonylfluoride, 170µl/5ml, Sigma, St. Louis, USA) was added to each well. Cells were then scraped into a 1.5 ml Eppendorf tube. The lysates sat on ice for 20 minutes before being centrifuged at 12000 g for 20 minutes to remove cellular debris. The supernatant containing cellular protein was transferred to another 1.5 ml Eppendorf tube. Total cellular protein was quantified using the Dc Protein Assay (Bio Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions. The samples were stored at -80º C. Frozen heart and pancreas tissues were powdered and lysed using Cell Extraction Buffer, homogenized, sat in the ice for 20 minutes and centrifuged at 12000g for 20 minutes to remove cellular debris. Later, the supernatant containing cellular protein was transferred to another 1.5 ml Eppendorf tube. Total cellular protein was quantified using Dc Protein Assay (Bio Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions. The samples were stored at -80º C.
CHAPTER 4

S100B: ROLE IN CARDIAC REMODELING AND FUNCTION FOLLOWING MYOCARDIAL INFARCTION IN DIABETES

*The results of this study are reported in a manuscript in the journal of Life Sciences, Mohammadzadeh F, Desjardins JF, Tsoporis JN, Proteau G, Leong-Poi H, Parker TG,
4.1 Abstract

Existing data suggest an adverse impact of diabetes mellitus (DM) on cardiac function and clinical outcomes. However, the effect of diabetes on post-MI ventricular remodeling remains controversial. Therefore, we explored the impact of different duration of diabetes on cardiac function and left ventricular (LV) remodeling in clinically relevant animal models. DM was induced in CD1 mice by streptozotocin (STZ) then followed for 9, 15 and 19 weeks. Coronary artery ligation or sham surgery was performed 15 weeks after STZ or vehicle injection. To confirm and validate the finding from the STZ model, coronary artery ligation was performed on C57BL/6 and Akita mice as a non-pharmacological type 1 diabetic model. In diabetes, post-MI remodeling exhibited less increase in LV mass, dilation, and myocyte hypertrophy in association with increased apoptosis. Altering elastic properties of LV contributes to overall cardiac remodeling. AGE may directly enhance collagen formation (333). In this study we demonstrated increased fibrosis and reduced matrix metalloproteinase-2 (MMP-2) activity in post-MI diabetic myocardium. In addition, AGE levels were higher in diabetes following MI compare to MI alone, demonstrating possible contribution of cross-linked AGE with collagen in limited LV dilation of post-MI diabetes. Despite reduced LV dilation, impairment of cardiac function was similar to non-diabetic controls.

S100B, with restricted expression to neuronal cells, is anonymously expressed in myocardium following MI and regulates LV remodeling by preventing cardiac hypertrophy and induction of cardiac myocyte apoptosis. Since S100B and its receptor for advanced glycation end products (RAGE) expressions are induced in diabetic vasculature, we examined the expressions of S100B, RAGE and relevant signaling pathways in post-MI
diabetic mice. Both diabetes and MI alone induced myocardial RAGE and S100B expression and phosphorylation of extracellular signal-regulated kinase (ERK), a phenotype recapitulated by advanced glycation end product (AGE) stimulation of cultured cardiac myocytes. By contrast, in post-MI diabetic myocardium, S100B and NFκB expression were attenuated and ERK phosphorylation augmented. Since, the data presented above demonstrated the paradoxical expression of S100B in the setting of MI alone and post-MI diabetes, we investigated the role of S100B on LV remodeling and cardiac function by using post-MI diabetic wild-type (WT), S100B knockout (BKO) and S100B transgenic (BTG) mice. Following MI, BKO demonstrated increased ventricular dilation and greater impairment of cardiac function compared to WT and BTG. This was associated with increased ERK phosphorylation and systemic AGE levels with concomitant reduction in myocardial GLUT4 mRNA levels.

In total, these data demonstrate that in direct contrast to non-diabetics, the abrogation of S100B post-MI is associated with adverse ventricular remodelling and additional functional impairment. Thus they suggest that S100B expression may paradoxically serve to modulate cardiac metabolism and the adverse consequences of AGE expression in diabetic post-MI remodeling and function.

In addition, S100A1, another RAGE ligand, is important in regulating cardiac function, however it has not been examined in diabetes. Therefore, we investigated the cardiac expression of S100A1 in respond to diabetes alone and post-MI diabetes. S100A1 expression was altered opposite with data shown for S100B, demonstrating the complex and antithetical regulation of multiple RAGE ligands in post-MI diabetic hearts with potential implication for signalling pathways important for overall cardiac remodeling.
4.2 Introduction

Diabetes mellitus (DM) is a major risk factor for development of coronary artery disease and is associated with greater mortality from cardiovascular causes (225). Diabetes alone is associated with LV hypertrophy, and reduced systolic and diastolic function (73, 115). Although the adverse impact of diabetes on post MI survival is recognized (208), the underlying cellular and molecular mechanisms are not fully elucidated.

Chronic hyperglycemia contributes to diabetic complications, in part, through formation of advanced glycation end products (AGE), which are irreversibly formed by non-enzymatic glycosylation and oxidation of proteins and lipids (234). AGE exerts its effects via its receptor, RAGE. Members of the calcium binding S100 protein family can also stimulate RAGE signaling. S100 proteins entail a multigenic family of small (10-12 kDa) EF- hand calcium binding proteins that exert both intracellular and extracellular functions. Intracellularly, they function as dimeric calcium sensors and interact with intracellular effector proteins to regulate diverse functions such as contraction, cell growth and differentiation, cell cycle transcription, cell survival, apoptosis, and protein phosphorylation (84). Extracellularly, a subset of S100 proteins are secreted and function as RAGE-ligands, activating associated intracellular signaling pathways (15). S100B is among the best characterized of these multifunctional proteins. Although under basal conditions, S100B is predominantly expressed in astrocytes, oligodendrocytes, and Schwann cells, we have previously demonstrated that S100B is induced in the peri-infarct region of the human heart after myocardial infarction and in rat heart following experimental coronary artery ligation (321). Moreover in non-diabetic models of infarction, we have elucidated the intracellular and extracellular mechanisms by which S100B modulates cardiac hypertrophy, apoptosis,
remodeling, and function (320). Elevated S100B serum levels in dilated cardiomyopathy patients also suggest a correlation between S100B and LV remodeling (203). Of interest, diabetic RAGE-null mice were significantly protected from the adverse impact of ischemia/reperfusion injury in heart (42). Upregulation of S100B protein in diabetes (22) and its interaction with RAGE (136) result in oxidative stress, induction of stress signals, and activation of mitogen-activated protein kinases (MAPK) in vasculature (172). Thus, both RAGE and S100B may play a role in LV remodeling in diabetes following MI.
4.3 Objectives of this study

The objectives of the present study were to: 1) characterize the impact of diabetes on cardiac structure and function following MI in mice; 2) examine patterns of expression of S100B, RAGE and associated signaling pathways in diabetic versus non-diabetic myocardium post-MI; 3) define the contribution of AGE to the expression of the S100B/RAGE axis in cultured cardiac myocytes; 4) assess the impact of S100B on diabetic post-MI remodeling in knockout (BKO) and S100B transgenic (BTG) mice.

4.3 Hypotheses of this study

The experiments outlined in this thesis tested two distinct hypotheses:

1- Hyperglycemia will augment the expression or functional impact of S100B and RAGE post-MI.

2- Abrogated or forced expression of S100B will produce alterations in post-MI cardiac structure and remodelling that is specific to diabetes.
4.4 Methods

4.4.1 Animals

See “General Methods”. Akita mice were obtained from Jackson Laboratory (Maine, USA) and S100B KO, S100B TG and S100A1 KO mice were bred from an existing colony based at St. Michael’s Hospital.

4.4.1.1 S100B Knockout Mice

See “General Methods”.

4.4.1.2 S100B Transgenic Mice

BTG mice containing multiple copies (~8) of the human S100B gene under the control of its own promoter were derived from CD1 stock as described previously (103). The genotypes S100B TG and WT mice were determined via PCR for the human S100B (~213bp) and mouse S100B (~200bp) using the following primer pairs: (Human S100B Forward) 5’ – ACTACTGCCTGCCACGAGTT - 3’ and (Human S100B Reverse) 5’ – CCGTTAAAACAGCCTTTGGA 3’; (Mouse S100B Forward) 5’- CCTGGTAGGCCTCTCTACCC- 3’, and (Mouse S100B Reverse) 5’– TTCAGCTTGTGCTTGTCACC– 3’. The amplification conditions were as follows: 1 cycle at 94°C for 3 min, followed by 35 cycles consisting of denaturing at 94°C for 40 sec, annealing at 58°C for 40 sec, and extension at 72°C for 1 min.

4.4.1.3 S100A1 Transgenic Mice

S100A1 gene-targeted (S100A1 knockout (KO)) mice with a null mutation of murine S100A1 were derived on a C57BL/6 background as previously described and characterized (85).

4.4.1.4 Akita
Insulin-2-Akita (Ins2<sup>Akita</sup>) mouse mutant model of type 1 diabetes. These mice develop pancreatic β cell failure as a result of β cell selective proteotoxicity resulting from misfolding of insulin 2 (The Jackson Laboratory). Also the heterozygous mice are viable and fertile. This mutation exists on the C57BL/6 background. To determine the Ins2 Akita +/− Fnu4HI (5U/ml, NE Biolabs) was used to digest PCR product.

PCR conditions were as follows: The amplification conditions were as follows: 1 cycle at 94°C for 3 min, followed by 12 cycles consisting of denaturing at 94°C for 20 sec, annealing at 64°C for 30 sec, and extension at 72°C for 35 sec, and another 25 cycles consisting of denaturing at 94°C for 20 sec, annealing at 58°C for 30 sec, and extension at 72°C for 35 sec, followed by 72°C for 2 min. The genotypes Akita +/+ and Akita +/− and WTC57 mice were determined using the following primers for the Ins2 exon 3 which amplifies a 280 bp DNA fragment from both WT and mutant alleles. PCR products from the mutant allele are not digested by the Fun4HI restriction enzyme. PCR products from the WT allele are digested to 140 bp by the Fun4HI:

(Ins2 forward) 5’-TGCTGATGCCCTGGCCTGCT – 3’ and (Ins2 Reverse) 5’-TGGTCCCACATATGCACATG– 3’.

4.4.2 Induction of Diabetes- See “General Methods”.

4.4.3 Blood Glucose- See “General Methods”.

4.4.4 Experimental Design
4.4.4.1 Study 1

Six week old male CD1 mice were intraperitoneally injected with streptozotocin (STZ, Sigma) in citrate buffer (40 mg/kg body weight/day) for five days to induce hyperglycemia (total dose of STZ was 200 mg/kg). To examine the impact of diabetes alone, at 9, 15, and 19 weeks, post-STZ-treated mice were compared with age matched vehicle-treated controls. To examine the impact of myocardial infarction, left anterior descending (LAD) coronary artery ligation or sham operation was performed on STZ and vehicle-treated mice at 15 weeks, and sacrificed 35 days later (Fig.4.4.1).

![Flow diagram of various groups of CD1 mice according to experimental design.](image)

Fig. 4.4.1 Flow diagram of various groups of CD1 mice according to experimental design.
### 4.4.4.2 Study 2

To further evaluate the effects of S100B on the post-MI diabetic cardiac function a separate group of animals including diabetic BKO, BTG and littermate diabetic WT was created. Similar to the previous group, six week old male BKO, BTG and WT were injected with STZ and LAD ligation or sham operations was performed at 15 weeks, and sacrificed 35 days later (Fig. 4.4.2).

![Flow diagram of the BKO, BTG and their littermate control groups according to experimental design.](chart.png)
4.4.5 Echocardiographic Analysis

Transthoracic echocardiography was performed in mice anaesthetized with 2% isoflurane. Isoflurane anesthesia was induced in a sealed chamber and was maintained using a nose cone connected to the vaporizer (Ohmeda Isotec4 vaporizer, Benson medical industries Inc., Markham, ON). The chest hair was removed with an electric razor and a topical depilatory agent. Two-dimensional echocardiography (Hewlett-Packard Sonos 5500, Philips Ultrasound, Bothell, Washington, USA) was performed using a broadband 5-12 MHz ultrasound probe (S12 transducer). The animals were imaged in the supine position. A standoff was created by using a small water-bath. Imaging depth was set at 2 cm and magnified to 1 cm, allowing a frame rate of 100-120 Hz. A short-axis two-dimensional view of the left ventricle (LV) at the midpapillary muscle level was digitally acquired and stored on a magneto-optical drive for off-line analysis. End-diastole was defined as the frame with the largest cavity size and end-systole as the frame with the smallest cavity size. LV end-diastolic internal diameter (LVIDd) and LV end-systolic internal diameter (LVIDs) were measured on the 2-dimensional image, using the standard leading edge to leading edge technique. Each measure was made by a single observer blinded to the identity of the mice. For each measurement, 3 consecutive cardiac cycles were measured and then averaged. Fractional shortening (FS) in percent was calculated as (LVIDd-LVIDs)/LVIDd*100, and ejection fraction (EF) as (LVIDd^3-LVIDs^3)/LVIDd^3*100 (72).

4.4.5.1 Myocardium Infarct Size Measurement

For measurement of the circumferential extent of myocardial infarction, an image loop from end-diastole to end-systole was created (3 to 5 frames on average) for analysis using custom-designed software. Eight to 12 epicardial and endocardial targets were then defined by the
observer in each frame from end-diastole to end-systole, which were automatically connected using cubic-spline interpolation to derive the epicardial and endocardial contours. In order to correct for cardiac rotation, the junction of the posterior LV free wall and the right ventricular free wall was defined over the epicardium in each frame. The computer program generates 100 equidistant chords between the 2 contours starting at this point, with each chord representing the shortest distance between the epicardial and endocardial contours. Plots of % wall thickening (WT) over the entire systolic contraction sequence were generated (178). The circumferential extent of infarction was defined as the extent of severe hypokinesis or akinesis, defined as WT<10%.

4.4.6 Hemodynamic Measurement

Hemodynamic assessment was performed in mice under 2% isoflurane. Anesthesia was induced and maintained as described above. Mice were placed in the supine position on a heating pad to maintain adequate body temperature. Systemic systolic and diastolic arterial pressures were measured using a Millar Mikro-tip catheter transducer (model SPR-671; Millar Instruments, Inc., Houston, TX) inserted into the right carotid artery. The catheter was then advanced into the left ventricle (LV) for the measurement of LV pressures, and the maximum rate of pressure rise \( (+\frac{dP}{dt})_{\text{max}} \) and decline \( (-\frac{dP}{dt})_{\text{max}} \). Pressure signal was recorded using a PowerLab data acquisition system and analysed via the software Chart 5 (ADInstruments Inc., Colorado Springs, CO)(72).

4.4.7 Cardiac Catheterization

Cardiac catheterization was performed as previously described (62). Briefly, mice were anaesthetized with 2% isoflurane, intubated using a 22-gauge catheter and ventilated with a volume-controlled ventilator (Hugo Sachs Elektronik, Harvard Apparatus, type 845) with
tidal volumes set at 200-225 ml at a frequency of 4 Hz. Mice were placed in the supine position on a water circulating heating pad with the temperature set at 38°C and a 1.4F micromanometer-tipped pressure or, in some experiments, conductance catheter (Models SPR-671 and SPR-839, respectively, Millar instruments, Houston, TX) was inserted into the carotid artery and advanced into the left ventricle and pressure-volume (PV) loops were generated. Data were acquired (Chart v5, AD Instruments) under steady state and following inferior vena cava occlusion (preload reduction). The following parameters were then calculated using a cardiac PV analysis program (PVAN 3.4, Millar instruments): end-systolic volume (ESV), end-systolic pressure (LVSP), end-diastolic volume (EDV), end-diastolic pressure (LVEDP), stroke volume (SV), peak rate of pressure rise (+dP/dt max), peak rate of pressure decline (-dP/dt max) and the slope of the end-diastolic PV relationship (EDPVR), an index of LV stiffness. The relaxation time constant (Tau), an index of diastolic function, was calculated using two methods: Weiss (tau (w)), regression of log (pressure) vs. time, and Glantz (tau (G)), regression of dP/dt vs. pressure. In mice post-MI, caval occlusion resulted in deviations in catheter position in the LV that precluded acquisition of reliable dynamic loops resulting in the inability to calculate EDPVR in infarcted mice.

4.4.8 Tissue and Blood Collection

Animals were given a further dose of anesthetic (pentobarbitone sodium 60mg/kg IP), then underwent excision of the heart, kidney, and lungs. After brief rinse in a saline solution and blotted dry, they were weighted. The heart was then dissected into right ventricle and left ventricle. Each was weighed separately and the weight recorded. The LV was divided into two parts by a cross-sectional cut perpendicular to the long axis apical to the insertion of papillary muscles; the base of the heart was snap frozen in liquid nitrogen for subsequent protein and RNA analysis and the apex was fixed in 10% formalin phosphate buffer for 24 hours on a shaker at
room temperature then rinsed in phosphate-buffered saline (PBS), and stored in 70% ethanol until the day of paraffin-embedding.

4.4.9 Histopathology and Immunohistochemistry

4.4.9.1 Hematoxylin and Eosin (H& E) Staining - See “General Methods”.

4.4.9.2 Picrosirius Red Staining

Following deparaffinization and rehydration of cardiac sections, slides were rinsed with distilled H2O and stained in Celestine for 5 minutes. After washing in water, Harris' Hematoxylin was then applied to each slide and incubated for 5 minutes. Then they were washed in water, and differentiated in acid alcohol. Following this, the slides were washed in water, and stained blue in scott’s tap water for 1 minute. Following this, the slides were rinsed in running water and stained with Picrosirius Red for 30 minutes, blot dried and dehydrated in 95% ethanol, 100% ethanol (4 x 10 dips) and xylene (3x 10 dips) and cover-slipped and mounted in permount. Collagen was shown in red and nuclei were shown in black.

4.4.9.3 Collagen Density

Cardiac fibrosis was quantified after Picrosirius red (PSR) staining of heart cross sections with use of an upright microscope at ×40 objective. PSR stains thin collagen fibers and in bright field microscopy appears red. Collagen content was calculated as a percentage of the area of each image (expressed as pixels). Using Image J (Rasband et al. http://imagej.nih.gov/ij/, 1997-2012), the red colour of the PSR staining was sampled, the red, green and blue (RGB) contributions to each pixel were noted and thresholding was applied with those values. Using this method and the same levels of thresholding, the
percentage of collagen area fraction was quantified in 16 randomly picked areas within non-
infarct, and peri-infarct regions and the results averaged.

4.4.10 Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick- End- Labeling
(TUNNEL)
Paraffin sections (5µm) were deparaffinized in xylene and rehydrated in graded ethanol
(100%, 90%, and 70%). Sections were permeabilized using proteinase K (10 µg/ml), washed
in dd water for 5 minutes, then in PBS (2 x 5 min), endogenous peroxidase was quenched
with 3% hydrogen peroxide in methanol for 30 minutes at room temperature.
After washing in PBS (3 X 5 min) slides were blocked with 2% BSA/20% NBS in PBS for
15 minutes. Following pre-incubation with 1x One –Phor-All buffer for 10 minutes, slides
were incubated in the TdT labeling solution, containing biotin-16- dUTP, tDt enzyme and
nucleotide mix for 2 hour at 37°C. Following PBS rinses (3x 5 min) and blocking with 2%
BSA/20% NBS in PBS for 15 minutes, slides were incubated with an avidin-biotin
peroxidase ABC Complex, both A and B 1:50 (Vector, Burlingame, CA) for 90 minutes at
37°C. After washing in 0.1% Triton X-100 in PBS (2x 5 min), and PBS (2x 5 min) color
development occurred by incubation with 3, 3’-Diaminobenzidine (DAB) (DAKO, CA,
USA) for 15 minutes. Slides were counterstained with Hematoxylin for 20 seconds,
dehydrated, cleared and mounted. Apoptotic cells were shown in dark brown and nuclei in
blue. The number of TUNEL-positive nuclei was determined in a blinded-fashion by
examining 4 randomly selected microscopic fields from each of 4-5 mice from each
experimental group. The % TUNEL-positively was calculated as (number of TUNEL-
positive nuclei)/(total number of nuclei) * 100%.

4.4.11 Myocyte Hypertrophy
The extent of cardiac myocyte hypertrophy was determined on Haemotoxylin-Eosin stained sections as adapted from the methods described by Kai and colleagues (140). In brief, spindle-shaped cardiac myocytes with elliptical nuclei in transverse section were selected. The transverse diameter was measured 3 times per cell at the level of the nucleus, and the values were averaged. Approximately 20 cells per field at x200 magnification were found with 3 to 4 fields randomly selected per sample slide. The average diameter of at least 50 myocytes was then calculated for 6 animals in each group.

4.4.12 AGE-Bovine Serum Albumin (BSA) preparation

AGE-BSA was prepared by incubating 20% BSA (Sigma, St. Louis, USA) and 1.67 M glucose in 0.5 M phosphate buffer (pH 7.4) for 12 weeks at 37°C. AGE formation was confirmed by measurement of fluorescence at 440 nm of wavelength. Control non-glycated BSA was obtained by incubation of the same solution in the same manner omitting glucose. Endotoxin levels were checked using a kit for endotoxin test (Limulus J Single Test, Wako). The AGE-BSA solutions at the concentrations used in this study were confirmed to be endotoxin-free (< 2.5 U/ml of endotoxin).

4.4.13 Enzyme-Linked Immunosorbent Assay (ELISA)

To determine the quantity of advanced glycated end products, we purchased and used the OxiSelect™ Advanced Glycation End Product (AGE) ELISA Kit (Cell Biolabs Inc., Ca, USA). The kit is an enzyme immunoassay developed for rapid detection and quantification of AGE protein adducts. The quantity of AGE adduct in protein samples is determined by comparing its absorbance with that of a known AGE-BSA standard curve. Each kit provides sufficient reagents to perform up to 96 assays, including standard curve and unknown protein samples.
The principle of the AGE assay consists of adsorbing AGE-BSA standards or protein samples (10 µg/mL) onto a 96-well plate and incubating for 2 hrs at 37°C. The AGE protein adducts present in the sample or standard was then probed with an anti-AGE antibody, followed by horseradish peroxide (HRP) conjugated secondary antibody. The AGE protein adduct content in an unknown sample is determined by comparing with a standard curve that is prepared from AGE-BSA standards. The reagents were prepared and the assay was performed following the manufacturers instructions. The unknown samples were thawed and their protein content was measured using the Bio-Rad Standard Procedure for Microtiter Plates (Bio-Rad Bradford Bulletin 9004). An aliquot of each sample was then diluted to a final concentration of 10 µg/ml protein. A standard curve of AGE-BSA was prepared. Duplicate aliquots of 100 µL of each sample and standard were then pipetted in the wells of the plate and incubated for 2 hr at 37°C. The wells were washed 2 times with 250 µL 1X PBS per well. Assay Diluent (200 µL) was added to each well and incubated for 1 hour at room temperature on an orbital shaker. The wells were then washed 3 times with 250 µL of 1X Wash Buffer. After each wash, the plate was tapped on paper towel to remove excess wash solution. Anti-AGE Antibody was added to all the wells and incubated for 1 hour at room temperature on an orbital shaker. The plate was washed 3 times as previously described. Secondary Antibody-HRP Conjugate was added to all wells and incubated for 1 hour at room temperature on an orbital shaker and the plate was washed 3 times as previously described. Substrate Solution was added to each well and incubated at room temperature for 10 minutes on an orbital shaker. Then the reaction was stopped with the addition of the Stop Solution to each well and the plate was read at 450 nm as the primary wave length and 570 nm for correction.

4.4.14 Cell culture
4.4.14.1 Primary neonatal rat cardiac myocytes

Neonatal cardiac myocytes (CM) were isolated from the ventricles of 2-day-old Sprague-Dawley rats by enzymatic dissociation in 0.1% trypsin, 0.1% collagenase, and 0.025% DNAaase (Worthington Biochemical Corp., Freehold, NJ). CM were grown in DMEM/F-12 media (Hyclone’s modified Eagle’s medium, GIBCO, Invitrogen, Carlsbad, Canada) supplemented with 10% Fetal Bovine serum (FBS, Invitrogen, Canada), 0.11mg/ml sodium pyruvate, 0.3mg/ml glutamax, 50µ/ml Penicillin, 50µg/ml streptomycin. Cells were maintained at 37°C and 2 % CO₂. Cells were plated onto 50mm-well dishes at a density of 1 x 10⁶ cells/well. After 24 hours cells were serum-starved in basal medium with 0.1% BSA for 24 hours. Following this, cells were treated with several concentrations of either Glucose (5, 25, and 50 mM) or AGE (1, 5, 10 µg/ml) for 4 days. BSA and mannitol were used as a control for AGE and glucose treatment, respectively.

4.4.14.2 Primary adult mouse cardiac myocytes

Primary adult cardiomyocyte isolation was performed as previously described (257), and cells were used, on the same day since they cannot be maintained beyond 24 h (266) and do not proliferate. Briefly, anesthesia was administered, the murine aorta was cannulated, and the heart was perfused with isolation buffer containing (in mM) 120.4 NaCl, 4.7 KCl, 1.2 MgSO4, 0.6 Na2PO4, 0.6 KH2PO4, 4.6 NaHCO3, 5.6 glucose, 10 HEPES, 10 2,3-butanedione monoxime (BDM, Sigma, St. Louis, USA), and 32 taurine (Sigma, St. Louis, USA, pH 6.9), for 2.5 min, followed by a 10- to 12-min perfusion with an enzyme digestion buffer containing 12.5 mM CaCl2 and 1 mg/ml Collagenase Type II (Worthington Biochemical Corporation, Lakewood, NJ). The heart was cut from the cannula just below the atria and transferred into stop buffer (isolation buffer containing 12.5 mM CaCl2 and 5%
FBS), and the ventricles were gently teased into small pieces with forceps. Pipettes of decreasing size (1 to 0.5 mm diameter) were used to bring the tissue to a suspension. The Cellector Tissue Sieve filter system (Bellco Biotechnology, Vineland, NJ) removed any undigested tissue, and the filtrate was brought to a final volume of 10 ml in stop buffer. In a nonstick 100-mm plate the calcium concentration was adjusted using increasing volumes of 10 mM CaCl2 to bring the final calcium concentration from 12.5 M to 1 mM. Cells were transferred into a 15-ml tube and allowed to settle for 8 min. The supernatant was spun for 6 min (at 100 g), and both pellets were suspended in DMEM medium (GIBCO, Invitrogen, Carlsbad, Canada) and supplemented with 2.5% FBS and 10 mM BDM. Cells were counted in suspension and optimally plated for 120,000 –150,000 rod shaped cells per 60-mm tissue culture plates coated with 5 g/ml laminin. Cells were then incubated at 2% CO2 for 2 h to allow attachment of the rod-shaped cells. After 2 hours cells were serum-starved in basal medium with 0.1% BSA and were treated with AGE (10 µg/ml) overnight.

4.4.15 Immunofluorescence
Briefly cultured myocytes after fixation, permeabilization and blocking were incubated with primary antibodies against RAGE (Cell signaling, Beverly, MA, USA) and S100B (Abcam, Cambridge, USA). After washing, cells were incubated with relevant secondary antibodies. DAPI staining was used to determine the number of nuclei and to assess gross cell morphology.

4.4.16 RNA Extraction and cDNA Synthesis- See “General Methods”.

4.4.17 Quantitative Real Time RT-PCR- See “General Methods”.

4.4.18 Protein extraction- See “General Methods”.

4.4.19 Western Immunoblotting
Equal amounts of protein (50µg) were diluted with 2x sample buffer (125mM Tris-HCl at pH 6.8, 4% SDS, 20% glycerol, 0.004% bromophenol blue, and 10% -mercaptoethanol), boiled for 5 minutes, and loaded onto gradient (4-12%) NOVEX® Tris-glycine polyacrylamide gels (Invitrogen, Canada). A predetermined molecular mass standard was also loaded as a protein marker (Fermentas International Inc, Burlington, Canada). Once the electrophoresis was completed, the proteins were transferred onto a BioTrace™ NT Nitrocellulose Transfer Membrane (Pall Canada Ltd.-Quebec, Quebec, Canada) and blocked in 5% skim milk in TBS-T (TBS + 0.05% Tween) for 1 hour at room temperature (RT). Upon completion of the immunoblotting protocol (see below), the signals were detected by exposure of the membranes to Kodak film (VWR, West Chester, PA, USA). The bands were scanned with an imaging densitometer, and optical densities of the protein bands quantified.

**4.4.19.1 S100B, RAGE, ERK, GAPDH, Bax and Bcl2 Immunoblotting**

Membranes were incubated overnight at 4°C with the following primary antibodies diluted in 5% skim milk in TBS-T: rabbit anti-S100B (1:1000; Abcam, Cambridge, Massachusetts, USA), goat anti-RAGE (1:100; Santa Cruz biotechnology, Santa Cruz, CA, USA), rabbit anti-ERK1/2 (1:1000 dilution; Cell signaling, Beverly, MA, USA), rabbit anti-pERK1/2 (1:1000 dilution; Cell signaling, Beverly, MA, USA), rabbit anti-S100A1 (GeneTex Inc., Irvine, CA, USA), rabbit anti-AKT (1: 1000, Cell signaling, Beverly, MA, USA), rabbit anti-pAKT (1: 1000, Cell signaling, Beverly, MA, USA), rabbit anti-Bax (1: 1000, Cell signaling, Beverly, MA, USA), and rabbit anti-Bcl2 (1:2000, Cell signaling, Beverly, MA, USA). The following day, the membranes were incubated for 1 hour at RT with an appropriate horseradish peroxidase-conjugated either goat anti-rabbit (1:5000; Santa Cruz biotechnology, Santa Cruz, CA, USA) or donkey anti- goat (1:400; Santa Cruz biotechnology, Santa Cruz, CA, USA) or donkey anti- mouse (1:10000, Santa Cruz biotechnology, Santa Cruz, CA,
USA) secondary antibody in 5% skim milk in TBS-T. Detection of signals was performed through enhanced chemiluminescence reaction, Western Lightning Plus-ECL (PerkinElmer Inc., Waltham, MA, USA). To verify total protein levels, membranes were stripped then probed with mouse anti- GAPDH antibody (1:200, Chemicon International, Billerica, MA, USA).

4.4.19.2 Immunoprecipitation

2µg of RAGE antibody (Santa Cruz biotechnology, Santa Cruz, CA, USA) was added to 100-150 µg protein and incubated for 1 hour at 4°C with gentle rocking. Protein A/C plus-Agarose beads (Santa Cruz biotechnology, Santa Cruz, CA, USA) were then added to the lysates and allowed to bind to the RAGE Ab for 1 hour at 4°C with agitation. Next, the Ab-antigen /A-Agarose complex was collected by centrifugation at 12000g for 20 seconds at 4°C. After removing the supernatant by aspiration the pellet was washed with 1ml wash Buffer (0.05% Nonidet P-40) for 20 minutes at 4°C with rocking (repeated it three times). Final washed protein A-antigen/antibody complex were collected by centrifugation at 12000g for 20 seconds at 4°C. Finally the pellet was dissolved in 30µl of 1X reducing sample buffer. After denaturing the protein at 100°C for 3 minutes protein A-Agarose was removed by centrifugation at 12000g for 20 seconds and the aliquots of supernatants were electrophoresed using SDS-PAGE gel.

4.4.20 Gelatin Zymography

Cell lysates (10µg) were separated on 10% SDS-PAGE gel embedded with gelatin (Invitrogen, Canada) using non reducing sample buffer for 3 hours at 40 mA/m²gel at 4°C to limit degradation of gelatin by MMPs during electrophoresis. Next, incubation of the gel with TritonX- 100 helped to remove SDS for 1 hour at room temperature. The gels were washed
with dd H₂O twice and incubated for 24 h at 37°C in 50 mM Tris-HCl, 5 mM CaCl₂ to start activation. The next day gels were incubated in 100 mM EDTA to stop the activation of the enzyme. Finally, after fixing the gels in destaining solution for 1 hour, gels were stained with Coomassie blue staining. Gelatinolytic bands were detected and quantified by VersaDoc Imager (Biorad Laboratories, Hercules, CA, USA) as clear bands. Active bands were quantified as a percentage of total MMP-2. Total MMP-2 was measured as a sum of latent and active MMP-2.

4.4.21 Statistical Analysis

All data are presented as mean ± SEM. Statistical analysis was performed using SPSS, software version 11.5. Means of two groups were compared using an unpaired two-tailed Student’s t-test. Differences amongst multiple means were determined by analysis of variance (ANOVA), and when overall differences were detected, the post-hoc Tukey testing was used to determine differences between individual means. A value of p<0.05 was considered statistically significant.
4.5 Results

4.5.1 Plasma Glucose, Survival, and Infarct Size

Plasma glucose levels were elevated in all diabetic mice at 16 days after STZ injection and Akita mice (Tables 1, 2, 3 and 4). In infarcted mice operative mortality was ~20% with no difference between groups and there were no deaths in non-infarcted or sham-operated control and diabetic animals. Infarct size at 35 days post-MI was comparable between diabetic and non-diabetic mice (38.7 ± 4 % vs. 36.2 ± 4 % of LV circumference), non-diabetic WT and Akita mice (40.7 ± 3 % vs. 39.2 ± 4 % of LV circumference), diabetic BKO, BTG and WT mice (41.2 ± 2, 37.2 ± 4 and 41.2 ± 7 % of LV circumference; p=NS).

4.5.2 Echocardiography and Hemodynamics

Echocardiography up to 19 weeks after STZ injection in the absence of MI demonstrated preserved LV systolic function and no alterations in LV diameter (Table 4.5.1). Coronary artery ligation resulted in LV dilation that was significantly attenuated in diabetic animals with, however, a comparable reduction in ejection fraction (EF) and fractional shortening (FS; Table 4.5.2). To test whether these changes in LV structure and function were due to STZ toxicity effect, coronary artery ligation was performed on C57BL/6 and Akita mice. Echocardiography results indicated, similar to STZ-treated mice, a significant reduction of LV dimension in Akita mice (Table 4.5.3). By contrast, in the absence of S100B expression, diabetic BKO exhibited greater post-MI LV dilation than diabetic WT and BTG, with additional impairment of systolic function as assessed by FS and EF (Table 4.5.4). We reconfirmed that this augmented dilation in post-MI diabetic BKO stands in contrast to the reduction in post-MI dilation that occurs in non-diabetic BKO (LVEDD; 5 ± 0.01 in non-
diabetic BKO vs. 6 ± 0.02 mm in non-diabetic WT, LVESD; 4 ± 0.02 in non-diabetic BKO vs. 5 ± 0.02 mm in non-diabetic WT, n= 6, p<0.01) that we have previously described (319).

Representative steady state pressure-volume loops demonstrate the alterations in hemodynamics and ventricular size in response to diabetes and/or MI (Figure 4.5.1). Up to 19 weeks post-STZ without MI, only a reduction in left-ventricular systolic pressure (LVSP) is observed with no significant differences in all measures of LV systolic and diastolic function (Tables 4.5.1 and 4.5.2). Post-MI, diabetic mice had a significant further reduction in LVSP with no differences in diastolic functional indices, LV end diastolic pressure (LVEDP) and peak rate of pressure decline (-dP/dt\textsubscript{max}) compared to non-diabetics. However, in keeping with additional impairment in ventricular relaxation, a significant increase in relaxation time constant, Tau (G), was observed in diabetic post-MI (Table 4.5.2).
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DM</th>
<th>Control</th>
<th>DM</th>
<th>Control</th>
<th>DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Glucose (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9WD</td>
<td>7.4 ± 0.3</td>
<td>32.0 ± 1.2 *</td>
<td>6.4 ± 0.4</td>
<td>28.5 ± 1.5 *</td>
<td>6.3 ± 0.4</td>
<td>28.0 ± 1.8 *</td>
</tr>
<tr>
<td>15WD</td>
<td>32.0 ± 1.2 *</td>
<td>6.4 ± 0.4</td>
<td>28.5 ± 1.5 *</td>
<td>6.3 ± 0.4</td>
<td>28.0 ± 1.8 *</td>
<td></td>
</tr>
<tr>
<td>19WD</td>
<td>32.0 ± 1.2 *</td>
<td>6.4 ± 0.4</td>
<td>28.5 ± 1.5 *</td>
<td>6.3 ± 0.4</td>
<td>28.0 ± 1.8 *</td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>9WD</td>
<td>41 ± 1</td>
<td>36.8 ± 1 *</td>
<td>49.5 ± 2</td>
<td>42.3 ± 1 *</td>
<td>47.1 ± 2</td>
<td>40.7 ± 1 *</td>
</tr>
<tr>
<td>15WD</td>
<td>36.8 ± 1 *</td>
<td>49.5 ± 2</td>
<td>42.3 ± 1 *</td>
<td>47.1 ± 2</td>
<td>40.7 ± 1 *</td>
<td></td>
</tr>
<tr>
<td>19WD</td>
<td>36.8 ± 1 *</td>
<td>49.5 ± 2</td>
<td>42.3 ± 1 *</td>
<td>47.1 ± 2</td>
<td>40.7 ± 1 *</td>
<td></td>
</tr>
<tr>
<td>TL (mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>20.5</td>
<td>19.8</td>
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</tr>
<tr>
<td>15WD</td>
<td>20.5</td>
<td>19.8</td>
<td>20.1</td>
<td>19.8</td>
<td>20.1</td>
<td>19.8</td>
</tr>
<tr>
<td>19WD</td>
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<td>19.8</td>
<td>20.1</td>
<td>19.8</td>
<td>20.1</td>
<td>19.8</td>
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<tr>
<td>Heart weight/TL (mg/mm)</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>9WD</td>
<td>5.7 ± 0.2</td>
<td>5.1 ± 0.1 *</td>
<td>6.8 ± 0.1</td>
<td>5.8 ± 0.2 *</td>
<td>7.6 ± 0.3</td>
<td>5 ± 0.5 *</td>
</tr>
<tr>
<td>15WD</td>
<td>5.1 ± 0.1 *</td>
<td>6.8 ± 0.1</td>
<td>5.8 ± 0.2 *</td>
<td>7.6 ± 0.3</td>
<td>5 ± 0.5 *</td>
<td></td>
</tr>
<tr>
<td>19WD</td>
<td>5.1 ± 0.1 *</td>
<td>6.8 ± 0.1</td>
<td>5.8 ± 0.2 *</td>
<td>7.6 ± 0.3</td>
<td>5 ± 0.5 *</td>
<td></td>
</tr>
<tr>
<td>LVSP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9WD</td>
<td>98 ± 4</td>
<td>86 ± 1 *</td>
<td>100 ± 2</td>
<td>83 ± 5 *</td>
<td>100 ± 4</td>
<td>91 ± 3 *</td>
</tr>
<tr>
<td>15WD</td>
<td>86 ± 1 *</td>
<td>100 ± 2</td>
<td>83 ± 5 *</td>
<td>100 ± 4</td>
<td>91 ± 3 *</td>
<td></td>
</tr>
<tr>
<td>19WD</td>
<td>86 ± 1 *</td>
<td>100 ± 2</td>
<td>83 ± 5 *</td>
<td>100 ± 4</td>
<td>91 ± 3 *</td>
<td></td>
</tr>
<tr>
<td>LVEDP</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9WD</td>
<td>7.2 ± 1.5</td>
<td>4.5 ± 0.4</td>
<td>4.9 ± 0.4</td>
<td>5.3 ± 0.3</td>
<td>6.0 ± 0.6</td>
<td>5.8 ± 0.6</td>
</tr>
<tr>
<td>15WD</td>
<td>4.5 ± 0.4</td>
<td>4.9 ± 0.4</td>
<td>5.3 ± 0.3</td>
<td>6.0 ± 0.6</td>
<td>5.8 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>19WD</td>
<td>4.5 ± 0.4</td>
<td>4.9 ± 0.4</td>
<td>5.3 ± 0.3</td>
<td>6.0 ± 0.6</td>
<td>5.8 ± 0.6</td>
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</tr>
<tr>
<td>+dP/dt max (mmHg/s)</td>
<td>6535 ± 232</td>
<td>6385 ± 435</td>
<td>7350 ± 339</td>
<td>6258 ± 546</td>
<td>6525 ± 508</td>
<td>6191 ± 396</td>
</tr>
<tr>
<td>-dP/dt max (mmHg/s)</td>
<td>-6565 ± 854</td>
<td>-6196 ± 1522</td>
<td>-7517 ± 462</td>
<td>-6714 ± 691</td>
<td>-6006 ± 672</td>
<td>-5007 ± 277</td>
</tr>
<tr>
<td>Echocardiographic Data</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>4.3 ± 0.01</td>
<td>4.3 ± 0.01</td>
<td>4.2 ± 0.01</td>
<td>4.4 ± 0.01</td>
<td>4.3 ± 0.0</td>
<td>4.3 ± 0.01</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>3.2 ± 0.01</td>
<td>3.1 ± 0.01</td>
<td>2.7 ± 0.02</td>
<td>2.9 ± 0.01</td>
<td>3.2 ± 0.01</td>
<td>3.1 ± 0.01</td>
</tr>
<tr>
<td>Fractional shortening %</td>
<td>27 ± 1.4</td>
<td>28 ± 1.5</td>
<td>34 ± 2.7</td>
<td>33 ± 1.6</td>
<td>25 ± 1.9</td>
<td>29 ± 2.8</td>
</tr>
<tr>
<td>Ejection Fraction %</td>
<td>61 ± 2.3</td>
<td>62 ± 2.3</td>
<td>70 ± 3.3</td>
<td>70 ± 2.0</td>
<td>57 ± 3.3</td>
<td>63 ± 4.6</td>
</tr>
</tbody>
</table>

Table 4.5.1. Time course of heart weight, Hemodynamic and Echocardiographic data of non-diabetic and diabetic mice (9, 15 and 19 weeks). Group abbreviations: 9WD, 9 weeks post-STZ; 15WD, 15 weeks post-STZ; 19WD, 19 weeks post-STZ; N, number of mice; TL, tibial length; LVSP, left ventricular systolic pressure; +dP/dtmax, maximal rate of pressure
rise; \( -\frac{dP}{dt}\text{max} \), maximal rate of pressure decline; LVEDP, LV end-diastolic pressure; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter. Data are presented as mean ± SEM, *p<0.05: vs. non-diabetic control.
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DM</th>
<th>MI</th>
<th>DM+ MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>13</td>
<td>19</td>
<td>13</td>
<td>21</td>
</tr>
<tr>
<td>Blood Glucose (mmol/L)</td>
<td>5.0 ± 1</td>
<td>28.4 ± 5*</td>
<td>5.5 ± 4</td>
<td>28.7 ± 3*</td>
</tr>
<tr>
<td>Organ weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>47 ± 2</td>
<td>41 ± 2*</td>
<td>46 ± 2</td>
<td>41 ± 1†</td>
</tr>
<tr>
<td>TL (mm)</td>
<td>20.2</td>
<td>20.3</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>LV weight/TL (mg/mm)</td>
<td>6.5 ± 0.2</td>
<td>5.6 ± 0.3*</td>
<td>7.2 ± 0.3</td>
<td>6.1 ± 0.3†</td>
</tr>
<tr>
<td>Heart weight/TL (mg/mm)</td>
<td>8.2 ± 0.3</td>
<td>7.1 ± 0.2*</td>
<td>9.1 ± 0.5</td>
<td>7.9 ± 0.4†</td>
</tr>
<tr>
<td>Hemodynamic data</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESV (µL)</td>
<td>31 ± 3</td>
<td>30 ± 2</td>
<td>44 ± 3*</td>
<td>38 ± 2†</td>
</tr>
<tr>
<td>EDV (µL)</td>
<td>17 ± 2</td>
<td>13 ± 2</td>
<td>26 ± 4*</td>
<td>25 ± 1†</td>
</tr>
<tr>
<td>SV (µL)</td>
<td>13 ± 2</td>
<td>17 ± 2</td>
<td>17 ± 2</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>LVSP</td>
<td>94 ± 2</td>
<td>86 ± 3*</td>
<td>84 ± 3*</td>
<td>71 ± 4*†</td>
</tr>
<tr>
<td>+dP/dtmax (mmHg/s)</td>
<td>7308 ± 358</td>
<td>6772 ± 302</td>
<td>5669 ± 501</td>
<td>4844 ± 343‡</td>
</tr>
<tr>
<td>LVEDP</td>
<td>6 ± 1</td>
<td>7 ± 1</td>
<td>12 ± 1*</td>
<td>13 ± 1*</td>
</tr>
<tr>
<td>-dP/dtmax (mmHg/s)</td>
<td>-7094 ± 439</td>
<td>-5999 ± 380</td>
<td>-4577 ± 306*</td>
<td>-4370 ± 377*</td>
</tr>
<tr>
<td>Tau (W) (ms)</td>
<td>5.7 ± 0.3</td>
<td>6.7 ± 0.7</td>
<td>9.4 ± 1.5*</td>
<td>12.6 ± 0.9*</td>
</tr>
<tr>
<td>Tau (G) (ms)</td>
<td>9.0 ± 0.8</td>
<td>8.5 ± 0.8</td>
<td>12.7 ± 1.2*</td>
<td>19.4 ± 1.7†</td>
</tr>
<tr>
<td>EDPVR (mmHg/mL)</td>
<td>0.237 ± 0.047</td>
<td>0.237 ± 0.033</td>
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<td>—</td>
</tr>
<tr>
<td>Echocardiographic Data</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>4.0 ± 0.02</td>
<td>3.8 ± 0.02</td>
<td>6.3 ± 0.02*</td>
<td>5.1 ± 0.02†</td>
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<td>LVESD (mm)</td>
<td>2.6 ± 0.01</td>
<td>2.7 ± 0.03</td>
<td>5.6 ± 0.03*</td>
<td>4.5 ± 0.03†</td>
</tr>
<tr>
<td>Fractional shortening%</td>
<td>34 ± 2.8</td>
<td>29 ± 1.9</td>
<td>11 ± 1.8*</td>
<td>13 ± 1.6*</td>
</tr>
<tr>
<td>Ejection Fraction%</td>
<td>71 ± 3.9</td>
<td>64 ± 2.9</td>
<td>30 ± 4*</td>
<td>33 ± 3.9*</td>
</tr>
</tbody>
</table>

Table 4.5.2. Heart weight, Hemodynamic and Echocardiographic data of non-diabetic and diabetic mice with or without MI. Group abbreviations: Control, nondiabetic sham-operated control; DM, 19 weeks diabetes in the absence of MI; MI, nondiabetic 35 days
following MI; DM+MI, diabetic 19 weeks following STZ injection and 35 days following MI; N, number of mice; TL, tibial length; +dP/dtmax, maximal rate of pressure rise; –dP/dtmax, maximal rate of pressure decline, LVSP, left ventricular end-systolic pressure; LVEDP, LV end diastolic pressure; Tau (W), relaxation time constant (Weiss method); Tau (G), relaxation time constant (Glantz method), ESV, end-systolic volume; EDV, end-diastolic volume; SV, stroke volume; EDPVR, end-diastolic PV relation slope, an index of LV stiffness; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter. Data are presented as mean ± SEM, *p<0.05: vs. Control, †p<0.05: vs. MI, ‡P<0.05 vs. DM.
<table>
<thead>
<tr>
<th></th>
<th>WT + MI</th>
<th>Akita + MI</th>
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</thead>
<tbody>
<tr>
<td>(N)</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>(Blood\ glucose) ((mmol/L))</td>
<td>5 ± 1</td>
<td>29 ± 5*</td>
</tr>
<tr>
<td><strong>Organ weight</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Body\ weight) ((g))</td>
<td>26.0 ± 1.4</td>
<td>21.8 ± 2.5</td>
</tr>
<tr>
<td>(LV weight/TL) ((mg/mm))</td>
<td>5.0 ± 0.7</td>
<td>4.2 ± 0.6</td>
</tr>
<tr>
<td>(Heart\ weight/TL) ((mg/mm))</td>
<td>6.3 ± 0.6</td>
<td>5.4 ± 0.9</td>
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<tr>
<td><strong>Hemodynamic data</strong></td>
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<tr>
<td>(LVEDP) ((mmHg))</td>
<td>12 ± 7</td>
<td>11 ± 6</td>
</tr>
<tr>
<td>(LVSP) ((mmHg))</td>
<td>93 ± 27</td>
<td>92 ± 10</td>
</tr>
<tr>
<td>(+dP/dtmax) ((mmHg/s))</td>
<td>4427 ± 1332</td>
<td>4229 ± 1297</td>
</tr>
<tr>
<td>(-dP/dtmax) ((mmHg/s))</td>
<td>-2989 ± 1427</td>
<td>-2989 ± 941</td>
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<td><strong>Echocardiographic Data</strong></td>
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<td></td>
</tr>
<tr>
<td>(LVEDD) ((mm))</td>
<td>4.5 ± 0.03</td>
<td>3.9 ± 0.05*</td>
</tr>
<tr>
<td>(LVESD) ((mm))</td>
<td>3.9 ± 0.05</td>
<td>3.3 ± 0.05*</td>
</tr>
<tr>
<td>(Fractional\ shortening\ %)</td>
<td>14.1 ± 0.02</td>
<td>19.3 ± 0.09</td>
</tr>
<tr>
<td>(Ejection\ Fraction\ %)</td>
<td>36.2 ± 0.05</td>
<td>46.5 ± 0.17</td>
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</table>

**Table 4.5.3. Heart weight, Hemodynamic and Echocardiographic data of post-MI non-diabetic WT and diabetic Akita mice following 4 weeks of observation.** Group abbreviations: WT+MI, nondiabetic C57BL/6 4 weeks following MI; Akita + MI, diabetic Akita mice 4 weeks following MI; N, number of mice; TL, tibial length; \(+dP/dtmax\), maximal rate of pressure rise; \(-dP/dtmax\), maximal rate of pressure decline, LVSP, left ventricular end-systolic pressure; LVEDP, LV end diastolic pressure; LVEDD, LV end-
diastolic diameter; LVESD, LV end-systolic diameter. Data are presented as mean ± SEM, *p<0.05: vs. WT
### Table 4.5.4. Heart weight, Hemodynamic and Echocardiographic data of diabetic WT, BKO and BTG with or without MI.

<table>
<thead>
<tr>
<th></th>
<th>DM + WT</th>
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<th>DM + BKO</th>
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<th>DM + BTG</th>
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<tbody>
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<td>Sham</td>
<td>MI</td>
<td>Sham</td>
<td>MI</td>
<td>Sham</td>
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<td>6</td>
<td>11</td>
<td>6</td>
<td>10</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td><strong>Blood Glucose (mmol/L)</strong></td>
<td>26 ± 3</td>
<td>37 ± 4</td>
<td>24 ± 5</td>
<td>30 ± 1</td>
<td>29 ± 1</td>
<td>22 ± 3</td>
</tr>
<tr>
<td><strong>Organ weight</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BW (g)</strong></td>
<td>34 ± 1</td>
<td>36 ± 5</td>
<td>35 ± 1</td>
<td>31 ± 1</td>
<td>31 ± 1</td>
<td>31 ± 1</td>
</tr>
<tr>
<td><strong>TL (mm)</strong></td>
<td>20</td>
<td>21</td>
<td>21</td>
<td>19.5</td>
<td>20</td>
<td>20.1</td>
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<tr>
<td><strong>LV weight/TL (mg/mm)</strong></td>
<td>5.03 ± 0.2</td>
<td>6.4 ± 0.5</td>
<td>4.7 ± 0.1</td>
<td>4.9 ± 0.1</td>
<td>5.3 ± 0.5</td>
<td>4.9 ± 0.1</td>
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<tr>
<td><strong>Heart weight/TL (mg/mm)</strong></td>
<td>7.0 ± 0.1</td>
<td>8.1 ± 1†</td>
<td>7.0 ± 0.1</td>
<td>7.3 ± 0.1*†</td>
<td>7.0 ± 0.1</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td><strong>Hemodynamic data</strong></td>
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<td></td>
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</tr>
<tr>
<td><strong>LVSP (mmHg)</strong></td>
<td>87 ± 9</td>
<td>76 ± 6†</td>
<td>84 ± 6</td>
<td>72 ± 4†</td>
<td>83 ± 2</td>
<td>77 ± 2†</td>
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<tr>
<td><strong>SBP</strong></td>
<td>84 ± 8</td>
<td>75 ± 6</td>
<td>78 ± 4</td>
<td>66 ± 4</td>
<td>79 ± 1</td>
<td>73 ± 3</td>
</tr>
<tr>
<td><strong>+dP/dtmax (mmHg/s)</strong></td>
<td>6972 ± 676</td>
<td>4346 ± 924</td>
<td>6843 ± 900</td>
<td>3564 ± 412†</td>
<td>5360 ± 261</td>
<td>4607 ± 289</td>
</tr>
<tr>
<td><strong>-dP/dtmax (mmHg/s)</strong></td>
<td>-6874 ± 895</td>
<td>-4320 ± 900</td>
<td>-6063 ± 582</td>
<td>-2803 ± 287†</td>
<td>-6458 ± 347</td>
<td>-3969 ± 310†</td>
</tr>
<tr>
<td><strong>Echocardiographic Data</strong></td>
<td></td>
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<tr>
<td><strong>LVEDD (mm)</strong></td>
<td>3.9 ± 0.01</td>
<td>5.0 ± 0.01†</td>
<td>4.0 ± 0.02</td>
<td>5.6 ± 0.01*†‡</td>
<td>4.3 ± 0.02</td>
<td>5.0 ± 0.02†</td>
</tr>
<tr>
<td><strong>LVESD (mm)</strong></td>
<td>2.7 ± 0.02</td>
<td>4.3 ± 0.02†</td>
<td>2.5 ± 0.01</td>
<td>5.2 ± 0.01*†‡</td>
<td>3.1 ± 0.02</td>
<td>4.5 ± 0.03†</td>
</tr>
<tr>
<td><strong>Fractional shortening %</strong></td>
<td>32.5 ± 3.2</td>
<td>12.9 ± 0.8†</td>
<td>36.2 ± 2.4</td>
<td>8.7 ± 0.6†</td>
<td>28.8 ± 25.5</td>
<td>11.8 ± 2.8†</td>
</tr>
<tr>
<td><strong>Ejection Fraction %</strong></td>
<td>68 ± 4.4</td>
<td>33.9 ± 1.8†</td>
<td>73.9 ± 2.9</td>
<td>23.2 ± 1.6†</td>
<td>62.6 ± 3.7</td>
<td>30.9 ± 6.6†</td>
</tr>
<tr>
<td><strong>Wall Thickness (mm)</strong></td>
<td>0.71 ± 0.02</td>
<td>0.68 ± 0.02</td>
<td>0.70 ± 0.03</td>
<td>0.78 ± 0.02</td>
<td>0.71 ± 0.02</td>
<td>0.88 ± 0.03*†</td>
</tr>
</tbody>
</table>

Group abbreviations: BKO, S100B knockout; BTG, S100B transgenic; DM, 19 weeks following STZ injection; N, number of mice; TL, tibial length; LVSP, left ventricular systolic pressure; SBP, systolic blood pressure; +dP/dtmax, maximal rate of pressure rise; −dP/dtmax, maximal rate of pressure decline, LVEDP, LV end diastolic pressure; LVEDD, LV end diastolic diameter; LVESD, LV end systolic diameter.
diastolic pressure. Data are presented as mean ± SEM, † p<0.05: vs. counterpart sham, * p<0.05: vs. post MI diabetic WT, ‡ p<0.05: vs. post MI diabetic BTG.
Fig. 4.5.1

Representative in vivo steady-state LV pressure-volume loops in control (A), diabetes (B), MI (C) and post-MI diabetes (D) mice, demonstrating reduced LV systolic pressure in diabetes and increased ventricular volumes, end-diastolic pressures, and lower end-systolic pressures (LVSP) post-MI.
4.5.3 Myocardial Hypertrophy

As previously observed (75), STZ resulted in reductions in body weight not further altered post-MI (Tables 4.5.1 and 4.5.2). Relative LV weight (corrected to tibial length) was significantly reduced in all diabetic groups (Table 1) and the increase in LV mass post-MI was attenuated in diabetes (Table 4.5.2). Similarly, increases in cardiomyocyte diameter were greater post-MI in non-diabetic compared to diabetic mice (1.3 ± 0.06, vs. 1.1 ± 0.04 fold relative to sham, respectively, p<0.05, Fig. 4.5.2A and B). In the absence of S100B, this reduction in myocyte diameter in diabetes was not observed as myocyte diameter was higher in non-diabetic post-MI BKO (1.2 ± 0.06 fold vs. WT, p<0.05; (319) but it was not different between diabetic post-MI BKO and WT (1.0 ± 0.05 fold vs. WT).
Fig. 4.5.2

Effects of diabetes, MI, and post-MI diabetes on myocardium hypertrophy; H & E stained left ventricular sections (A). Quantitative assessment of cardiac myocyte size in noninfarcted area (B). The histograms are presented as mean ± SEM., n=13-21; p<0.05: †vs. Control, * vs. MI alone.
4.5.4 Apoptosis

While increased apoptosis was observed in the LV of diabetic and MI animals the greatest frequency was seen in diabetic post-MI mice (2.7 ± 0.2 % vs. 1.8 ± 0.09 % in MI alone, p<0.05, Fig. 4.5.3A and B). Confirming the TUNEL results, the ratio of bax/bcl-2 was highest in post-MI diabetic mice (Fig. 4.5.3C & D). qRT-PCR results demonstrated less bax mRNA expression in post-MI BKO with or without diabetes compared to post-MI WT (1.0 ± 0.1 vs. 1.5 ± 0.1 in non diabetes and 0.7 ± 0.1 vs. 1.4 ± 1.3 in diabetes, relative to sham WT, p<0.05).
Fig. 4.5.3

Effects of diabetes, MI and post-MI diabetes on myocardium apoptosis; TUNEL assay in left ventricular sections (A). Arrows show TUNEL positive cells. Quantitated TUNEL positive cells in non-infarcted area (B). Representative Western blots of left ventricular Bax, Bcl2 and GAPDH as a loading control (C). The blots were quantified by densitometry and expressed as a ratio of Bax/Bcl2 (D). The histograms are presented as mean ± SEM., n=13-21; p<0.05: †vs. Control, * vs. MI alone, ‡ vs. Control, DM and MI
4.5.5 Collagen Content and MMP-2 Activity

Collagen content was calculated as a percentage of the area of each image (expressed in pixels). Collagen area fraction assessed in non-infarct regions was significantly increased in diabetic and post-MI mice with the greatest increase in post-MI diabetics (8.7 ± 0.9 % vs. 4.0 ± 0.4% in diabetes alone and 3.4 ± 0.4 % in post-MI alone, p<0.05; Fig. 4.5.4A and B). In agreement, the presence of diabetes inhibited the increase of MMP-2 production and activity seen post-MI alone (Fig. 4.5.5), however this alteration in the MMP-2 production and activity was not seen in diabetic post-MI BKO. The expression of TIMP-2, a tissue inhibitor of MMP-2, was not altered in any group compared to controls (data are not shown).

![Fig. 4.5.4](image)

Effects of diabetes, MI and post-MI diabetes on myocardium fibrosis; Picrosirius red stained left ventricular sections (A). Quantitated % collagen area in non-infarcted region (B). The histograms display mean ± SEM, n=13-21; p<0.05: † vs. Control, * vs. MI alone.
Fig. 4.5.5

Effects of diabetes, MI and post-MI diabetes on myocardium MMP-2 activity; Representative gelatin zymography gels of MMP-2 production and activity (A). PC, positive control for MMP-2. Band intensities were normalized to control (B and C). The histograms display mean ± SEM, n=13-21; p<0.05: † vs. Control, * vs. MI alone.
4.5.6 RAGE and S100B Expression in Diabetes

As assessed by qRT-PCR, diabetes was associated with increased transcript levels for RAGE (2.6 ± 0.4, 1.6 ± 0.1, and 2.6 ± 0.2 fold at 9, 15, and 19 weeks respectively vs. control, p<0.04) and S100B (1.6 ± 0.1, 2.4 ± 0.3 and 1.6 ± 0.1 fold at 9, 15, and 19 weeks, respectively vs. control, p<0.03; Fig. 4.5.6A and B). Similar increase in S100B protein was seen in these groups (2.8 ± 0.2, 4.1 ± 1.3 and 4.0 ± 1.1 fold at 9, 15, and 19 weeks, respectively vs. control, p<0.05; Fig. 4.5.6C).
Fig. 4.5.6

Effects of 9, 15, 19 weeks diabetes on RAGE and S100B relative mRNA and protein levels; Group abbreviations: 9WD, 9 weeks post-STZ; 15WD, 15 weeks post-STZ; 19WD, 19 weeks post-STZ; RAGE and S100B mRNA were measured by qRT-PCR and results were normalized to RPL32. Representative Western blots of left ventricular S100B and GAPDH as
a loading control (C). The blots were quantified by densitometry. The histograms are presented as mean ± SEM, n=8-14; p<0.05: † vs. control.
4.5.7 Impact of Diabetic Post-MI on RAGE and S100B Expression

RAGE mRNA was comparably increased in sham-operated diabetics, non-diabetics post-MI, and post-MI diabetics (4.0 ± 0.6, 3.7 ± 0.7, 4.9 ± 0.7 fold, respectively, vs. control, p<0.05; Fig. 4.5.7). In contrast, the increase in S100B mRNA seen in diabetics and non-diabetic post-MI alone was attenuated in post-MI diabetic LV myocardium (8.2 ± 2.1, 13.3 ± 1.2, and 3.4 ± 1 fold relative to control, p<0.05, respectively; Fig. 4.5.8A). A similar attenuation of the increase of S100B protein was also seen in diabetic versus non-diabetic mice post-MI (1.6 ± 0.2 vs. 2.4 ± 0.1 fold relative to control, p< 0.001, respectively; Fig. 4.5.8C). In contrast, significant alteration of circulating S100B levels was not observed in any group (Fig. 4.5.8B). There was no change in RAGE mRNA level in post-MI diabetic BKO, BTG and WT mice and myocardial RAGE mRNA level was similarly upregulated in nondiabetic post-MI BKO myocardium (3.1 ± 0.7 and 3.7 ± 0.9 fold vs. non infarcted counterpart controls, p = NS)

![Graph showing RAGE mRNA/RPL32 levels](image)

**Fig. 4.5.7** Effects of diabetes, MI and post-MI diabetes on RAGE. RAGE mRNA was measured by qRT-PCR and results were normalized to RPL32. The histograms are presented as mean ± SEM, n=13-21; p<0.05: † vs. Control.
Effects of diabetes, MI and post-MI diabetes on S100B. S100B mRNA was measured by qRT-PCR and results were normalized to RPL32. Representative Western blots of left ventricular S100B and GAPDH as a loading control (C). The blots were quantified by densitometry (C). Serum level of S100B was measured by Elisa (B). The histograms are presented as mean ± SEM, n=13-21; p<0.05: † vs. Control, * vs. DM+MI.
4.5.8 ERK1/2 Phosphorylation

Western blot analysis revealed that ERK1/2 phosphorylation was induced at 9 weeks following STZ injection (4.5 ± 0.9, 9.0 ± 2.6 fold vs. control, p< 0.02), but despite ongoing hyperglycemia, levels returned to baseline at 15 and 19 weeks (Fig. 4.5.9A). Post-MI ERK1/2 phosphorylation was highest in diabetic myocardium (9 ± 1.7 fold relative to control compared to 5.7 ± 1.0 fold in MI alone, p< 0.05; Fig. 4.5.9B). Deleting S100B augmented phosphorylation of post-MI ERK1/2 in diabetic myocardium (2.1 ± 0.3 fold relative to WT, p< 0.05; Fig. 4.5.9C).
Fig. 4.5.9

Effects of 9, 15, 19 weeks diabetes (A), MI, post-MI diabetes (B) and S100B (C) on ERK phosphorylation. Group abbreviations: 9WD, 9 weeks post-STZ; 15WD, 15 weeks post-STZ; 19WD, 19 weeks post-STZ. Representative Western blots of left ventricular pERK1/2 and
ERK1/2 (A, B and C). The blots were quantified by densitometry and normalized to levels of total ERK protein (A, B and C). The histograms are presented as mean ± SEM, n=8-14; p<0.05: † vs. counterpart control, * vs. DM and ‡ vs. WT+DM+MI and BTG + DM + MI.
**4.5.9 AKT and NFκb**

To investigate possible downstream signaling of S100B/RAGE we studied AKT phosphorylation and NFκb1 (p50 subunit) mRNA expression. As assessed by Western blotting, AKT (Ser 473) phosphorylation was comparably increased in sham operated diabetics, non-diabetics post-MI, and post-MI diabetics (1.6 ± 0.2, 1.6 ± 0.1, 1.7 ± 0.2 fold, respectively, vs. control, p<0.05; Fig. 4.5.10A). In contrast, NFκb mRNA was attenuated in diabetic post-MI myocardium (0.3 ± 0.03 fold relative to control, p<0.05; Fig. 4.5.10B).
Fig. 4.5.10

Effects of diabetes, MI, and post-MI on AKT phosphorylation and NFκb relative mRNA levels. Representative Western blots of left ventricular pAKT and AKT (A). The blots were quantified by densitometry and normalized to levels of total AKT protein (A). NFκb mRNA was measured by qRT-PCR and results were normalized to β-Actin (B). The histograms are presented as mean ± SEM, n=13-21; p<0.05: † vs. Control, * vs. MI.
4.5.10 AGE level

In contrast to S100B expression, increased tissue AGE level in diabetes and post-MI diabetes was significantly reduced in response to MI alone (Fig. 4.5.11A). However, serum AGE levels were unaltered among these groups (0.35 ± 0.01 vs. 0.34 ± 0.01 µg/10 µg protein).

To study the impact of S100B on AGE production, serum AGE level was measured in the absence of S100B, demonstrating significantly greater elevation in diabetic post-MI BKO mice compared to diabetic WT (0.15 ± 0.03 vs. 0.08 ± 0.01 µg/10 µg protein, p<0.05, Fig. 4.5.11B).
Fig. 4.5.11

Effects of diabetes, MI, post-MI diabetes (A) and S100B (B) on AGE levels. Cardiac tissue AGE level (A) and serum AGE level was measured by ELISA. The histograms are presented as mean ± SEM, n=13-21; p<0.05: † vs. Control, * vs. DM and DM+MI and ‡ vs. WT+DM+MI and BTG + DM + MI.
4.5.11 Impact of S100B on GLUT4 Expression

To investigate the myocardial insulin resistance, GLUT4 expression was studied. Results indicated a significant reduction of GLUT4 mRNA level in diabetic post-MI BKO compared to WT (Fig. 4.5.12).

![Bar chart](image)

**Fig. 4.5.12**

Effects of S100B on GLUT4 mRNA. mRNA level was measured by qRT-PCR and results were normalized to RPL32. The histograms are presented as mean ± SEM, n=6-11; p<0.05: ‡ vs. WT+DM+MI and BTG + DM + MI.
4.5.12 RAGE, S100B and ERK1/2 Expression in Isolated Cardiac Myocytes

To confirm the capacity to regulate S100B and signaling pathways specifically in cardiac myocytes and to explore mechanisms, rat neonatal and mouse adult myocytes were treated with glucose or AGE. Both protein and mRNA levels of RAGE were upregulated in neonatal myocytes by AGE-BSA treatment (1µg-10µg) for 4 days compared with vehicle (Fig. 4.5.13A, D). Similar to RAGE, S100B protein expression was stimulated by AGE-BSA but not glucose (4.1 ± 1.3 fold relative to BSA in 10µg AGE-BSA, p<0.05, Fig. 4.5.13B). Similarly, AGE stimulated ERK1/2 phosphorylation compared with BSA while glucose had no effects comparable to the osmotic control (7.5 ± 0.8 fold relative to BSA in 10µg AGE-BSA, p<0.05, Fig. 4.5.13C). Confirming comparable responses in fully differentiated myocardium, 10µg AGE-BSA similarly upregulated RAGE and S100B mRNA expressions in adult mouse cardiac myocytes (25.9 ± 3 and 6.2 ± 1 fold, respectively, relative to BSA, p<0.05, Fig. 4.5.14A & B). Immunofluorescence assay was used to confirm the upregulation of RAGE and S100B in respond to AGE-BSA, or glucose treatment. Increasing of RAGE and S100B expression was observed in myocyte cell culture treated with AGE-BSA (Fig. 4.5.15).
Fig. 4.5.13

Effects of BSA-AGE and glucose on RAGE and S100B relative mRNA, protein levels and ERK1/2 phosphorylation in rat neonatal cardiomyocytes. Representative Western blots of neonatal cardiac myocyte RAGE protein (A), S100B protein (B), ERK1/2 phosphorylation (C) histogram for RAGE mRNA (D). Rat neonatal CMs were treated with either (5-50mmol) glucose or (1-10 µg) AGE-BSA for 4 days. BSA and mannitol were used as controls for AGE and glucose treatment, respectively. RAGE and S100B mRNA were measured by qRT-PCR and results were normalized to RPL32. The histograms are presented as mean ± SEM, n=3-5; † vs. BSA, * vs. mannitol.
Fig. 4.5.14

Effects of BSA-AGE on RAGE and S100B relative mRNA expressions in mouse adult cardiomyocytes. Histogram for RAGE (A) and S100B (B) mRNA. Mouse adult CMs were treated with 10 µg/ml AGE-BSA for 5 hours. BSA was used as a control for AGE treatment. RAGE and S100B mRNA were measured by qRT-PCR and results were normalized to RPL32. The histograms are presented as mean ± SEM, n=3-5; p<0.05: † vs. BSA.
Fig. 4.5.15

Representative immunofluorescence slides of cardiac myocyte RAGE and S100B proteins in respond to BSA-AGE treatment *in vitro*. Rat neonatal CMs were treated with either (25 mmol) glucose or (10 µg/ml) AGE-BSA for 4 days. BSA was used as control. Primary antibodies against RAGE and S100B were used to detect the RAGE and S100B. DAPI staining was used to determine the number of nuclei and to assess gross cell morphology.
4.5.13 Impact of Diabetes on S100A1 Expression

S100A1 mRNA levels were decreased in sham-operated diabetics, non-diabetics post-MI, and post-MI diabetics (0.5 ± 0.1, 0.2 ± 0.1, 0.25 ± 0.1 fold, respectively, vs. control, p<0.05; Fig. 4.5.16A). A similar attenuation of S100A1 mRNA was also seen in diabetic with further reduction in mice post-MI. However, despite reduction in mRNA levels in diabetic post-MI myocardium, protein expression was augmented compared to MI alone (Fig. 4.5.16B). In agreement, protein levels of S100A1 were reduced in neonatal myocytes in response to AGE-BSA treatment (1µg-10µg) or glucose treatment (5-50 mmol) for 4 days compared with control or vehicle (Fig. 4.5.16C).
Fig. 4.5.16

Effects of diabetes, MI and post-MI diabetes and in vitro BSA-AGE treatment on S100A1 relative mRNA (A) and protein (B and C) levels. Rat neonatal CMs were treated with either (5-50 mmol) glucose or (5-15 mg) AGE-BSA for 4 days. S100A1 mRNA was measured by qRT-PCR and results were normalized to RPL32. Representative Western blots of left ventricular S100B and GAPDH as a loading control (B and C). The histogram is presented as mean ± SEM, n=6-7; p<0.05: † vs. Control, * vs. DM.
4.6 Discussion

The main findings presented in chapter 4, are that 1) despite comparable infarct size and impairment of cardiac systolic function, myocardial infarction in the presence of diabetes was associated with less LV dilation, reduced cardiac myocyte hypertrophy, and greater apoptosis; 2) the combination of diabetes and MI increased ventricular collagen content associated with diminished MMP-2 activity; 3) experimental diabetes led to enhancement of RAGE and S100B expression in myocardium, whereas in association with myocardial infarction there was paradoxical blunting of S100B expression; 4) ERK1/2 phosphorylation was transiently induced by induction of hyperglycemia and its re-induction by MI was amplified in the setting of diabetes in association with downregulation of NFκb; 5) diminished AGE levels following MI was increased in the post-MI diabetes; 6) knocking out S100B in diabetes was accompanied with increased dilation and worsening cardiac function following MI associated with reduction of GLUT4 mRNA levels and increased serum AGE levels and ERK phosphorylation.

As previously reported in rats, the cardiac phenotype in STZ-induced DM in mice encompassed mild abnormalities including reduction in LV weight and systolic blood pressure in the absence of demonstrable abnormalities in systolic function by echocardiographic and hemodynamic analysis (5, 26, 137, 254). Such a significant LV mass reduction has been shown in previous studies using experimentally induced, insulin-independent diabetic animals (137). This might be due to metabolic disturbances in diabetic models, which shifts energy production from glucose utilization to the oxidation of free fatty acids, leading to caloric deprivation (256). The systolic blood pressure decreased in these mice, suggesting a decrease in peripheral vascular resistance or intravascular volume as
previously reported (5, 26, 122, 256). Although diabetic rats may manifest LV diastolic dysfunction (137, 209), at 19 weeks post-STZ, we observed no abnormalities in ventricular relaxation or filling as assessed by pressure volume analysis, including sensitive measures of the time constant of relaxation (Tau) or the end-diastolic pressure volume relationship (EDPVR). These differences might be due to differences in the species examined (mice vs. rats), dose of STZ injected, lack of requirement for insulin therapy for animal survival, the time-point examined or the sensitivity of these hemodynamic measures to detect subtle alterations in LV relaxation and compliance; we can’t exclude that sustained hyperglycemia beyond 19 weeks could be associated with changes in cardiac function in the mouse.

By contrast, adverse ventricular remodeling and augmented functional abnormalities have been reported following myocardial infarction in other animal models of diabetes (282). We observed less ventricular dilation post-MI in the diabetic group. Although increased ventricular dimensions are the best predictor of adverse outcomes following myocardial infarction in humans (286), this reduction in dilation was not an indicator of improved remodeling in the diabetic mouse as it was associated with comparable reduction in all measures of myocardial systolic performance. Moreover, compared with non-diabetics, the diabetic mouse exhibited a greater reduction in systolic pressure and a greater prolongation of Tau, a measure of active ventricular relaxation (348). We recognize that the use of the STZ may introduce nonspecific toxicity that we tried to limit by multiple low dose injections and, in addition, is not representative of the more common type 2 diabetic phenotype in humans with CAD (183, 184). We saw similar absence of ventricular dilation in the Akita model suggesting that this is not a finding unique to STZ-induced diabetes and future examination in other models of diabetes is justifiable. In total, our findings parallel the observation in
human diabetic patients that clinical heart failure can develop following infarction in the absence of ventricular dilation (275, 286). The results from this thesis and previous findings (90, 275, 286) highlight the importance of mechanisms other than greater LV enlargement and contractile dysfunction in mediating the adverse effect of diabetes. Of interest, as described previously, MI in the setting of diabetes is associated with increased myocardial apoptosis (17, 282) that may contribute to the dissociation between ventricular dilation and functional impact. Similarly we also described reduced myocyte hypertrophy as assessed by cardiomyocyte size in post-MI diabetes and have previously demonstrated that absence of a post-MI hypertrophic response may contribute to functional impairment in a non-diabetic mouse model (319).

By altering elastic properties of the left ventricle, both limited dilation and diastolic functional abnormalities could be a consequence of the enhanced deposition of collagen post-MI in diabetes. Moreover, in diabetes, AGE deposition results from long-standing hyperglycemia that may affect LV structure and function directly by cross-linking with collagen (48, 132). This could alter the biophysical property of collagen, limit ventricular dilation and increase diastolic stiffness. AGE may also directly enhance overall collagen formation (333). Intriguingly, AGE may also directly enhance overall collagen formation (333). In fact, we observed enhanced collagen formation in response to MI in diabetes. This is in agreement with previous finding demonstrating increase of collagen deposition in the myocardium of STZ-diabetic mice (90). Accumulation of cardiac fibrosis can result from excessive production of collagen as well as from decreased degradation of collagen by MMPs. We focused on MMP-2 as it is expressed in myocardium and degrades fibrillar collagen peptides and newly synthesized fibers (350). It has been previously established that
MMPs activities are decreased in high glucose treated cardiac fibroblasts (16) as well as in STZ treated rat myocardium (33). MMP-2 is expressed in myocardium (350) and in diabetes following MI, myocardial MMP-2 activity was significantly reduced in the presence of both diabetes and infarction consistent with the increased cardiac fibrosis observed. The level of TIMP-2, a MMP-2 inhibitor was not altered. We recognize that our use of whole LV homogenate limits our capacity to define regional variations in expression of MMPs and TIMPs in myocardium following MI.

Intriguingly the lack of ventricular dilation was associated with impaired myocyte hypertrophy as assessed by cardiomyocyte size. While the pathophysiologic significance of reduced myocyte hypertrophy in diabetic post–MI remodeling is unclear, hypertrophy may provide additional adaptation post-MI and its absence may contribute to adverse consequences.

An important role for RAGE in excessive cellular activation and enhanced inflammatory pathways in diabetic tissues has been demonstrated by pharmacologic blockade (234, 343) and diabetic RAGE null mice exhibit reduced ischemia/reperfusion injury (42). We demonstrated that RAGE in myocardium was induced comparably in both diabetes (short and long term) and MI alone. However, post-MI diabetic myocardium did not demonstrate further augmentation of RAGE suggesting that RAGE expression alone does not account for the post-MI phenotype in diabetes. In addition to cardiomyocytes, there are several other cell types in the heart that may contribute to the phenotypic responses to cardiac injury and remodeling. Although RAGE expression in DM after ischemia/reperfusion was localized particularly to both vascular endothelial and mononuclear cells (42), our results demonstrate
that isolated neonatal and adult cardiac myocytes are capable of responding to AGE and glucose stimulation and activate RAGE.

AKT and mitogen activated protein kinase (MAPK) pathways, including ERK 1/2 are involved in the mitogen action of insulin (154, 289), activated by RAGE ligands (175), and regulate cell survival (147). As in other cell types, we showed in vitro that AGE induces ERK phosphorylation in cardiac myocytes (182) and our in vivo results were consistent with the transient impact of hyperglycemia on ERK1/2 in diabetic myocardium (217). Although ERK is known as a survival factor, it is involved in induction of myocyte apoptosis via RAGE (318). In agreement, we showed post-MI diabetic hearts demonstrated increased ERK phosphorylation in association with increased apoptosis. By contrast, phosphorylation of AKT was not differentially regulated but the relative downregulation of NFκb in post-MI diabetic hearts was paralleled by attenuated expression of the alternate RAGE ligand, S100B. S100 proteins entail a multimember subfamily of EF-hand Ca^{2+}-binding proteins, with putative roles in regulating cellular metabolism, cell growth and differentiation. S100B basal expression is restricted to neuronal cells, however it is anomalously expressed in myocardium following both myocardial infarction in humans and coronary artery ligation or pressure overload in rats (319). The involvement of extracellular S100B and its receptor RAGE has been implicated in diabetic vasculature diseases such as atherosclerosis (262) and in post-MI remodeling in the absence of diabetes (318). We have previously demonstrated that S100B is expressed in the left ventricle in both experimental models and humans post-MI and modulates post-MI remodeling and function by both intracellular and RAGE-dependent extracellular mechanisms in non-diabetic hearts (318-320). We now demonstrate sustained expression of S100B in STZ-induced diabetic myocardium and recapitulate this
induction in isolated neonatal and adult cardiac myocytes by treatment with AGE. Despite upregulation of S100B in diabetes or MI alone, diabetic myocardium post-MI surprisingly exhibited attenuated S100B expression in myocardium S100B protein nullifying our original hypothesis. In contrast AGE levels were augmented in post-MI diabetes, suggesting a possible mechanistic link between expressions of these distinct RAGE ligands. In addition, we demonstrated that S100B canonical receptor, RAGE, in myocardium was induced comparably in both diabetes and MI alone. However, post-MI diabetic myocardium did not demonstrate further augmentation of RAGE suggesting that RAGE expression alone does not account for the post-MI phenotype in diabetes.

While the functional consequence of this reduced S100B expression in diabetes remains unclear, we demonstrated that complete abrogation of S100B expression in diabetes in BKO resulted in significant dilation post-MI and significantly worsened LV function. This observation is dependent on the presence of diabetes as we reconfirmed our previous observation that BKO post-MI in the absence of diabetes actually exhibits a antithetical reduction in dilation compared with WT controls. Thus, loss of S100B must impact on other diabetes-dependent pathways that regulate LV remodeling and, indeed, higher circulating AGE levels and greater phosphorylation of ERK 1/2 were seen in diabetic BKO post-MI. In addition to alterations in S100B and AGE interaction with RAGE post-MI in diabetes, other RAGE ligands in myocardium could be involved including S100A1, a regulator of myocardial contractility (72) and High-Mobility Group Box-1 (HMGB1), an activator of NFκb induction of inflammatory responses (331). Such ligands are amenable to future testing. Further emphasizing potential complexity, distinct RAGE ligands may differentially regulate RAGE-dependent signaling by interaction with distinct extracellular domains of the
receptor (175); S100B-induced nitric oxide production does not require RAGE transducing activity but depends on RAGE extracellular domains (3); S100B can interact with distinct receptors, including TLR4 (331); AGE may induce reactive oxygen species (13); and crosslinking of AGE with SERCA2 may decrease intracellular Ca\(^{2+}\) uptake and impair cardiac relaxation (13). As an additional limitation, we measured predominantly CML-derived AGEs in this study and can’t exclude regulation of AGEs derived from other sources that may be differentially regulated by S100B in diabetes.

S100A1 is important in regulating myocyte calcium cycling not only through modulating the activity of Ryanodine receptor, but also by influencing SERCA2 and protein kinase A activity (163, 214). The ability of S100A1 to restore cell shortening in cardiomyocytes has made this protein an exciting prospect for therapeutics. Despite down regulation of protein expression of S100A1 in diabetes and in non-diabetics post–MI, diabetic myocardium post-MI exhibited upregulation of S100A1 to the levels seen in diabetes alone. No discordant changes in S100A1 protein and mRNA levels were observed in myocardium as reported previously (365). This suggests that this gene is regulated at the transcriptional/posttranscriptional levels by insulin. While the functional consequence of upregulation of S100A1 in post-MI diabetes is unclear, paradoxical expression of myocardium S100A1 and S100B in the setting of post-MI diabetes suggest the possible inter-regulatory link between these two genes and also strengthens the observation of distinct and differential regulation of S100 proteins in diabetic myocardium. S100B/S100A1 heterodimer combination may also result in distinct pathological outcomes. Both, S100A1 and S100B may regulate enzymes implicated in cardiac energy metabolism such as glucose-6-phosphatase (81). Moreover, S100A1 may play a role in silencing S100B in the context of the maintenance of the genetic program that underlies diabetic cardiac metabolism (323).
Adipose tissue has already been highlighted as an important extra cerebral source of S100B (134, 207, 310) where, its secretion is reduced by insulin (310). Adipose tissue alterations, particular insulin resistance, appear to be involved in many diseases, including type 2 diabetes (195). MI results in greater expression of GLUT4, facilitating glucose entry and glycolysis to generate ATP (304). In diabetes, however, glycolysis and glucose oxidation are suppressed (101), in part due to depressed levels of GLUT4 that results in reduced availability of intracellular glucose (296). These metabolic perturbations result in depressed ATP production, generation of oxygen free radicals, increased myocardial oxygen consumption, and myocardial contractile dysfunction. Furthermore, STZ induced-diabetic mice showed a significantly decreased level of cardiac glucose uptake after ischemia reperfusion (90). It is not surprising that reduced GLUT4 mRNA levels accompany additional myocardial damage in post-MI BKO hearts. It has been demonstrated that cellular nitric oxide (NO) AMPK- dependent mechanism enhances GLUT4 expression (57) and S100B by increasing inducible NO synthase (iNOS) and NO production may be involved in the GLUT4 upregulation (190). Moreover, RAGE activation by AGE in cultures of adipocytes inhibited glucose uptake through the over generation of intracellular reactive oxygen species and this could contribute to insulin resistance (330). Therefore increase of AGE levels in post-MI diabetic BKO mice may cause further impairment of cardiac metabolism.
4.7 Conclusion

In conclusion, in experimental diabetes, less ventricular dilatation is observed post-MI in association with increased fibrosis and apoptosis and blunted hypertrophy. Components of S100B/RAGE signaling were differentially regulated in post-MI diabetic myocardium and knocking out S100B had a deleterious effect on cardiac function in diabetes post-MI partly attributed to increased ventricular dilation and increased AGE formation. The impact of S100B abrogation was in direct contrast to that seen in non-diabetics. These data have implications for our understanding of the impact of diabetes on post-MI remodeling, the signaling pathways that may be differentially regulated, and suggests that these pathways, particularly those associated with ligand-receptor interaction, may be amenable to therapeutic manipulation.
CHAPTER 5
DEFICIENCY OF S100B CONFERS RESISTANCE TO EXPERIMENTAL DIABETES MELLITUS IN MICE
5.1 Abstract:
S100B, a calcium binding protein is predominately expressed in astrocytes, and Schwann cells. It has been implicated in diabetic neuronal and vascular complications but has not been examined in the development of diabetes. Moreover, in the process of establishing a diabetic model in S100B deficient mice, our preliminary data suggested that S100B might play a role in the development and progression of STZ-induced diabetes itself. S100B knockout mice (BKO) and wild-type mice (WT) were injected with 40 or 50 mg/kg body weight streptozotocin (STZ) for 5 days followed by 4 weeks. Blood plasma and pancreas were extracted to study the profile of glucose, insulin, RAGE and S100B. Immunohistochemistry and immunofluorescence staining of islets were analyzed to study the islets function and structure. Insulin secreting cell lines (MIN6) were used to test the apoptotic effect of S100B. BKO mice were significantly resistant to lower dose STZ (40 mg/kg) induced-diabetes with lower values for food and water intake and urine volume and increased body weight compared to WT mice. Intraperitoneal glucose tolerance test (GTT) and insulin tolerance test (ITT) indicated BKO displayed enhanced glucose tolerance and insulin sensitivity both prior to, and after, STZ injection. This was associated with a higher amount of circulating insulin in the plasma of BKO mice at 4 weeks after STZ injection. Histopathological examinations of islets showed that S100B deficiency prevented β-cell disruption and islet destruction seen in WT mice. Immunohistochemistry showed that expression of S100B, which is normally limited to the peri-islet Schwann cells, disappeared concomitant with the progression of hyperglycemia in WT. This coincided with expression of S100B inside islets, which was not colocalized with β cell or Schwann cell markers. To understand the mechanism by which S100B prevents β cell disruption we treated a β cell line (MIN6) with progressive doses of
S100B protein. Caspase-3 activity was increased in response to 500nM concentration of S100B and results of flow cytometry confirmed the apoptotic effect of S100B on β cells \textit{in vitro}.

These findings indicate that at least in a STZ animal model, abrogation of S100B both enhances insulin sensitivity and reduces pancreatic islet, and more specifically, β cell, destruction. S100B may be a promising target for pharmacological interventions aimed at repressing diabetes initiation or progression.
5.2 Introduction

S100B, a 10-12kDa EF hand calcium-binding protein, is a member of a multigenic family highly expressed in the astrocytes, oligodendrocytes, and schwann cells, implicated in the regulation of a number of intracellular activities by interacting with effector proteins. It exerts a paracrine or autocrine function following secretion from glial cells on several neuronal populations depending on concentration (166). In brain, S100B at nanomolar concentration stimulates neurite outgrowth whereas at micromolar concentrations it causes neuronal death (142). There is emerging evidence suggesting S100B involvement in diabetes and vasculature (320, 365). Level of S100B protein increases in brain, white fat, and testes tissues of streptozotocin (STZ)-treated animals (365). Increased S100B release from fat pad of adipose tissue in response to diabetes, was reduced following insulin treatment, indicating the regulation of S100B expression in diabetes (310).

Chronic hyperglycemia contributes to diabetic complications, in part, through the formation of advanced glycation end products (AGE), which are irreversibly formed by non-enzymatic glycosylation and oxidation of proteins and lipids (234). AGE could exert their effects via the canonical receptor, receptor for AGE, RAGE. Moreover, RAGE signaling can also be stimulated by S100B ligation (136). The involvement of extracellular S100B and RAGE has been implicated in diabetic vasculature diseases such as atherosclerosis (358) and in the neuronal system via stimulation of inducible nitric oxide synthase and secretion of nitric oxide (142). Moreover as shown in chapter 4 complete abrogation of S100B expression in diabetes paradoxically resulted in significant dilation post-MI and worsening LV function.

As discussed in chapter 1, it has been demonstrated that in non-obese diabetic (NOD) mice, progression of type 1 diabetes causes breaching in the glial sheath surrounding the pancreatic islets of Langerhans. This leads to the infiltration of T cells inside the islet and invasion of
islet interior. Targeting pancreatic Schwann cells (pSC), which form the glial sheath, was associated with S100B and filament GFAP specific auto reactive T cells. Interestingly, immunotherapy with these two antigens significantly delayed diabetes development, establishing their involvement in disease progression (354). S100B and GFAP are used as markers for diabetes-induced glial and neuronal injury (22). Thus, S100B may play a role in the progression to overt type 1 diabetes. Data presented herein demonstrates, for the first time, that absence of the S100B gene protects mice against STZ-induced hyperglycemia, while wild type (WT) mice are susceptible to developing hyperglycemia and β cell death. The main characteristic of S100B knockout (BKO) mice was a better ability to respond to glucose increase by preserving pancreatic β cell’s function to secret insulin and increased insulin sensitivity. This suggests the essential engagement of S100B as a key molecule in STZ-induced diabetes and this might occur, in part, via direct effects of S100B on pancreatic β cells.
5.3 Objectives of this study

The objective of this study was to investigate the impact of S100B deficiency in mice on STZ-induced diabetes and the associated regulation of insulin sensitivity and pancreatic islet cell structure and function.

5.3 Hypothesis of this study

S100B plays a role in limiting islet cell damage in response to STZ and/or enhancing insulin sensitivity.
5.4 Methods

5.4.1 Animals- See “General Methods”.

S100B heterozygous mice for S100B allele were back–crossed to CD1 mice in order to obtain the littermate controls.

5.4.1.1 S100B Knockout Mice- See “General Methods”.

5.4.2 Induction of Diabetes- See “General Methods”.

5.4.3 Blood Glucose- See “General Methods”.

5.4.4 Experimental Design

For this study, heterozygous mice for S100B allele were back–crossed to CD1 mice to generate the congenic strain. Five-six weeks old male WT and S100BKO mice were intraperitoneally injected with streptozotocin (STZ, Sigma, St Louis, Missouri) in citrate buffer (40mg/kg or 50 mg/kg body weight/day) for 5 days to induce hyperglycemia. Before STZ injection and 1, 2 and 4 weeks after STZ injection, mice were sacrificed and the pancreas dissected for analysis.

Plasma glucose level of each mouse was obtained every 3 days during the observation period. Body weight was monitored before STZ induction and every week thereafter.

5.4.5 Tissue and Blood Collection- See “General Methods”.

Pancreas tissues were cut into small pieces and paraffin embedded in a randomized pattern. Blood samples were collected by puncturing the left lateral saphenous vein with a 25-gauge needle after shaving the tarsal area of the left leg and mixed with two protease inhibitors; Aprotinin (5000 KIU, Sigma, St. Louis, USA) and Diprotin A (0.1 mM, Sigma, St. Louis, USA).

5.4.6 Histopathology and Immunohistochemistry

5.4.6.1 Hematoxylin and Eosin (H& E) Staining- See “General Methods”.

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5.4.6.2 Immunohistochemistry

Paraffin sections (5µm) were deparaffinized in xylene and rehydrated in graded ethanol (100%, 90%, and 70%) and immersed in PBS. After antigen retrieval by 10mM sodium Citrate and heat for 200 minutes and blocking of endogenous hydrogen peroxide with 3% hydrogen peroxides in methanol for 30 minutes, sections were blocked in serum free Dako protein block for 30 minutes. Sections were then incubated with rabbit anti-S100B antibody (Abcam, Cambridge, USA) diluted 1:400 in blocking solution over night at 4°C. On the following day, sections were thoroughly washed in PBS (3 x 5 minute changes) and incubated with biotinylated goat anti-rabbit IgG antibody (Vector lab, Burlingame, CA, USA). Sections were then rinsed with PBS (3 x 5 min) followed by incubation with an avidin-biotin peroxidase ABC complex, both A and B 1:50 (Vector, Burlingame, CA). Following rinsing with PBS (2 x 5 min), localization of the peroxidase conjugates was achieved using 3,3’- diaminobenzidine tetrahydrochloride (DAB, DAKO, CA, USA) as a chromagen, for 10 minutes. Color development was stopped by rinsing in dd water for 5 minutes, slides were then counterstained in haemotoxylin, dehydrated, cleared, mounted in permount and examined under a microscope. Sections incubated with 1:10 normal goat serum (NGS), instead of the primary antiserum, served as the negative controls.

The above method was used for detection of insulin using Guinea Pig anti-insulin antibody (Invitrogen, Canada) diluted 1:100, GFAP using Rabbit anti-GFAP antibody (SIGNE, Cambridge, USA) diluted 1; 100, RAGE using Rabbit anti RAGE antibody (Abcam, Cambridge, USA) diluted 1:100, and S100B using Rabbit anti- S100B antibody (Abcam, Cambridge, USA) diluted 1: 400. Sections after labeling with specific antibody were counterstained with Hematoxylin and digital images of sections were used for image analysis.
5.4.7 Islet Mass Measurement

An estimate of islet size per mm$^2$ pancreas was obtained by morphometric analysis of fixed pancreatic sections labeled for insulin by immunohistochemistry. The entire paraffin-embedded pancreas from each animal was cut into 4 µm sections and the cross-sectional area occupied by all of the beta-cells and the cross-sectional area of all pancreatic tissue was quantified. Total beta-cell area and total pancreas mass for each animal was calculated as the sum of the determinations from each of the 8–10 segments of pancreas. Sections, after labeling with insulin antibody (Invitrogen, Canada), were counterstained with Hematoxylin. Entire sections were imaged using a ScanScope XT microscope slide scanner (Aperio technologies, Vista, CA, USA). Diameters of area of insulin labeled cells within islets on each section and total tissue were measured using thresholding option with the aid of Aperio ImageScope software. To determine the fractions of the β-cell areas, total insulin area was divided by total tissue area. Cell mass was calculated as the fraction of insulin positive areas of the total pancreatic area multiplied by gross pancreas weight (339).

5.4.8 Insulin and Glucagon Measurement

Blood samples from non fasting S100B KO and WT mice from chapter 5 were centrifuged at 10000 g. Serum insulin and glucagon were measured by rat insulin and glucagon RIA kits according to manufacture’s protocol (Linco Research, St. Charles, MO, USA).

5.4.9 Glucose and Insulin Tolerance Test

Mice were fasted overnight prior to glucose tolerance test. Both Control and S100B KO mice were injected with glucose intraperitoneally (i.p)(1.5 g/kg of body weight). Glucose levels were measured from tail bleeds by a Glucometer Elite testing system (Ascensia: Bayer) at
specified time pointe after glucose administration. Insulin test was performed similar to glucose (2U/K of body weight) tolerance test with a fasting period of 2 hours.

5.4.10 Food and Water Intake and Urine Output Measurement

For food and water intake and urine collection experiments mice were housed individually in metabolic cages at the end of study for 24 hours. Free access to food and water was provided.

5.4.11 Cell culture

5.4.11.1 Insulin Secreting Cells

Rat pancreatic β cell line (INS-1) were grown in RPMI-16 media (Invitrogen, Canada) supplemented with HEPES, Penicillin/ Streptomycin (Pen/Strep, 5ml/500ml), Sodium Pyruvate (55mg/500), β- mercaptoethanol (1.7 µl/500 ml) and 10% FBS. Mice pancreatic β cell line (MIN6) were grown in Dulbecco's Modified Eagle Medium (Invitrogen, Canada) supplemented with Pen/Strep (5ml/500ml) and 10% FBS.

5.4.12 RNA Extraction and cDNA Synthesis -See “General Methods”.

5.4.13 Quantitative Real Time RT-PCR- See “General Methods”.

5.4.14 Annexin-V/Propidium Iodide Staining and Flow Cytometry

Following treatment of MIN6 cells with 500 nM concentration of S100B protein (from bovine brain, Calbiochem, Gibbstown, NJ, USA) cell death was determined by flow cytometric analysis using the FITC Annexin V-Apoptosis detection kit (BD biosciences, Santiago, CA, USA). Following manufacturer’s protocol, adherent cells were trypsinized (0.05% trypsin with 0.53mM EDTA) and combined with the free floating cells, washed twice with cold PBS, and re-suspended in 100µl of Annexin-V binding buffer alone or in combination with 2µl of FITC-conjugated Annexin-V antibody and/or 2µl of PI. The cells were then gently mixed and incubated for 15 minutes in the dark at RT. Following this, the
samples were diluted in 400µl of the Annexin-V binding buffer and analyzed by flow cytometry (Cytomics FC500, Beckman Coulter, Fullerton, CA).

5.4.15 Assay of Caspase-3 Activity

MIN6 cells were starved for 12 hours and then in the presence of 0.05 BSA were treated with either 200 or 500 nM S100B protein and then trypsinized (0.05% trypsin with 0.53mM EDTA), collected and analyzed by Enzchek™ caspase-3 Assay kit#2 (Molecular Probes, Inc. Eugene), using HITACH-F4010 fluorescence spectrophotometer (496nm/520nm). This kit provides the substrate of caspase-3 Z-DEVD-R110, which can be lysed by active caspase-3 and release R110, the fluorescence of which was analyzed by fluorescence spectrophotometer.

5.4.16 Statistical Analysis

Repeated measure ANOVA design was used for body weight, and glucose data collected over time from the same mouse. Treatment was between subject factor and time was within subject factor. One-way ANOVA design followed by post-hoc Tukey testing was used for caspase -3 activity. ANOVA calculations were made by using the general linear model procedures of the Self-Propelled Semi-Submersible Software (SPSS). The two-tailed Student’s t-test was used for WT and S100BKO groups. When a mean effect or an interaction was significant, the least significant difference was used to locate the significant differences. The data are expressed as mean ± SEM. A value of \( p < 0.05 \) was considered to define statistical significance.
5.5 Results

5.5.1 S100B Knockout Mice were Resistant to STZ-Induced Diabetes.

We characterized the metabolic status of S100B KO and WT mice by injecting a diabetogenic dose of (40 mg/kg /day) STZ for 5 days, and their blood glucose concentrations were measured. There was a gradual increase in blood glucose concentration in WT at day 7 after injection to day 20. After day 20 till the end of study course, the values of blood glucose were constantly higher than pre-injected WT mice.

In contrast, injection of 40 mg/kg STZ did not induce hyperglycemia in BKO at any time point over the study course (Fig. 5.5.1A). This demonstrates BKO are resistance to induction of hyperglycemia in response to a diabetogenic dose of STZ.

Demonstrating a dose-depended impact of STZ on the development of diabetes in BKO, elevated glucose levels were achieved in response to a higher dose of STZ (50mg/kg/day) albeit attenuated in comparison with WT (Fig.5.5.1B). For the remaining experiments, 40 mg/Kg/day STZ injection was used.

While WT showed body weight reduction after STZ injection through the study period, BKO demonstrated significant weight increase (data not shown).

Metabolic studies at day 28 demonstrated a significant increase in dietary and fluid intake and urine output in WT mice compare to BKO (Fig. 5.5.2A, B, C, respectively).
Fig. 5.5.1

Effects of S100B on blood glucose levels in STZ-treated mice. Mice were injected either with 40 mg/kg/day (A) or 50 mg/kg/day (B) STZ for 5 days. Blood glucose levels were measured before and after injection for either 20 or 28 days. The data are presented as mean ± SEM., n=8-20; p<0.05: * vs. WT.
Fig. 5.5.2

Effect of S100B on food and water intake and urine volume in STZ-treated mice. Mice were housed individually in metabolic cages four weeks after STZ treatment (40mg/kg) for 24 hours to collect food (A) and water (B) intake and urine outtake (C). The histograms display mean ± SEM, n=6-12; p<0.05: * vs. WT.
5.5.2 S100B Knockout Mice Exhibit Enhanced Glucose Tolerance and Insulin Sensitivity.

Intraperitoneal glucose tolerance test (GTT) indicated that pre-injected BKO displayed a blunted elevation of blood glucose compared to the levels in WT at all time points (Fig. 5.5.3A). Similarly, glucose levels were significantly lower in BKO at all time points 4 weeks post injection (Fig. 5.5.3B). At 30 min after glucose administration, the glucose level in the WT mice was > 22 compared to <13 mmol/L in BKO.

The insulin tolerance test (ITT) showed that pre-injected BKO mice were more sensitive to insulin challenge, experiencing lower blood glucose (3.7 ± 0.3 vs. 4.7 ± 0.2 mmol/L in WT) (Fig. 5.5.4A). At 4 weeks post injection, BKO demonstrated a similar insulin sensitivity to lower the glucose levels. However, WT mice were not able to lower the glucose levels in response to insulin injection at any time point (Fig. 5.5.4B). Thus, abrogation of the S100B gene results in higher insulin sensitivity and, moreover, STZ induced insulin resistance (IR) in WT but not in BKO mice.
Fig. 5.5.3

Effect of S100B on glucose tolerance in STZ-treated mice. Intraperitoneal glucose tolerant test (GTT) in WT and BKO mice before STZ treatment (A) and after STZ treatment (B). The data are presented as mean ± SEM, n=8-10; p<0.05: * vs. WT.
Fig. 5.5.4

Effect of S100B on insulin tolerance test in STZ-treated mice. Insulin tolerance in WT and BKO mice before STZ treatment (C) and after STZ treatment (D). The data are presented as mean ± SEM, n=8-10; p<0.05: * vs. WT.

5.5.3 S100B Deficiency Prevents β-cells Disruption
Histopathological examination of islets showed extensive changes, including β-cell death and atrophy two weeks post injection in WT with further disappearances of islets after 4 weeks. In contrast, islet cells of BKO mice exhibited reduced β-cell death and atrophy at those time points (Fig.5A).

Islet mass measurements revealed that islet development at 4 weeks post injection was reduced in WT compared to BKO (5B).
**Fig. 5.5.5**

Effect of S100B on islet size in STZ-treated mice. Pancreata were removed 4 weeks after STZ injection. Cross–sections of pancreas were then stained with H&E (A). Islet β cell mass was measured based on fraction of insulin positive area out of the total pancreatic areas multiplied by gross pancreatic mass at 4 weeks after STZ injection (B). The histograms are presented as mean ± SEM, n=4 mice in each group; p<0.05: * vs. WT, normalized to a value of 1.

*This figure illustrates the impact of S100B on islet size in STZ-treated mice. The images show cross-sections of pancreas stained with H&E, with two groups: WT (wild-type) and S100B KO (knockout). The bar graph compares the relative islet β-cell mass between WT and S100B KO groups, showing a significant difference at 4 weeks post-STZ injection.*
5.5.4 S100B Deficiency Protects Mice from Developing STZ-Induced Diabetes.

Immunohistochemistry combined with morphometric analysis revealed loss of insulin producing cells in WT at 2 weeks, which was increased at 4 weeks post injection. In contrast, integrated islets were observed in BKO even at 4 weeks post injection (Fig. 5.5.6A). Similarly, plasma insulin level was significantly reduced in WT at the end of study course compare to pre-injected BKO mice. However, this was associated with preservation of plasma insulin in BKO (Fig. 5.5.6B).

Plasma glucagon level was not altered in WT but it was reduced in post-injected BKO compared with what was observed in pre-injected animals (Fig. 5.5.6C).
Fig. 5.5.6

Detection of insulin in islets of STZ-treated BKO and WT mice and effect of S100B on plasma insulin and glucagon STZ-treated mice. Pancreata were removed 1, 2 and 4 weeks
after STZ injection and cross-sections were stained with insulin antibody (A). Plasma insulin (B) and glucagon (C) levels in WT and BKO mice before and 4 weeks after STZ treatment. The histograms are presented as mean ± SEM, n=4 mice in each group; p<0.05: † vs. untreated, * vs. WT.
5.5.5 S100B, GFAP and RAGE Expression in the Islets of Pancreas.

As assessed by immunohistochemistry, S100B expression was limited to the peri-islet Schwann cells (pSC) surrounding islets and disappeared concomitant with the progression of hyperglycemia in WT. This coincided with the appearance of S100B inside the islets, which did not co-localize with insulin expressing β cells (Fig. 5.5.7A,B). To examine possible expression of S100B from internalized pSC in the islets, GFAP staining as a marker for pSC was used. Results from immunohistochemistry and immunofluorescence revealed that there is less GFAP staining at 4 weeks in STZ treated WT or S100BKO islets indicating that most of the pSC have been lost by this time point (Fig. 5.5.7C & 5.5.7D).

RAGE expression was not significantly changed among the groups (Fig. 5.5.8). qRT-PCR results demonstrated very low level of S100B in MIN6 cells. In contrast there was a detectable level of RAGE expression in these cell types.
Detection of S100B in islets (A), colocalization of S100B and insulin (B), detection of GFAP in islets (C), and colocalization of S100B and GFAP (D) in vehicle or STZ-treated WT and BKO mice. Slides were stained with S100B (A) and with S100B and insulin (B) antibodies in WT mice before and after STZ injection. Insulin and S100B are shown in green and red, respectively. Then pancreas slides were stained with GFAP antibody in WT and BKO mice before and 4 weeks after STZ injection (C). S100B and GFAP proteins colocalization in the
islets of nondiabetic and diabetic WT mice (D). GFAP and S100B are shown in green and red, respectively. Pancreatic islet sections as evaluated by immunofluorescence microscopy. Arrows are included for orientation.

Fig. 5.5.8

Detection of RAGE in STZ-treated WT and BKO mice. Slides were stained with RAGE antibody in pancreatic islets of WT and BKO mice before, 1 and 4 weeks after STZ injection. Pancreatic islet sections as evaluated by immunofluorescence microscopy.
5.5.6 S100B Induces Apoptosis in Insulin Producing β Cells

To gain insight into the mechanisms and investigate whether S100B may induce β cells apoptosis, MIN6 cells were treated with two different concentrations of S100B (200 and 500 nM). Apoptosis was determined using Caspase -3 activity assay and flow cytometry. Caspase-3 activity was significantly induced by 500nM S100B compared with vehicle (Fig. 5.5.9A). Confirming these results, the proportion of Annexin V positive cells (apoptotic cells) was higher in S100B treated MIN6 compare to non-treated cells (Fig. 5.5.9B).
**Fig. 5.5.9**

Effect of S100B on insulin secreting cells; Caspase-3 activity and β cell apoptosis *in vitro.* Caspase-3 activity after 24 hour treatment of MIN6 cells with either 200nM or 500nM S100B (A). Relative % Annexin positive cells after 24 hours treatment of MIN6 cells with 500nM S100B (B). The histograms are presented as mean ± SEM, n=5; p<0.05: * vs. Control.
5.6 Discussion

Our study highlights physiological and pathophysiological roles for S100B in vivo as a regulator of insulin-producing cell growth and survival, insulin sensitivity, and the impact of STZ on these determinants of glucose metabolism. The main findings presented in chapter 5 are that 1) S100B deficient mice in response to STZ treatment exhibited less hyperglycemia and reduction in insulin levels, enhanced glucose tolerance and insulin sensitivity and less pancreatic β cells destruction; 2) S100B expression increased in non β or Schwann cells in the islets of STZ-induced diabetes; and 3) S100B induced β cells apoptosis in vitro.

It is well characterized that multiple low–dose injections of STZ result in severe hyperglycemia, insulitis, body weight loss, major glucose and insulin intolerance, and destruction of pancreatic β cells in vivo (177). It has been shown that disruption of certain genes such as glucagon, poly (ADP-ribose) polymerase and KATP channel-deficient β cells increase resistance to the development of hyperglycemia (61, 201, 357). In this study we demonstrated that absence of S100B significantly attenuated the adverse impact of STZ. Compared with WT, after low dose STZ injection BKO mice had normal fasting and non-fasting plasma glucose, normal food and water intake and urine output, no significant decrease in plasma insulin and no weight loss. Pancreatic islets appeared normal in BKO with preservation of insulin content. When challenged with multiple high doses of STZ, BKO mice developed an intermediate level of hyperglycemia, demonstrating that the STZ-resistance was dose-dependent. These results indicate that S100B expression appears to be a key factor involved in STZ-induced diabetes development.

Impaired insulin sensitivity is a key pathogenetic feature of type 2 diabetes. STZ-treated mice more closely resemble type 1 than type 2 diabetes because they are primarily insulin
deficient. However, several studies have shown STZ-treated animals demonstrate insulin resistance (167, 313, 316). Our data demonstrate a role for S100B in both insulin production and resistance.

Adipose tissue has already been highlighted as an important extra cerebral source of S100B (116). S100B secretion from adipocytes is reduced by insulin, and is regulated in response to glucagon and catecholamines via cAMP-mediated pathway (310). Further evidence for the involvement of S100B in dysfunction of adipose tissue suggests that S100B serum concentration is closely correlated with body mass index (BMI) as well as levels of leptin and adipocyte-type fatty acid binding protein (299). Adipose tissue alterations, particularly insulin resistance, appear to be involved in many diseases, including type 2 diabetes (195). Interestingly, insulin resistance in schizophrenic patients may be closely linked to serum S100B released from brain and adipose tissue as indicated by impaired insulin sensitivity and C-peptide/glucose ratios (300). In this study we showed that disrupting the S100B gene led to increased insulin sensitivity and better glucose tolerance with or without hyperglycemia as evidenced by the lower blood glucose levels and improved performance as determined by GTT, and ITT. Increased insulin sensitivity in pre STZ-injected BKO mice was paralleled by lower circulating insulin and greater glucagon levels. Glucagon is secreted into the portal blood supply and acts as a counterregulatory hormone to insulin (111). The relative hypoinsulinemia in BKO mice indicates that they are insulin hypersensitive and therefore require less systemic insulin to maintain euglycemia. This observation may also contribute to the protection against hyperglycemia following STZ administration.

Pancreatic Schwann cells marked by the expression of GFAP (315) and S100B could play an important role in the development of type 1 diabetes (354). GFAP and S100B immunotherapy delayed diabetes development in NOD mice. Animals were protected despite
showing strong T helper cells (TH1)-biased peri-insulitis along with the removal of pSC mantle and they were resistant to islet invasion and β cells apoptosis (54, 354). Our data indicated that in STZ treated WT mice; S100B expression persisted within the islets despite loss of pSC. Also we showed that β cells were not the source for S100B expression in these mice. Since the STZ model is also associated with immune system activation in later stages, infiltrating immune cells, both T and non T cells (54, 354), may contribute to S100B expression in islets in addition to secreted or released extracellular S100B from pSC. This is supported by the previous observation that transferring diabetogenic spleen cells into NOD/SCID mice caused an induction of S100B and its receptor RAGE expression in the islet cells as well as in a population of T cells and B cells (54).

In this study, the pSC structure was preserved in the BKO mice. It has been shown that S100B inhibits GFAP phosphorylation in response to cAMP or calcium/calmodulin stimulation (104). Since GFAP is a major cytoskeletal protein in glial cells and provides docking sites for several enzymes involved in the generation of different glial responses, blunting GFAP level results in diminished pSC destruction.

Effects of hyperglycemia include increases in free calcium levels in different tissues (174) as well as glucose–mediated increases in oxidative stress and inflammation (108). Intracellular S100B, works as an intermediate linkage between agents altering intracellular calcium levels and proteins generating cell responses. In addition to the intracellular role of S100B, it is secreted from the cells and, in nanomolar concentrations, behaves as a pro-survival factor whereas, in micromolar concentrations it behaves as a pro-apoptotic factor acting on glial cells and the neuronal system (166, 274). The results from the current study have demonstrated that BKO mice are resistant to STZ-induced β cell apoptosis and dysfunction. More importantly, significant increases of caspase-3 activity and Annexin binding in insulin
secrating cells in response to S100B treatment indicate that higher concentration of S100B induces β cell apoptosis. One possible mechanism to explain the deleterious effect of S100B on β cells may be the induction of cyclooxygenase-2 (PTGS2), which has been shown to occur in response to S100B treatment or diabetes in the pancreatic islet. PTGS2 modulates autoimmunity and thereby causes islet dysfunction (277). In addition, S100B stimulates generation of intracellular reactive oxygen species and nitric oxide (93, 278), which they may result in the induction of apoptotic signaling in the pancreatic β cells. Although we demonstrated that S100B induces β cell apoptosis, the potential implications of such pancreatic protective effects against STZ, a chemical toxin, on the development and treatment of diabetes require further investigation. The mechanism for such resistance to STZ remains unclear. It is possible that resistance of BKO mice to STZ may be at least in part mediated via increased circulating glucagon-like peptide-1 (GLP-1). This is consistent with the observation that GLP-1 treatment prevents STZ-induced apoptosis in INS-1 cell line in a dose dependent manner (247).

Chronic hyperglycemia contributes to diabetic complications, in part, through the formation of advanced glycation end products (AGE), which are irreversibly formed by non-enzymatic glycosylation and oxidation of proteins and lipids (234). AGE could exert their effects via the canonical receptor, receptor for AGE (RAGE). RAGE induces several intracellular signaling cascades in response to ligation with AGE/S100B, such as activation of inflammatory factors and generation of \( \text{O}_2^- \) in diabetes (77), which results in β cells apoptosis (186). Therefore, S100B may play a role in induction of a chain of events in the pancreatic β cells, including interaction with RAGE and induction of inflammatory responses. Interestingly, RAGE activation by AGE in cultures of adipocytes inhibited glucose uptake through the over generation of intracellular reactive oxygen species (330) and this could lead to insulin
resistance. While our data did not show significant differences in pancreatic RAGE expression between STZ treated BKO and WT mice, additional studies are needed to clarify the relationship of S100B and RAGE in the diabetic islets. We cannot exclude the possible effect of S100B on the hippocampus and neuronal system controlling the development of diabetes as hippocampal S100B level is significantly increased at an early time point after STZ injection in rat models (174). Additional studies are required to fully determine the possible linkage between S100B progression and complication of overt type 1 and type 2 diabetes. Crossings of S100B deficient mice and genetic models of type 1 and type 2 diabetes will be beneficial.
5.7 Conclusion

In conclusion, our studies demonstrated that BKO mice were resistant to STZ-induce diabetes and also demonstrated preserved glucose tolerance by preserving pancreatic β cell’s function to secret insulin and increased insulin sensitively. While elaboration of the cellular mechanisms require further studies, these observations imply that S100B can modify the course of diabetes, via pancreatic β cells apoptosis and possible regulation of insulin sensitivity. Our demonstration of resistance to STZ induced diabetes in BKO mice supports the idea that S100B can be used as a novel treatment target in diabetes.
CHAPTER 6

DISCUSSION/CONCLUSION
Basal expression of S100B is restricted to glial and neuronal cells and anomalously expressed in myocardium following MI. Forced expression of S100B, in non diabetes, prevents the development of hypertrophy and induces cardiac myocyte apoptosis resulting in modulation of LV remodeling. In addition, it has been implicated in diabetic neuronal and vascular complications. This thesis has been focused on understanding the role of S100B in post-MI cardiac function in diabetes as well as in the development and progression of diabetes. We have identified novel and specific determinants of S100B’s function and expression in the setting of diabetes.

Complete abrogation of S100B expression in diabetes resulted in significant dilation post-MI and worsening LV function. This result is completely dependent on the presence of diabetes as the previous data showing that post-MI BKO in the absence of diabetes actually exhibits a reduction in dilation compared with WT controls. Thus, this paradoxical effect of S100B in diabetes suggests that it must specifically regulate other diabetes-dependent pathways involved in cardiac function and LV remodeling.

Moreover, despite upregulation of S100B in MI or diabetes alone, diabetic myocardium post-MI exhibited attenuated S100B expression. This attenuation was associated with the noted differential LV remodeling. In human diabetic patients, clinical heart failure following infarction occurs in the absence of LV dilation, an observation paralleled in our mouse infarct model. By using Tau as measure of active ventricular relaxation parameter we were able to show that post-MI diabetic mice exhibited diastolic dysfunction and despite attenuated dilation, there was a comparable reduction in systolic performance. This was associated with increased myocardial apoptosis that may contribute to the dissociation between ventricular dilation and functional impact.

As it was discussed in chapter 4, increased fibrosis in post-MI diabetes could play an
important role in limiting ventricular dilation and increase a diastolic stiffness. Since knocking out S100B did not alter collagen content or cardiomyocyte hypertrophy compared to WT or BTG, we can speculate that S100B does not primarily regulate fibrosis and alternate mechanisms such as AGE crosslinking of collagen may contribute to altered properties of infarcted and scarred myocardium. Indeed, higher circulating AGE levels and greater phosphorylation of ERK 1/2 were seen in diabetic BKO post-MI. Normally, ventricular enlargement is the best predictor of adverse outcomes following myocardial infarction in patients. The greater LV enlargement associated with worse cardiac dysfunction in post-MI diabetic BKO suggest that in diabetes, S100B may contribute to the attenuated ventricular dilation and potentially serves a protective role by regulation of distinct signalling pathways, including AGE, alternate S100 proteins including S100A1, and cardiac metabolism.

Ventricular dysfunction in STZ induced hyperglycemic BKO mice was accompanied by a reduction of GLUT4 translation in the heart, suggesting that insulin-stimulated GLUT4 signaling was impaired. The in vivo animal and human studies have clearly shown that diabetes and insulin resistance aggravate myocardial ischemic injury (144). Our study provided in vivo evidence to show that diabetes in the absence of S100B results in further impairment of myocardial function following MI and a role for S100B in glucose handling may contribute to this phenotype. Our data presented in Chapter 5 on the role of S100B in the development of diabetes and insulin resistance support this hypothesis.

Adipose tissue alterations result in insulin resistance. Although the direct effect of S100B on the release of fatty acid has not been shown, it has been demonstrated that releasing S100B from adipocytes is controlled under conditions in which the fatty acids being produced are not released promptly and accumulate in the cells. The in vitro hormonal changes induced in
the adipose tissue, under stressing conditions, suggest that S100B release from adipocytes might be linked to lipolysis, but definitive evidence is lacking. Evidence for the involvement of S100B in dysfunction of adipose tissue is the correlation of S100B serum concentrations with body mass index (BMI) as well as levels of leptin and adipocyte-type fatty acid binding protein (299).

Moreover, reduced S100B protein expression in diabetic hearts post-MI could reflect systemic disturbances in cellular energy supply rather than cardiac specific pathologies. Based on protein-binding assays, at least three putative targets of S100B have been implicated in energy metabolism: phosphoglucomutase (171), fructose-1,6-bisphosphate aldolase (368) and glyceraldehyde 3-phosphate dehydrogenase (366). Together, these effects lead to an increase in the glycolytic pathway as it has been shown in adipocytes (116). Therefore, upregulation of S100B following MI may increase intracellular energy supply by activating glycolysis. In addition, accumulation of glycolytic products such as lactate and H⁺ may contribute to myocardial cell injury (219). Decreasing glycolysis by depleting the myocardial glycogen pool prior to ischemia results in a decrease in lactate accumulation during ischemia. This may explain the paradoxical finding in the previous studies demonstrating that knocking out S100B in the absence of diabetes augmented hypertrophy, decreased apoptosis and preserved cardiac function following myocardial infarction.

RAGE plays an important role in diabetic complications. Despite alteration of S100B in MI or diabetic MI, our results demonstrated sustained RAGE upregulation. This suggests that the effect of RAGE activation may be ligand-dependent; that is, AGE or S100B does not necessary induce the same response. The diversity of effects induced by RAGE activation is not only cell-specific, but also depends on the oligomeric organization of the ligand, as well as RAGE oligomerization.
In addition, increased AGE levels in diabetic BKO post MI indicate a reciprocal relationship between these ligands and that AGE/RAGE may also differentially impact cardiac metabolism. RAGE activation by AGE in cultures of adipocytes inhibited glucose uptake through the over generation of intracellular reactive oxygen species and may result in insulin resistance (330). How S100B could regulate AGE is not clear. The other possibility is that sRAGE, the natural scavenger of RAGE ligands, could be altered depending on the presence of ligands (S100B, S100A1, AGE), diabetes or injury.

Despite increased insulin sensitivity and better glucose tolerance as shown in chapter 5, we observed decreased GLUT4 expression and augmented AGE levels in diabetic post-MI myocardium in S100B KO. Thus S100B appears to have tissue-specific mechanisms that regulate both development of diabetes, at least in response to STZ, and the development of diabetic complications, not only in vasculature, but also in myocardium.

In conclusion the work presented in this thesis provides novel evidence for a role for S100B in diabetes and its complications. As a component of S100B action is related to ligand-receptor interaction, this signaling pathway is amenable to therapeutic manipulation that could provide the basis for treatment of diabetes and its cardiovascular complications. A schematic diagram of a proposed model for the role of S100B in the regulation of cardiac metabolism, function and structure in diabetes following MI is shown in figure 6.1.
Lack of S100B leads to cardiomyocyte dilation and dysfunction. Previously known pathways are shown in black. The novel pathways that my thesis has contributed are shown in red.

We demonstrated that absence of S100B in diabetic post-MI myocardium resulted in increased ventricular dilation, worsening of cardiac function and reduction of GLUT4 mRNA levels. Blunting GLUT4 expression can reduce glucose uptake, glycolysis, ATP production and cardiac contractility. Therefore, absence of S100B may have an adverse effect on cardiac function by disturbing cardiac metabolism.

Moreover, we showed that absence of S100B caused increased AGE production. Crosslinking of AGE with SERCA2 reduces cardiac relaxation via blunting Ca2+ transit. Also, higher levels of AGE may increase insulin resistance via RAGE ligation and alters cardiac glycolysis.

Figure 6.1. Schematic representation of proposed model that S100B regulates cardiac metabolism, function and structure in diabetes following MI. Previously known pathways are shown in black. The novel pathways that my thesis has contributed are shown in red. We demonstrated that absence of S100B in diabetic post-MI myocardium resulted in increased ventricular dilation, worsening of cardiac function and reduction of GLUT4 mRNA levels. Blunting GLUT4 expression can reduce glucose uptake, glycolysis, ATP production and cardiac contractility. Therefore, absence of S100B may have an adverse effect on cardiac function by disturbing cardiac metabolism. Moreover, we showed that absence of S100B caused increased AGE production. Crosslinking of AGE with SERCA2 reduces cardiac relaxation via blunting Ca2+ transit. Also, higher levels of AGE may increase insulin resistance via RAGE ligation and alters cardiac glycolysis.
CHAPTER 7

FUTURE DIRECTIONS AND LIMITATIONS
The pathogenesis of diabetes involves a complex cascade of events that result in long-term damage, dysfunction and failure of various organs, such as heart. There are numerous animal models to study the impact of diabetes. One of the most widely studied animal models uses STZ as a specific chemical toxic for the pancreatic β cells. We recognize that use of STZ may introduce nonspecific toxicity; therefore we tried to limit the toxic effect by multiple low dose injection. Although, we recapitulated some of our observations in the Akita mouse as a type 2 diabetic model, it would be valuable to characterize the impact of S100B in the development of diabetes and post-MI heart by crossing a genetic diabetic model and BKO mice. This would provide reassurance that observations presented in this thesis have not been contaminated with interplay between S100B and the toxic effect of STZ.

At present, RAGE is the only well characterized receptor for S100B. However, S100B may interact with an unidentified receptor as shown in myoblast cell apoptosis (293). One candidate for a non-RAGE receptor S100B interaction is the dopamine receptor, widely distributed in the central nervous system and another potential receptor for S100B ligation is TLR4 (297, 331). Thus, possible extracellular effects of S100B in diabetes, independent of RAGE, have not been assessed. The impact of diabetes on these alternate receptors and their role post-MI are similarly open to additional experimental investigation.

It has been shown that mouse sRAGE is produced by a mechanism distinct from human sRAGE, which is produced by alternative splicing. The carboxyl-terminal proteolysis of RAGE appeared to be the likely mechanism of sRAGE formation in the mouse. Matrix metalloproteinase-9 has been found to cause murine pulmonary epithelial cells to shed sRAGE into the culture medium (26), implicating this proteinase in sRAGE production from RAGE. Therefore, in our study, measurement of RAGE mRNA does not indicate the total protein level of RAGE. In addition, negative correlation between S100B and sRAGE in
schizophrenia demonstrates a possible self-limiting regulatory mechanism contributing to a normalization of elevated serum S100B levels. Further studies determining serum sRAGE levels in WT and BKO post-MI diabetic animals may provide better understanding of RAGE alteration and its link with the ligands (S100B, AGE and S100A1) in diabetic alone and post-MI diabetic conditions.

We and other studies have shown S100A1 expression was altered in diabetes (365). S100A1, as a major S100 isoform in normal adult myocardial, showed differential expression in response to STZ induced diabetes, MI alone and MI associated with diabetes. While S100A1 was counter-regulated in comparison to S100B, its differential regulation in diabetes versus non-diabetes provides additional evidence for distinct regulation of this multi-genic family of proteins and there associated pathways in the setting of diabetes. S100A1 has been implicated in the regulation of cardiac muscle Ca\(^{2+}\) homeostasis and contractility but its role in diabetes has not been defined. Future studies investigating the functional role of S100A1 under diabetic conditions and post-MI would be valuable to test the link between S100A1 and S100B and their possible competition for RAGE ligation. Moreover S100B and S100A1 regulate enzymes implicated in energy metabolism (81). Finding the regulation of S100A1 in diabetes and possible effect of that on cardiac function potentially due to cardiac energy metabolism regulation may open new routes of investigation in the field of S100 proteins.

Regulation of gene expression by free fatty acid has a significant impact on the development of insulin resistance and its related pathophysiology. Beyond their obvious role in metabolism, they can modulate gene transcription, attenuate insulin signaling and GLUT4 translocation (361). It has been suggested that S100B could serve as a carrier protein of fatty acid. So far, however no studies have investigated a biochemical interaction between S100B and fatty acids. Future studies determining the concentration of free fatty acids in the BKO
and WT hearts or functional studies of additional alterations in cardiac metabolism would elucidate the suggested link between S100B, fatty acids and energy metabolism.

Alteration of insulin–regulated glucose transporter GLUT4 in the heart was an observation in this present study. It is mainly expressed in insulin-responsive tissues such as adipose (342), and in heart and skeletal muscles (2, 370), where it mediates glucose uptake in response to acute insulin stimulation. Therefore, it would be valuable to study the molecular processes regulating glucose homeostasis and understand the potential effect of S100B associated with the GLUT4 transcriptional machinery, aiming at improving insulin sensitivity. Although GLUT4 mRNA is a helpful indication of myocardial insulin resistance, it is widely believed that insulin resistance in the myocardium, skeletal muscle and adipose tissue, is primarily associated with a down-regulation of GLUT4 translocation to the plasma membrane (50, 173, 223). Nonetheless, previous studies have also shown that diabetes is also associated with a reduction in GLUT4 protein content (40). In our studies we demonstrated that diabetic BKO following MI was associated with a reduction in GLUT4 mRNA levels in the myocardium. However, whether or not the reduction in GLUT4 mRNA following MI, resulted in similar changes in GLUT4 protein levels were not investigated. With respect to normal mice, quantification of the intracellular GLUT4 protein content and their ability to translocate to the plasma membrane would have been extremely helpful in determining whether or not S100B exerted any additional benefits on myocardial glucose utilization following MI. Also, quantification of GLUT4 protein levels and their translocation potential in cardiac tissue would have been useful in assessing the potential metabolic properties of S100B in the heart. Since there is strong evidence that S100B serum levels correlate with BMI and leptin, this topic may be explored in further detail by future studies on metabolic parameters such as BMI, glucose and insulin levels and their correlation with the level of S100B in the serum of
diabetic patients with cardiovascular disease. Moreover, cardiac glucose uptake after ischemia reperfusion is reduced in diabetes (90). Therefore it would be of interest to use FDG PET-CT method to quantify cardiac glucose metabolism in the post-MI diabetes in the absence of S100B.

Lastly, the data from this thesis demonstrate that S100B plays a paradoxical role in diabetic versus non-diabetic post-MI remodeling and a novel role in development of experimental diabetes overall. Conditionally, knocking down of S100B by using a Cre/loxp strategy in cardiac myocytes, Schwann cells, adipocytes, and the vasculature would enable us to distinguish the tissue-specific role of S100B in diabetes and its complications. They would also facilitate further understanding of the molecular mechanisms by which S100B impacts on these diverse phenotypes.
CHAPTER 9

REFERENCES


Carmeliet P. Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. NatMed 5: 1135-1142, 1999.


172. **Lander HM, Tauras JM, Ogiste JS, Hori O, Moss RA, and Schmidt AM.** Activation of the receptor for advanced glycation end products triggers a p21(ras)-dependent


glucose or ligands of the advanced glycation endproduct-specific receptor (AGER), and in islets from diabetic mice. *Diabetologia* 49: 100-107, 2006.


