# NOVEL MECHANISMS UNDERLYING

# DIABETES-INDUCED ATRIAL FIBRILLATION

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Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 2016

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# ACKNOWLEDGEMENTS

I would first like to thank the Almighty for giving me the patience and strength throughout the course of this degree.

I am immensely grateful to my adviser Dr. Véronique Lacombe for believing in me and for being a constant guardian every step of the way. I thank her for her support, enthusiasm, and patience.

I would like to thank my husband, Shahan for his constant faith and unwavering support till the end. I am grateful to my parents, Kazi and Azizun and my brother Murtaza, who have always encouraged me, prayed for me, and taught me that if you try hard enough nothing is impossible.

I would like to thank Dr. Ali Kaan Kalkan, Dr. Pamela Lloyd, and Dr. Arvind Santhanakrishnan for serving in my doctoral committee and providing me with their valuable feedback.

I have been extremely lucky to have Allison Campolo, Shannel Shoop, Alia Houser and Ellen Jackson as my colleagues and friends, as they have created an excellent environment to work in. I would also like to thank them for their technical expertise and their support during tight deadlines. I thank Dr. Emilie Martinez for familiarizing me with the specialized techniques when I first started my research. I thank Jill Murray for providing me with her veterinary expertise and recommendations. I would also like to thank my friends Kalyani, Harshini, Farzana and Sadia for their support during the time of my research. Lastly I want to take this opportunity to thank everyone who have helped and inspired my during my PhD.

Name: ZAHRA MARIA

Date of Degree: JULY, 2016

Title of Study: NOVEL MECHANISMS UNDERLYING DIABETES-INDUCED ATRIAL FIBRILLATION

Major Field: MECHANICAL ENGINEERING

Abstract: Diabetes results from a defect in insulin production (type-1) or action (type-2) causing dysfunctional glucose uptake. Diabetic patients are predisposed to cardiac complications such as atrial fibrillation (AF). The heart is a major organ to utilize glucose; however, little is known about atrial glucose metabolism. Glucose transport into the cell via Glucose Transporters (GLUTs) is the rate-limiting step of glucose utilization. Although GLUT4 is the major cardiac isoform, GLUT8 has emerged as a novel isoform. Atrial structural remodeling and advanced glycation end products (AGEs) have been suggested as precursors to AF. However, glucose and insulin disturbances during diabetes may directly affect atrial function, potentially leading to AF. We hypothesize that the dysregulation of glucose metabolism in the diabetic atria underlies diabetes-induced AF. The specific aims of this dissertation are to test the hypothesis that: 1) GLUT-4 and -8 translocation to the atrial cell surface is insulin regulated and is impaired during insulin-dependent diabetes; 2) GLUT-4 and -8 translocation will be impaired during insulin-resistance-induced AF; and 3) impaired glucose metabolism resulting from insulin dysregulation provides a metabolic substrate for the development of AF. GLUT protein expression was measured in atrial myocytes of healthy rats, in the atrial tissue of insulin-deficient (streptozotocin-induced) and insulin-resistant (high-fat-diet-induced) mice. Active cell surface GLUT content was measured using the biotinylated photolabeled assay in the perfused heart. AF was induced by transesophageal atrial pacing. Atrial fibrosis and AGE were measured using histological analysis. We reported that both GLUT4 and GLUT8 are insulin sensitive in the healthy atria and are regulated by the insulin signaling pathway (i.e., Akt/AS160). Our data demonstrated that GLUT translocation is downregulated in the atria during insulin dysregulation. In addition, insulin treatment rescued GLUT translocation to the atrial cell surface during insulin deficiency but not during insulin resistance. We reported the absence of atrial fibrosis and AGE accumulation in animals with insulin dysregulation. Our results also indicated that insulin dysregulation significantly increases the vulnerability to AF. Overall, the results suggest that in the absence of atrial structural remodeling, insulin dysregulation may induce perturbations in energy production which could underlie the development of diabetes-induced AF.

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# CHAPTER I

## INTRODUCTION

Diabetes mellitus is a serious metabolic disorder affecting 350 million people worldwide.[1] Diabetes has now reached epidemic levels and has been identified as the 7th leading cause of death in the USA.[2;3] Hyperglycemia, the hallmark of diabetes, results from an impaired glucose uptake due to either a lack of insulin production by pancreatic beta cells (type 1) or cellular insulin resistance (type 2). Diabetes can lead to multiple organ dysfunction including cardiomyopathy, coronary artery disease and atrial fibrillation (AF).[4] Atrial fibrillation (AF), the most common sustained cardiac arrhythmia in the world, has been identified as an increasing public health problem as well.[5] Approximately 2% of the entire world population suffers from AF. In the United States 3 million people are currently affected and it is predicted to increase to 8 million by 2050. It is also a 26 billion dollar economic burden in the US.[6] AF highly increases the risk factors for heart failure, ischemic heart disease and stroke.[7-10]

The development of AF is thought to be multifactorial, including structural and electrical remodeling.[11] AF also increases energy demand, which is characterized by increased ratios of AMP and ADP/ATP and elevated energy expenditure.[12] Importantly,

diabetes has recently been identified as a major and independent risk factor of AF.[13] Indeed, the risk factor of a diabetic patient to develop AF is 1.4-2.2 compared to a healthy non diabetic person.[14] However, the underlying pathophysiologic mechanisms of AF are not well understood. In addition to structural-electrical remodeling, glucose and insulin disturbances may also directly affect the function of the atrium potentially leading to AF.[13] However, it is unknown whether a metabolic substrate underlies AF. In support of this novel concept, it has been shown that blood glucose level is significantly elevated during an AF attacks.[5;15;16] It has also been reported that the risk of developing AF increases approximately 3% for each additional year of the duration of diabetes.[17] Therefore, we hypothesize that diabetes modulates the progression of AF and that alterations in glucose metabolism during diabetes could be a major contributing factor to AF.[14;18;19]

Since all cardiac myocytes contract for each heartbeat (with an estimated 1 billion heartbeats through the lifespan of most mammals), the energetic demands in the heart are extreme. In order to sustain this high energy demand, the rate of glucose utilization in the heart is greater than in skeletal muscle, adipose tissue and lung, despite the ability of the myocardium to use other substrates (i.e., fatty acids, lactate, ketone bodies and amino acids).[20] During physiological condition, the heart generates ~30% of its total energy from glucose oxidation.[21] Therefore, cardiac glucose uptake and utilization is crucial for proper cardiac function. This is germane to the fact that the atria, which contains the pacemaker of the heart, significantly contributes to the overall cardiac function. Although the rate of glucose utilization in the heart is greater than in other tissue, little is known about glucose metabolism in the atria during both healthy and disease states.[22] Dysfunctional glucose

uptake in the cardiac tissue during diabetes could be one of the main underlying pathophysiological substrates that connects diabetes and atrial fibrillation.

Glucose entry from the blood stream into cardiac myocytes is the rate-limiting step in glucose utilization and it is tightly regulated by a specialized family of membrane transporters known as glucose transporters (GLUT).[23] GLUT4 has been identified as the major insulin sensitive cardiac isoform (~70% of total myocardium GLUTs).[24] Although GLUT4 has been studied extensively in the striated muscle and adipose tissue, there is no study that reports the role and regulation of GLUT4 trafficking in the atria. Although GLUT4 is the major isoform in the heart, in a study conducted by Katz *et al*., it has been reported that GLUT4 knockout mice do not develop hyperglycemia, suggesting that other GLUT isoforms could be involved in the regulation of whole-body glucose homeostasis.[25] Recently, GLUT8 has emerged as a novel isoform for glucose transport in striated muscle and adipose tissue.[23;26-30] Although GLUT8 has been reported to be an insulin-dependent isoform in blastocysts,[31] its functional role in the myocardium is yet to be determined. Although the downstream insulin signaling pathway has been studied in the ventricle, it is yet to be characterized in the atria. The majority of the studies that have investigated cardiac energetics have studied the global or left ventricular changes.[18;32-37] Therefore, the role of glucose uptake and utilization in the atria has received little attention. Specifically, there has been little to no studies that focus on glucose transport across the atria.

Chronic hyperglycemia, the landmark of diabetes, accelerates the reaction between glucose and proteins that leads to the formation of advanced glycation end products (AGEs), which form irreversible cross-links throughout the lifetime of many large proteins (such as collagen and hemoglobin), covalently modifying their structure and function, starting at 8-12

weeks after the onset.[38-43] During diabetes and to a lesser extent during aging, AGEs accumulate at an accelerated rate in various cell types and produce multiple organ dysfunctions.[39;44] At organ level, AGEs structurally stiffen collagen backbones and further stimulate collagen deposition (by acting as agonists for AGE receptors), which ultimately leads to increased tissue fibrosis.[39;40;44-46] In the heart, AGE accumulation contributes to diastolic dysfunction, by inducing myocardial fibrosis and stiffness.[40;45-49] In addition, AGE activation by receptor binding to RAGE leads to the enhanced expression of pro-fibrotic markers and therefore can possibly contribute to myocardial fibrosis. However, its role in the development and progression of AF remains unclear. It has been reported that activation of AGE-RAGE axis leads to the cleavage of cell bound RAGEs and produce soluble RAGE (sRAGE). Increased plasma levels AGE and sRAGE have been reported in patients with AF.[50;51] However, whether AGEs accumulation during diabetes is the cause or simply correlate with the incidence of AF needs to be further investigated.

The major goal of this study is to identify a novel metabolic substrate that underlies the pathophysiology of diabetes-induced atrial fibrillation. In order to achieve this goal, we will use healthy, type 1 and type 2 diabetic rodent models to characterize the role and regulation of insulin sensitive GLUTs during diabetes and diabetes-induced atrial fibrillation. In addition, we will investigate the mechanisms by which diabetes could induce possible metabolic and structural changes that render the diabetic atria vulnerable to atrial fibrillation. Insights gained from this study could lead to the identification of novel metabolic therapeutic targets for patients affected by diabetes and/or AF.

***Central Hypothesis:*** Impaired glucose metabolism during diabetes will provide a metabolic arrhythmogenic substrate which underlies the development and maintenance of AF.

**Specific Aim 1:** To test the hypothesis that GLUT-4 and -8 translocation to the atrial cell surface will be regulated by insulin and impaired during insulin-dependent diabetes.

**Specific Aim 2:** To test the hypothesis that GLUT-4 and -8 translocation to the atrial cell surface will be impaired during insulin-resistance-induced AF.

**Specific Aim 3:** To test the hypothesis that impaired glucose metabolism resulting from insulin dysregulation provides a metabolic substrate for the development and maintenance of atrial fibrillation.

# CHAPTER II

# REVIEW OF LITERATURE

## 2.1. Diabetes

Diabetes mellitus affects almost 18.1 million people in the United States and accounts for 225,000 deaths per year.[2] According to The Centers for Disease Control and Prevention, 40 million people have been diagnosed with pre-diabetes with 2 to 4 million new cases occurring every year.[52] A diabetic condition is defined as the presence of an elevated plasma glucose concentration known as hyperglycemia.[53] In a healthy vasculature, during fasting conditions, the plasma glucose concentration is 80-140 mg/dL. In humans, a diabetic condition is diagnosed either if the fasting glucose level exceeds 140 mg/dL or if the glucose level is higher than 200 mg/dL two hours after the intake of 75g of oral glucose.[4] To understand the pathogenesis of diabetes it is important to understand the mechanisms for glucose homeostasis in the normal vasculature during the absorptive and the post-absorptive state.[2] During the absorptive state (after a meal), the ingested nutrients enter the blood from the gastrointestinal tract, whereas during the post-absorptive state (such as 10-12 hours overnight fast) the gastrointestinal tract is

empty and energy must be supplied from the glucose stores of the body, such as the liver and kidney.[54] These two organs are responsible for the production of endogenous glucose during the post-absorptive state. The rate of use of basal glucose (2.0mg/kg/min) in the body is exactly matched by the endogenous glucose production by the liver (85%) and the kidney (15%), and therefore maintains glucose homeostasis.[4]

During the absorptive state, the plasma glucose concentration gradually increases, the beta cells in the islets of Langerhans in the pancreas are sensitive to this alteration in glucose concentration and produce the hormone insulin.[54] The presence of insulin inhibits endogenous glucose production and facilitates the uptake of glucose into insulin sensitive tissues, such as striated muscle tissue (80-85% of the total glucose uptake), the liver and adipose tissue (where the glucose is stored as glycogen and triglycerides to be used during the post-absorptive state).[4] When tissues absorb glucose from the blood stream, the plasma glucose concentration decreases, causing a down-regulation in insulin production. It is important to note that the increase in plasma concentration of insulin is one of the major causes of all absorptive state events, and the decreased plasma concentration of insulin is the major cause of all post-absorptive state events.[4] However, in diabetes pathogenesis, insulin is either insufficient in quantity or functionally unable to facilitate the glucose transport into the tissue. Thus, based on the exact condition and the amount of insulin present in the blood, diabetes can be classified into two groups: insulin-dependent diabetes mellitus or type 1 diabetes and non-insulin dependent diabetes mellitus or type 2 diabetes. In type 1 diabetes, the islets of Langerhans do not produce sufficient insulin and thus insulin is largely absent in the plasma. In type 2 diabetes, insulin is present at near normal or above normal level in the plasma (caused by the

diseased state), but becomes ineffective in the normally insulin sensitive tissues due to constant exposure, resulting in insulin resistance. [54]

### 2.1.1 Type I Diabetes

Type 1 diabetes (T1Dx) is characterized by the body's inability to produce insulin due to immunological disruption of pancreatic beta cells.[55-58] Apart from sudden hyperglycemia, rising HbA1c levels and an impaired fasting glucose tolerance are also hallmarks of type-1 diabetes. During this disease the plasma blood glucose level rises above 300mg/dL and the subjects are severely dependent on external insulin for survival.[59] T1Dx constitutes of 5-10% of the total diabetic population.[60;61] In the United States approximately 1.25 million people are suffering from T1Dx, and approximately 40,000 new patients are diagnosed every year. This disease has also been associated with an estimated loss of life expectancy of 13 years.[60-62]

T1Dx is also known as juvenile diabetes as it is mostly diagnosed during childhood or early adulthood with a sudden and severe presentation.[62-64] 50-60% of those diagnosed with T1Dx are younger than 16-18 years and this disease occurs at low incidence during early adulthood (20-25 years).[63] Early symptoms of T1Dx include polyuria (excessive urination), polydipsia (increased thirst), xerostomia (dry mouth), polyphagia (increased hunger), fatigue, and weight loss.[65]

T1Dx is thought to be caused by a combination of genetic susceptibility and triggering environmental agents. Although there is yet to be determined a gene that is either necessary or sufficient to predict the development of T1Dx, the Human Leukocyte Antigen (HLA) complex has been identified to have the greatest contribution

(approximately 60%) to the disease. Among the three classes of the HLA gene, class II has been reported to have the strongest association with T1Dx. These genes take part in the immune response by participating in the presentation of beta cells' antigen peptides (either by promoting anti-self-reactivity or by the failure to regulate immune response).[66-69] Despite two decades of intensive research, the environmental factors that induce the pathogenesis of T1Dx are still debated. Some of the proposed factors include viral infection, early infant diet (breast feeding vs. early introduction to cow's milk components), toxins, and psychological stress. However, there is yet to be a firm conclusion.[70-75]

T1Dx is characterized by hyperglycemia (>350mg/dL) due to the lack of insulin production caused by the immune-mediated destruction of the pancreatic beta cells.[76;77] At the time of clinical diagnosis of T1Dx approximately 80% of the beta cell is destroyed, and the islet is affected by insulitis (infiltration of chronic inflammatory T cells, B cells and macrophages).[78;79] The first signs of T1Dx are the presentation of islets auto antibodies and their antigens which are reactive to the islet cells such as glutamic acid decarboxylase (GADAs), insulin (IAA), and tyrosine phosphatase (IA2As).[80] These autoantibodies are the earliest markers towards the autoimmunity of beta cells and precursor to beta cell destruction. Beta cell destruction (i.e. loss of beta cell mass) has been non-linearly correlated with the development of islet autoantibodies.[81] It is also believed that beta cell destruction during T1Dx is T-cell mediated.[58] It is thought that the beta cells themselves produce the beta cell antigens due to cellular turnover or damage. These antigens are processed and presented to the helper T cells by antigen presenting cells, namely macrophages and dendritic cells. The helper T cells recognize the beta cell

antigen peptides by the MHC (major histocompatibility complex) molecules on the macrophages and dendritic cells that have infiltrated the islet and become activated. Once activated the T cells penetrate the islets and differentiate into cytotoxic T cells. These activated T cells also release pro-inflammatory cytokines such as TNF-α (tumor necrosis factor-α), IL-1β (interleukin-1β), IFN-γ (interferon- γ) and soluble free radicals $O_2$, NO, and $H_2O_2$.[57;76;82-89] These cytokines act in synergy during the inflammation of pancreatic beta cells and eventually lead to the activation of NF-κB followed by the destruction of the pancreatic beta cells, which are highly susceptible to cytokine mediated killing.[76] The increases in soluble free radicals also increase the oxidative stress and add to the beta cell damage.[90-93] In this manner, macrophages, T cells, cytokines, and oxidative stress synergistically act on beta cell destruction leading to the development of autoimmune T1Dx. Once beta cell destruction has begun its progression, it can be quite variable depending on the subject's age, body mass index physical activity levels, etc.[94]

Long term T1Dx has been associated with both micro- and macro-vascular complications.[95] Some of the most common microvascular complications include diabetic nephropathy, microalbuminuria and diabetic retinopathy.[96-98] In the context of macrovascular disease, it has been reported that T1Dx can increase the relative risk factor for cardiovascular diseases such as hypertension, peripheral vascular disease, coronary artery disease and atherosclerosis compared to non-diabetic patients.[99-102] T1Dx has also been associated with reduced vascular repair, impaired endothelium dependent vasodilation, impaired wound healing, endothelial dysfunction, and reduction in collateral vessel formation during ischemia.[103-108] Cardiac dysfunction such as impairment in left ventricular function, has been reported in young T1Dx patients with diabetic

nephropathy.[109] So far it has been suggested that hyperglycemia and increased reactive oxidative stress during T1Dx could be the common cause of the cardiovascular disease associated with T1Dx.[110-112]

### 2.1.2 Type II Diabetes

In type 2 diabetes mellitus (T2Dx), as the fasting plasma glucose rises from 80 to 140 mg/dL, the fasting plasma insulin concentration rises progressively, reaching a peak that is 2-2.5 times the comparative levels found in non-diabetic controls.[4] However, even this rise in plasma insulin cannot offset the deterioration in glucose homeostasis, and the plasma glucose concentration continues to rise. As the fasting plasma glucose concentration exceeds 160 mg/dL, the pancreatic beta cells become unable to maintain the elevated rate of insulin secretion and the insulin concentration starts to decline.[113] As the insulin concentration begins to decrease, the liver senses this change in concentration and begins the production of endogenous glucose, resulting in an overall increase in the plasma glucose concentration.[113] Finally, as the fasting glucose concentration exceeds 200-220 mg/dL, the plasma insulin response to the glucose is markedly diminished resulting in a prolonged hyperglycemic condition.[114]

It has been well documented that patients with T2Dx cannot elicit a normal glucose metabolic response even with maximum stimulation of plasma insulin concentration.[2] This may be due to insulin resistance in the insulin sensitive tissues, loss of glucose sensitivity, reduction in mass or enhanced apoptosis of the pancreatic beta cells.[114] The number of beta cells in the pancreas is an important determinant of the total amount of insulin secreted. Several studies have reported a significant reduction in beta

cell mass (20-50%) and an approximate three-fold increase in beta cell apoptosis.[115] It seems likely that the loss of beta cell mass and functionality could contribute to the impairment of insulin secretion during T2Dx. It has been documented that, during the disease conditions, the of insulin sensitivity cause the liver to continue the production of glucose, even in the presence of insulin in the absorptive state.[4] As a result, two factors simultaneously contribute to the increase in plasma glucose concentration: 1) ingested glucose and 2) the endogenously produced glucose, causing the prolonged hyperglycemia in T2Dx.

The sustained hyperglycemia, caused by the ineffectiveness or the lack of sufficient insulin, induces other disease conditions and dysfunctions in the vasculature, such as intracellular accumulation of certain glucose metabolites (such as advance glycation end products), endothelial and mitochondrial dysfunction, increase an in oxidative stress and the possibility of glucose cross-link formation with proteins (for more details see 2.3.3).[38;116] As albumin is the most abundant plasma protein with a low turnover rate, it is very susceptible to such cross-link formation and protein glycosylations. As a result, patients with T2Dx become more susceptible to atherosclerosis, kidney failure, small vessel and nerve disease, infection and blindness.

### 2.1.3 Cardiac Complications Associated with Diabetes Mellitus

Diabetes Mellitus significantly increases the risk factors for cardiac complication and cardiovascular disease (CVDs). The American Heart Association identified diabetes as one of the 7 major risk factors for CVDs. It has been reported that risk of mortality with some form of cardiac disease is 2-4 times higher in a diabetic patient compared to a

non-diabetic person. The diabetic vasculature differs vastly from the normal vasculature but interestingly, resembles the pathologies of many CVDs.[117] The mechanisms for this are only partially understood. However, few of the major risk factors relating both of the disease conditions include hypertension, high cholesterol, obesity, high blood sugar, and smoking.[38] Some of the most common cardiac complications associated with diabetes are coronary artery disease, ischemic heart disease, diabetic cardiomyopathy and atrial fibrillation.[2]

## 2.2 Atrial Fibrillation

Atrial fibrillation is the most common and sustained cardiac arrhythmia in the world. Approximately 2% of the entire world population suffers from atrial fibrillation (AF). It is predicted that, the affected population will reach 16 million in the United States by the year 2016.[118] The onset of atrial fibrillation has been attributed to disorganized and irregular electrical impulses originating in the left atria, thus initiating electrical impulses from the left atria to the right (reverse of the physiological signal path) and causing initiation and perpetuation of reentry circuits in the right atria. These multiple circuits eventually induce chaotic rhythm in the atria, known as AF.[119;120]

Atrial fibrillation is characterized by 3 stages: paroxysmal, persistent, and permanent. The paroxysmal state of AF is a silent state; it is self-terminated within 48 hours and is non-symptomatic. Approximately 15% of patients with paroxysmal AF are predicted to progress to a more persistent state, where AF sustains for at least 7 days and requires cardioversion. In most cases, this eventually leads to a more permanent state of AF where sinus rhythm is completely absent.[118] Permanent AF is a condition in which

sinus rhythm cannot be restored with treatment.[121] The mechanisms involved in the progression of paroxysmal-AF to the more persistent states are highly variable among individual subjects and are not well understood. It has been reported that chronic atrial pacing in dogs and goats results in the development of sustained AF.[122]

There has been ongoing research during the last 50 years to understand the underlying mechanisms that promote AF.[123-126] There have been three proposed models: 1) ectopic foci 2) single circuit reentry and 3) multiple circuit reentry.[127-131] In the past two decades, scientists have come to a consensus that AF is the result of a combination of all the three models. The ectopic foci (possibly located near the pulmonary veins of the left atrium)[127] can initiate or drive AF by rapid firing.[128] This rapid firing of the ectopic foci (which is at a regular rhythm faster or slower than firing of the SA node) can cause fibrillary activity as it disrupts the conduction of the wave generated at the sinoatrial node (SA node) of the right atrium.[130] However, if the ectopic firing rate is slower than the SA node firing rate, no fibrillatory activity can occur.[130;131] The ectopic foci can also act as a substrate where reentrant circuits can occur.[132] In single circuit reentry model, only one circuit which is continuously firing (against the SA node activity) causes irregular fractionated atrial response which leads to fibrillation of the atrium.[123] The multiple circuit model is similar to the previous model, with the exception of the existence of multiple reentry circuits.[122;129] These circuits cycle through the atria simultaneously and interact with each other, disrupting the atrial conduction system and promote an arrhythmogenic state.[127] Although there is debate in the literature, the current consensus is that, the initial state of paroxysmal AF results from the formation of ectopic foci and single reentry circuits.[130] As the disease progresses, due to atrial structural and electrical

remodeling (caused by the tachycardia) there is the introduction of multiple circuits which lead to the permanent forms of AF.[122;130;131]

Factors that influence and cause the reentry of the circuits include wavelength of the generated impulse, conduction velocity, circuit length, action potential duration and refractory period (the period in which the cardiac myocyte remains inexcitable).[122;129-131] It has been reported that during AF there is decrease in action potential duration and shortening of the refractory period and wavelength as well as an overall decrease in conduction velocity (due to electrical and structural remodeling of the atrial tissue.)[130;131]

Wavelength is defined as the distance traveled by a single impulse during a single refractory period (wavelength= refractory period x conduction velocity).[130;131] The reentry circuit is the smallest circuit that can maintain continuous activity and reactivate the excitable core substrate of the atrial tissue by initiating continuous centripetal impulses.[124;125] Wavelengths determine the shortest path length for the reentry circuits.[130;131] Therefore, factors that reduce the size of atrial wavelengths also decrease the dimension of reentry currents, which in turn increases the potential number of simultaneous circuits.[122;130;131] In order to form the reentry circuits, the conduction time of a single wavelength (i.e. circuit time) must be greater than the refractory period. If the conduction velocity is low (i.e. increase in conduction time) then it becomes more likely that all the potential excitable points in the atrium have had the time to recover from excitation and are able to be reactivated by the invading reentry impulses.[122;127;133] Therefore, the shorter the refractory period, the higher the probability of reactivation by the reentry impulses. It is suggested that refractory period is the sole determinant of circuit time and the rate of tachycardia during AF. Wijffels *et al.* reported that during the

development of chronic atrial fibrillation, a progressive shortening of the refractory period was observed with the decrease in fibrillation interval. The shortening of the refractory period to a critical value was required to stabilize AF during atrial pacing in their goat model. In the same study it was also reported that both the wavelength dimension and conduction time was reduced as AF became more stable.[122]

The decrease in action potential duration (APD) of atrial myocytes has been strongly associated with the pathology of AF. Action potential duration is determined mainly by the duration of phase 2 (inward $Ca^{2+}$ current, keeping the myocyte depolarized and outward $K^{+}$ rectifier current, tend to repolarize the myocyte) or the plateau phase. It has been reported that during AF shortening of the APD occurs as there is a reduction in inward $Ca^{2+}$ current and enhancement in the outward $K^{+}$ current, resulting in a shorter plateau period. Faster repolarizations in the cardiac myocyte make it more susceptible/excitable to a potential reentry current as well and play a major role in AF development and progression.[130;131;134-136]

Although the mechanisms behind the shortening of the refractory period still remains unclear, a few of the possible causes are changes in the autonomic nervous system, ischemia, and the high rate of electrical activation. The role of the autonomic nervous system has often been associated with AF. Coumel *et al.* reported that initiation of AF was associated with high vagal tone and adrenergic stimulation of the heart.[137;138] Studies have reported that adenosine and acetylcholine are pro-fibrillatory as they shorten the refractory period by activating outward $K^{+}$ currents[139] without having an effect on the conduction velocity. Ischemia also reduces the refractory period by activating ATP-regulated $K^{+}$ channels.[122;140] The high rate of electrical activity during the initial stages of

AF can cause tachycardia modulated atrial remodeling that affects the synthesis and assembly of ion channels that control the atrial repolarization and thus affect the atrial refractory period.[141;142]

Inflammation and atrial dilation also make the atrium more susceptible to AF. The number of wavelets that can coexist simultaneously is dependent on the mass and surface area of the atrial tissue.[122;139;143] Atrial dilation increases the total surface area atria of the atrial tissue which can now accommodate higher number of reentry circuits. On the other hand the wavelengths of the impulses are not directly proportional to the size of the atria.[139] Since the wavelengths are considerably shortened during AF and circuit length is directly proportional to wavelength, dilated atria can thus allow a higher number of reentry circuits simultaneously and make the atrium susceptible to AF.[144] Hearts of larger size (those of large animal models and humans) are therefore more susceptible to AF.

### 2.2.1 Atrial Remodeling

As, "Atrial fibrillation begets atrial fibrillation",[122] AF itself induces electrical, contractile and molecular remodeling in the atrial myocytes that further propagate the disease pathology. This remodeling can occur within minutes and is thought to be the transitory mechanism behind the development of persistent AF from paroxysmal AF. Clinical trials and various animal models have identified two components of atrial remodeling: 1) remodeling due to rapid tachy-arrhythmia (atrial flutter and paroxysmal AF); 2) Atrial structural remodeling. It has been suggested that electrical remodeling occurs first, followed by a decrease in atrial contractility and finally structural remodeling that eventually leads to persistent AF.[122;145]

Tachycardia has been shown to induce rapid atrial activation in the paroxysmal stages of AF, in turn inducing electrical remodeling in atrial tissue.[130] At the onset of AF the atrial rate increases approximately 8 fold (SR 60/min vs AF 340-350/min in humans). To accommodate this higher rate there is an increase in $Ca^{2+}$ load in the myocytes. Since $Ca^{2+}$ is cytotoxic, the myocytes respond by reducing $Ca^{2+}$ influx via the L-type $Ca^{2+}$ current. However, this results in decreased APD (and in turn the refractory period) and wavelength which favors the perpetuation of AF (since smaller wavelength allows more atrial reentry circuits).[130;135;146] As AF becomes more sustained it has been reported that there is persistent decrease in the L-type $Ca^{2+}$ current via not only the inactivation but also the downregulation of mRNA expression (50-60%) of subunits that form the L-type $Ca^{2+}$ channels. However the detailed mechanism of the molecular basis of abnormal $Ca^{2+}$ handling is still not well understood.[147] Although the expressions of ryanodine receptors and phospholamban activity unaltered during AF, there is a suggested upregulation of NCX (Na/Ca ion exchanger).[147;148] Increase in NCX activity could increase the inward Na+ current and increase the propensity of delayed after depolarization (DADs) and contribute towards the pathogenesis of AF.[130;131;135]

Abbreviation of APD and shortening the refractory period during AF has also been attributed to the increased inward-rectifier $K^+$ current. Increased vagal tone association with the autonomic alterations during AF results in the release of acetylcholine, which in turn activates the acetylcholine activated $K^+$ ($I_{K,Ach}$). Atienza *et al.* reported that activation of $I_{K,Ach}$ by infusing adenosine increased reentry frequency in human patients of both paroxysmal and persistent AF. The increase of $I_{K,Ach}$ causes

shortening of the APD and cell membrane hyperpolarization of the atrial myocytes, and enhances the susceptibility of AF.[149;150]

In addition to the shortening of the refractory period, atrial tachycardia slows down intra-atrial conduction. It has been reported that conduction velocity is decreased during deletion of connexins (gap junction components).[151;152] Although there has been debate regarding the role of gap junction activity in the pathogenesis of AF. Verheule *et at.* reported decreased conduction velocity (by 30%) and AF inducibility in Connexin 40 knockout mice.[153] In a different study, Hagendorff *et al.* reported that during atrial pacing there were conduction disturbances in sinoatrial, intra-atrial and atrioventricular conduction in Connexin 40 deficient mice.[154] Kostin *et al.* reported a decrease in expression of Connexin 40 and 43 in the right atrial appendages (RAA) in human patients with AF. This study also reported a lateralization of the connexins in the AF patients, deviating from their physiological location at the junctions of the intercalated discs.[155]Together the combination of heterogeneity within the conduction pathway and decreased wavelength promotes multiple circuit reentry and increases the propensity of AF.

Atrial structural remodeling, particularly interstitial fibrosis has been identified as one of the important contributors to the AF substrate.[130] It has been reported that atrial fibrosis and AF occur in a concurrent manner, however whether or not there exists a direct cause and effect relationship remains elusive.[156] Atrial fibrosis is hard to reverse and therefore has been considered as major contributor in the progression from paroxysmal to persistent and permanent AF.[157] Myocardial fibrosis occurs due to an imbalance between the extracellular matrix (ECM) proteins that results in the

proliferation of fibroblast and excessive ECM protein build up in the interstitial space between the two adjacent myocytes. The increased space between the cardiac myocytes due to the fibrotic deposit and expansion of ECM can cause conduction delays and allow alternate pathways of conduction, thereby increasing the propensity of AF.[156;158] Interestingly, it has been reported that the atrium is more vulnerable to fibrosis compared to the ventricle.[159] Frustaci *et al.* reported the existence of patchy fibrosis in endomyocardial biopsies from the right atrium of human patients with paroxysmal lone AF (AF in the absence of other cardiac complications).[160;161]

Transforming growth factor 1 (TGFβ-1), secreted by both myocytes and fibroblasts, is one of the well-established pro-fibrotic molecules. It has been suggested that cardiac overexpression of active TGFβ-1 causes atrial fibrosis, disruption in atrial conductivity, and promotes AF.[162;163] Nakijima *et al.* reported that overexpression of TGFβ-1 in the atria was sufficient to induced atrial fibrosis in transgenic mice. Venteclef *et al.* reported increased expression of TGFβ-1 in rat atrial myocyte expressing higher interstitial fibrosis *in vitro*.[164] Verheule *et al.* reported that selective overexpression of TGFβ-1 in the atrium was sufficient to increase AF inducibility during rapid atrial pacing in a transgenic mice model.[162] However, these mice did not develop AF spontaneously. In a large animal model, Polejaeva *et al.* reported that overexpression of TGFβ-1 in the heart resulted in atrial fibrosis which in turn increased inducibility of AF in transgenic goats.[165] Chen *et al.* reported enhanced expression of TGFβ-1 in the porcine atrial tissue that was induced to rapid atrial pacing.[166] Together, the reports indicate that atrial fibrosis is a common factor in animal models of AF and in human patients with AF.

TGFβ-1 acts through the SMAD pathway to stimulate fibroblast activation and collagen deposition.[167] The presence of TGFβ-1 dramatically increases fibroblast function upregulating ECM protein synthesis (collagen production) without offsetting the collagen degradation.[168] Ventclef *et al.* reported fibrotic deposits in the atrial biopsies from AF patients mostly consisted of collagen I, III and VI.[164] In a similar study, Mukherjee *et al.* reported increased collagen deposits in the atrium of patients with AF.[169] Collagen fibers represent electrical barriers and therefore cause dysregulation of atrial impulse transduction and in turn facilitate the formation of reentry circuits.[170]

The regulatory mechanisms that underlie extracellular matrix (ECM) remodeling are not well understood. An important contributing factor to ECM remodeling in the myocardium is matrix metalloproteinases (MMPs). MMPs are a family of proteolytic enzymes involved in the metabolism of ECM proteins (such as collagen) and can cause tissue remodeling.[171] Endogenous tissue inhibitors of MMPs (TIMPs) moderate the activity of MMPs and maintain balance between ECM protein turnover rates. The myocardial ECM is a dynamic structure that plays a crucial role in providing structural supporting network during cardiac cycle and is critical for the maintenance of overall structural integrity of the cardiac chambers.[169;171] Therefore, alteration and disruption in the ECM integrity results in loss of structural support, continuity and impairment in signal transduction. MMPs, responsible for the degradation of ECM proteins, are expressed at a very low level in the healthy myocardium. However substantial upregulation of MMPs have been associated with heart failure and AF.[171] It has been reported that activation of MMPs and downregulation of TIMPs results in the enhancement of atrial fibrosis.[169;171] In a study conducted by Moe *et al.*, it was reported

that inhibition of MMPs cause a reduction in atrial fibrosis and subsequent decrease in AF inducibility in a canine model.[172] These findings lead to the identification of MMPs as a potential target for atrial fibrosis and AF.

Cardiac myocytes predominantly synthesize and release MMP2 and MMP9, among which upregulation of MMP9 has been identified as an individual risk factor for AF.[166] In a study designed to investigate the role of MMPs during AF, Chen *et al.* reported that during rapid atrial pacing in a porcine model, there was an increased expression of MMP9 in its pro (active) form. This study demonstrated increased gelatinase activity in ECM due to increased MMP9. They also reported increased TIMP-1 co-localization with MMP9 in addition to enhanced expression of glycosylated TIMP-1 in the atrial tissue. This study hypothesized that TIMP-1 was present to subsidize and inhibit the upregulated activity of MMP9.[166] In a similar study Hoit *et al.* reported that rapid atrial pacing in a canine model increased MMP9 activity in the atrium.[173] Nakano *et al.* also reported significant increase in MMP9 levels in atrial biopsies of human patients with both paroxysmal and persistent AF.[145] And reported that the level of MMP9 increased step by step with the severity of the disease (from paroxysmal to persistent AF). Interestingly, none of these studies report alteration in MMP2 expression, indicating that MMP9 is mostly associated with atrial remodeling during spontaneous and/or induced AF. In another study by Meiner *et al.*, it was reported that reduced myocardial fibrosis was associated with decreased activity of MMP9.[174] It has been postulated that MMP9 can modulate the activation of TGFβ-1 and thereby participate in atrial fibrosis. In a case-cohort based study Huxley *et al.* reported that increased MMP9 was associated with increase in risk factor for AF.[175] Therefore, collectively, the results from these

studies indicate that MMP9 significantly contribute towards atrial remodeling and render the atria susceptible to AF.

One of the predominant changes in the atria at the onset of AF is glycogen accumulation.[176-180] It has been reported that within 4 weeks after the onset of AF there is a marked increase in the glycogen accumulation in the atria, which was hypothesized to be responsible for the perpetuation of persistent AF at the end of 8 weeks. [177;181] Based on this hypothesis, Embi, *et al.* investigated the location and distribution of glycogen in the left and right atrial appendages in the healthy goat heart, and reported that the left atrial appendage (LAA) has a significant higher concentration of glycogen and it is located against the intercalated disc and at the myocyte junction, whereas in the right atrial appendage (RAA) the glycogen granules where scattered within the mycoytes.[179] This study also suggested that this regional difference in the glycogen accumulation between the LAA and the RAA could be the major contributing factor to the initiation of the arrhythmia in the LAA. The importance of the location of the glycogen molecules are of particular significance in the electrical signal transduction pathway, as the gap junction pores can only allow molecules of a given size to pass from cell to cell and given that the glycogen molecules are of higher molecular size create an obstacle in the path.[182] Furthermore, rapid atrial pacing causes remodeling of the gap junctional proteins (connexin 40) causing disturbance in the conduction pathway.[183] Therefore it has been suggested that locational increase in the glycogen molecule may cause fractionated intracellular communication, causing a disruption in atrial syncytium.[179] It is also reported that the LAA have lower conduction speed and higher resistance compared to the RAA due to the location and density of glycogen accumulation.[151;152] Therefore,

increase in glycogen accumulation is suggested to be a major pathophysiological factor in the propensity of sustained/ permanent AF.[179;180]

In the follow up study of Embi *et al.*, Zhang *et al.* reported significant increase in glycogen accumulation in the LAA against the intercalated discs extending into the myocyte and potentially creating a longitudinal and lateral conduction blockade between side by side myocytes after AF was induced in canine hearts.[180] There was significant increase in glycogen accumulation in the atria within 48 hours of rapid atrial pacing which eventually led to permanent AF.[180] There was also significant increase of glycogen in the right atria; although they were scattered molecules within the myocytes. However, there was significant increase in glycogen concentration in both atria 8 weeks after the induction of AF compared to baseline.[180] This study also reported significant increase in atrial fibrosis and an increase in collagen accumulation due to the rapid atrial pacing, which the group hypothesized as one of the deciding factors that led to sustained AF along with glycogen accumulation.[180]

In similar studies Ausma *et al.* reported that there was significant electrical (shortening of the atrial refractory period) and structural remodeling (in the form of glycogen accumulation, loss of contractile apparatus function and twofold increase in myocyte cell size) that occurred due to the induction of AF by rapid atrial pacing in healthy goat hearts. This study also reported that the electrical and structural remodeling initiate within the 48 hours of induction followed by a sustained AF within 1-2 weeks. This study also reported that the glycogen accumulation progressively increase for 8 weeks.[176;177;181]

### 2.2.2 Atrial Fibrillation and Diabetes

Diabetes has been identified as one of the most important risk factor for atrial fibrillation.[5;15] It has been reported that the risk factor for AF is 1.4-2.2 times higher in a diabetic patient compared to a healthy person.[14;19;184;185] In a population based study, *Du et al.* reported 7.6% of the diabetic patients had baseline AF.[186] Although the underlying mechanisms that cause diabetes induced AF remains elusive, a few of the proposed mechanisms include insulin resistance, poor glycemic control, and structural and electrical remodeling.

In a study by Ostgren *et al.*, a combination of insulin resistance and impaired glycemic control was identified as a potential mechanism for diabetes induced AF.[19] It was reported that AF patients had prominent insulin resistance whereas, the subjects in sinus rhythm did not.[19] In a similar study it was reported that high fasting blood glucose and insulin levels in diabetic patients were correlated with the risk factor for the development of AF but not with non-diabetic individuals.[15;184] The risk factor of AF was related to the duration of diabetes and level of glycemic control.[15] Indeed, Dublin *et al.* reported that the risk of developing AF for a diabetic patient increases approximately 3% for each additional year of diabetes duration.[17] Additionally, AF attacks have been associated with periods of hypoglycemia in insulin-dependent T1Dx patients.[187;188] Another study by Rigalleau *et al.* reported major hyperglycemia preceded by AF attack in insulin-independent T2Dx patients.[16] Together the results of these studies[15;17;19;185;189-192] suggest that disturbances in glucose and insulin could directly affect the function of the atrium, potentially leading to AF. The diabetic myocardium has increased susceptibility to ischemic injury which also makes the myocardium vulnerable to AF.[5] Atrial fibrosis

and remodeling is one of the major risk factors for AF, as demonstrated by Kato *et al.* when they reported widespread fibrotic deposits in the myocardium of diabetic rats. It was suggested that the fibrotic deposits could serve as anchoring points for reentry circuits and attenuate forward wave propagation in the atrium. In the same study a single electrical impulse was able to generate multiple atrial responses, which support the hypothesis that a diabetic myocardium is more vulnerable to AF.[14] Altered gap junction activity, which is an alteration in electrical coupling between two adjacent myocytes, has been reported as one of the hallmarks of AF. Interestingly, decreased phosphorylation of connexin-43 was reported in the atrium of diabetic rats.[193] This finding suggests that, the alteration in gap junction activity could lead to improper signal transduction between the atrial myocytes and cause atrial arrhythmia in the diabetic myocardium.

### 2.2.3 Atrial Fibrillation and Advanced Glycation End Products

During diabetes, Advanced glycation end products (AGEs), are formed by the non-enzymatic glycation of both intracellular and extracellular proteins in the presence of a high glucose concentration.[38] The level of hyperglycemia, the turnover rate of proteins and the extent of oxidative stress are the major factors that play a role on the formation of AGEs.[194;195] As the process of forming a glycated proteins occur over a period of few weeks, therefore the proteins with longer turnover rate are more susceptible to glycation (such as albumin and hemoglobin).[196] AGEs are formed when aldehyde or ketone groups of reducing sugars (such as glucose, fructose, ribose, etc.) react non-enzymatically and form covalent bonds with free amino groups of amino acids (such as lysine, arginine etc.) in the protein chain. This reaction is also known as the Millard reaction.[38] The Millard reaction begins with the formation of a reversible Schiff base (1-2 weeks glycation

period) which is considered reversible. With longer duration in a Schiff base, spontaneous rearrangement can be undergone to form the more stable Amadori products (6-8 weeks glycation period).[194] The proteins bearing the Amadori products are considered to be irreversible and are referred to as the glycation products.

Advanced glycation end products present in the diabetic myocardium also participate in major myocardial structural remodeling by upregulating growth factors, activating the AGE-RAGE (RAGE: Cellular receptor of AGE) axis and cause interstitial fibrosis. [197] Begieneman *et al*. reported increased expression of major AGEs ($N^{\varepsilon}$-carboxymethyl lysine, CML-AGE) in the left atrial appendage (LAA) of AF patients.[50] In addition, increased plasma AGEs have been reported to be associated with incidence of AF, independently of diabetes.[50;51] However, no significant correlation between AGEs and the incidence and development of AF have been reported in a recent large population based study.[198] Therefore, whether or not AGE accumulation plays a role in the progression of AF remains to be determined.

AGE also binds with its cellular receptor, RAGE, and activates pro-inflammatory pathway that results in the overexpression of pro-fibrotic markers (transforming growth factor -1 (TGFβ-1), matrix metalloproteinase -9 (MMP-9), collagen I and III) which has been identified as an independent risk factor of AF as it contributes to atrial structural remodeling and dilation.[145;166;174;175] Raposeiras-Roubin *et al.* conducted a study to correlate the activation of AGE-RAGE axis and atrial fibrillation in human AF patients. The study reported increased level of soluble RAGE, and AGE in the plasma of both diabetic and non-diabetic AF patients. This is probably due to the higher oxidative stress associated with other cardiac complications of the AF patients and the activation of AGE-

RAGE axis. Increased area and volume of the left atria in the AF patients were also reported, possibly due to the advanced glycation end products forming crosslinks with the extracellular matrix proteins.[51]

### 2.3 Cardiac Metabolism

As cardiac myocytes contract constantly, the myocardium requires constant source of energy (adenosine tri phosphate, or ATP). Under non ischemic conditions 95% of the ATP is generated in the mitochondria by oxidative phosphorylation. The heart utilizes 60-70% of the generated ATP for contractile function and the remainder is used for the function of various ion pumps. The myocardium has a high ATP hydrolysis and turnover rate and very little to no ATP reserve. Under physiological conditions, the rate of ATP generated by oxidative phosphorylation is slightly higher than the rate of ATP hydrolysis, and, therefore, the total content of ATP remains constant even with large energetic demands (intense exercise, acute stress). During resting conditions, a well perfused heart uses fatty acid (60-90%) and glucose (10-40%) as an energy substrate. However, in the presence of insulin this ratio is reversed, as the heart derives 60-70% energy from glucose. These substrates undergo β-oxidation and glycolysis respectively and produce Acetyl-CoA as an end product in the mitochondria. The produced Acetyl-CoA enters the TCA cycle and is converted to NADH. The electron transport chain within the mitochondria converts NADH to ATP using $O_2$ and produce $CO_2$ as a bi-product.[20;199-201]

Glucose for the heart is derived either from the blood stream or from the glycogen stores within the cardiac myocytes.[202] As exogenous glucose enters the cell they are

rapidly phosphorylated by hexokinase (first regulatory step in glucose metabolism) and converted to glucose-6-phosphate. This compound either enters the glycolytic pathway (majority) or is converted to glycogen as storage. However, a variable amount of exogenous glucose cycles through glycogen prior to entering the glycolytic pathway.[203] Glucose-6-phosphate is first converted to fructose-6-phosphate. At this stage phosphofructokinase (PFK-1), a key regulatory enzyme, phosphorylates fructose-6-phosphate to a more stable fructose-6-bisphosphate using one ATP molecule.[204;205] Conversion of fructose-6-bisphosphate from fructose-6-phosphate is the rate limiting step of the glycolysis process. Fructose-6-bisphospate is eventually converted to pyruvate at the cytosol and as pyruvate enters the mitochondria it is used in the production of ATP. The glycolysis pathway generates 2 pyruvate molecules from each glucose-6-phosphate molecule. Within the mitochondria, the activity of another key enzyme, pyruvate dehydrogenase, tightly regulates the production of acetyl-CoA from each of the pyruvate molecules. At the end of the oxidative phosphorylation process, 38 molecules of ATP are generated for each molecule of glucose.[20;199;204;205]

The amount of glycogen accumulation in the heart is dependent upon the availability of other metabolic substrates (glucose, fatty acid) or stimulation of hormone. During fasting, fatty acid oxidation is predominant which inhibits glycolysis and reroutes the exogenous glucose/glucose-6-phosphate towards the formation of glycogen.[202;206-208] Insulin also increases glycogen synthesis as it facilitates cellular glucose uptake and stimulates glycogen synthase activity.[206] Initiated by the requirement of extra energy and increase in work load (e.g. during exercise), glycogenolysis occurs due to the activation of glycogen phosphorylase (stimulated by epinephrine).[203;209;210] In healthy adults,

glycogen occupies 2% of the entire volume of the cardiac myocytes and in newborn or fetal cells it occupies 30% of the total volume of the cardiac myocytes.[211]

### 2.3.1 Abnormal Atrial Metabolism during Atrial Fibrillation

The mechanisms of persistent atrial arrhythmia and the related metabolic changes during atrial fibrillation are not well understood. Atrial fibrillation has been associated with modifications in atrial energy metabolism, however, whether alteration in glucose metabolism is the cause or consequence of AF remains inconclusive.[118;212-218] Kondrat've *et al.* studied the changes in energy metabolism in the myocardium during acute experimental AF on healthy male Wistar rats. They reported that the induced AF (5-10 mins for 8-10 times) depleted the atrial glycogen storage by activating the glycogen phosphorylase system. They hypothesized that this was caused by the increase in need of energy consumption to sustain the frequent, but yet inefficient contractions in the fibrillating myocardium.[219] However, in contrast to this study, glycogen accumulation in the atrial tissue has been identified as one of the structural remodeling factors that increase the pathogenesis of persistent/permanent AF. (Please find details regarding glycogen and AF in section 2.2.1).

Modifications of atrial energy demand have been associated with AF in both human and animal models.[213-215] However, while some studies reported increased energy demand[213;215] during episodes of AF, others reported impairment in energy production and consumption.[212;214;216] In a quantitative proteomics analysis, Tu *et al.* attributed the impaired energy production in the LAA of AF patients to the downregulation of carbohydrate metabolism related proteins.[220] Key enzymes of the glycolysis pathway

such as glucose-6-phosphate and 6-phospho-fructokinase were reported to be downregulated in the LAA of AF patients, suggesting an overall impairment of glucose metabolism during AF.[220] Irregularly irregular contraction of the atrial tissue has also been associated with increased ADP and AMP-to-ATP ratio in the cell as a marker of increased energy expenditure.[140;221;222] Human AF has also been associated with increased cytosolic $Ca^{2+}$ levels and $Ca^{2+}$/calmodulin dependent protein kinase II (CAMKII) levels. [223-227] Neef *et al.* reported higher activation of CAMKII resulting in increased sarcoplasmic reticulum $Ca^{2+}$ leaks resulting in elevated diastolic $Ca^{2+}$ concentrations in the right atrial myocardium of human patients with AF.[228] Activation of $Ca^{2+}$-dependent pathways and increased CAMKII [229;230] and AMP [231] levels have been reported to activate AMPK, the cellular regulator of stress related metabolic signaling.[232-234] Lenski *et al.* reported that irregular pacing resulted in the activation of CAMKII and AMPK, which enhanced fatty acid uptake, increased lipid accumulation and decreased glucose uptake in atrial myoctes.[12] AMPK activation decreases myocardial glucose uptake by decreasing the expression of SNAP-23 (synaptosomal-associated protein 23) which is a crucial regulator of GLUT4 (see section 2.5.1) vesicular trafficking to the cell surface.[235;236] It has been reported that in the left atria of AF patients, GLUT4 trafficking to the cell surface was impaired, whereas the expression of total GLUT4 expression was increased. The same study also reported increased glycogen accumulation and major myocardial remodeling, in the left atria of the AF patients. However, whether or not this increase was AMPK dependent remained inconclusive.[12] Despite the efforts of these studies, mechanisms of arrhythmia related to the altered metabolism in the atria are still not well understood.

### 2.3.2 Cardiac Glucose Transport

Glucose is a major energy substrate for the heart, which generates 30% of its total energy from glucose oxidation.[237] Therefore, cardiac glucose uptake is crucial for proper cardiac function. The first step of glucose uptake is the transport of glucose from the blood stream across the plasma membrane and into the cell. This is conducted by a specialized transporter of glucose known as the GLUT family. The GLUTs are stored intracellularly (inactive location) or located at the plasma membrane (active location). The intracellularly stored GLUT vesicles are translocated to the plasma membrane upon action from an external stimulus, such as insulin. Translocation of GLUTs to the plasma membrane to the cell surface is the rate limiting step of glucose utilization. The different members of the GLUT family and regulation of GLUT trafficking via insulin signaling pathway are discussed below in detail.

### 2.3.3 Glucose Transporters (GLUTs)

Glucose transporters (GLUTs), members of a specialized family of membrane transporters, are responsible for the disposal of exogenous glucose. It has been reported that, at least one GLUT isoform is present in every cell type of the body, therefore, GLUTs are recognized as key regulators of whole body glucose homeostasis.[23] Following glucose uptake, glucose is either stored as glycogen or oxidized to produce energy.[238] So far 14 sugar transporter proteins have been identified in the human genome.[23] The distribution of the glucose transporters is widespread, including the skeletal muscle, adipose tissue, liver, intestine, kidney, peripheral blood vessels, endothelial cells in the blood brain barrier, brain and the heart.[239] The kinetics of these transporters differs

according to their substrate location that reflects their specific roles in cellular and whole body glucose homeostasis.[240] The GLUT superfamily has been divided into 3 classes: Class I (GLUTs 1-4 and GLUT 14), Class II (GLUTs 5, 7, 9 and 11) and the novel Class III (GLUT 6, 8, 10, 12 and HMIT).[23] Of the Class I, transporters GLUT-1 and -4 have been extensively studied. GLUT1 is the major transporter of glucose across the endothelial cells in blood brain barrier and erythrocytes.[241] GLUT1 is mainly located at the cell surface and serves as a basal transporter. GLUT2 is mostly found in the liver and pancreatic beta cells, and functions as part of the glucose sensor system during the postprandial absorptive state.[239] GLUT3 is mostly present in neurons (both dendrites and axons) and testis.[240] GLUT4 is the major insulin-responsive isoform and present mainly in skeletal muscle, adipose tissue and the heart. Among these glucose transporters, GLUT2 has lower affinity for glucose compared to GULT 1, 3 and 4.[239] GLUT-5 and -11 are mainly the transporters of fructose in the small intestine, kidney and skeletal muscle.[238] Among the glucose transporters, insulin-dependent GLUT4 is the major mediator of glucose removal from the blood stream and is a key regulator of glucose homeostasis.[242] However, recent findings by Katz *et al.* report that GLUT4 knockout mice do not develop hyperglycemia, suggesting the presence of other insulin sensitive isoforms.[25] Therefore, there have been new studies that investigate the novel Class III isoforms, particularly GLUT-8 and -12. Both of these isoforms have been reported in skeletal muscle, adipose tissue and the cardiac tissue.[23] In the cardiac tissue, GLUT12 has been identified as a basal glucose transporter.[37] However, GLUT8 is not well characterized in the heart and whether or not this isoform is insulin responsive is yet to be

determined. In the current study, we investigate the role of GLUT-4 and -8 in atrial glucose uptake in the context of diabetes.

#### 2.3.3.a Glucose transporter 4 (GLUT4)

The insulin-sensitive GLUT4, 50-60 kDa glycoprotein containing 12 transmembrane domains, is the principal glucose transporter protein in the mammalian tissue.[243;244] GLUT4 is responsible for the glucose uptake into the cells, supplying cellular glucose for ATP production, and for a wide range of anabolic reaction.[243] In contrast to the other GLUT isoforms, which are primarily localized in the plasma membrane, only 3-10% of the total GLUT4 is present on the membrane during the basal or unstimulated state.[244] They are mainly sequestered into specialized storage vesicles that remain within the cytosol in the absence of insulin stimulation.[242] Upon insulin stimulation, these vesicles undergo exocytosis and are diffused onto the plasma membrane. In addition, the presence of insulin partially inhibits GLUT4 endocytosis and compartmentalization.[245-247] This results in a positive shift of GLUT4 localization in the membrane via which glucose can enter the cell through an ATP-independent, facilitative diffusion mechanism.[244] However, this process is reversed, when the circulating insulin level declines and the membrane bound GLUT4 is endocytosed into the storage vesicles.[244] Numerous studies have identified GLUT4 storage in the Trans Golgi network, clathrin coated vesicles and endosomes.[242] However, the majority of the GLUT4 is stored in the tubulovesicular elements that lie just beneath the plasma membrane in the cytoplasm and are identified as the specialized GLUT4 compartments.[248] Upon insulin activation, the PI3 kinase pathway is activated, leading to the phosphorylation of AS160 (see section 2.3.4.a). This step is essential for the vesicular trafficking of the GLUT4

vesicles. The phosphorylation of AS160 allows the activation of RabGTP which is necessary for the docking and diffusion of the GLUT4 vesicles on the plasma membrane.[249] It has been reported the caveolae lipid rafts serve as the major docking site for GLUT4 transporters as 85% of the total plasma membrane bound GLUT4 have been found to be localized in the caveolae rafts.[250]

During T1Dx, Huang et al. reported impairment in insulin stimulated GLUT4 trafficking in the myocardium of streptozotocin (STZ)-treated diabetic rats.[251] Similarly, Waller *et al.* reported a decrease in both total and membrane bound GLUT4 expression in the ventricles of STZ-induced diabetic mice. This study also reported impairment in GLUT4 translocation to the cell surface upon insulin stimulation.[37] During type 2 diabetes (T2Dx), there is a marked reduction in insulin-mediated glucose uptake caused by cellular resistance to insulin.[113] It has been reported in multiple studies that there is a substantial down regulation of GLUT4 protein and mRNA levels in adipocytes during T2Dx and obesity.[252;253] In the skeletal muscle, no change in total level of GLUT4 has been reported between diabetic and non-diabetic patients.[254-257] However, significant impairment of insulin-stimulated GLUT4 vesicular trafficking has been reported in patients with T2Dx.[258-260] It has been documented that, in skeletal muscle, insulin resistance alters sub-cellular localization of GLUT4 storage vesicles to dense membrane compartments from which insulin is unable to recruit GLUT to the cell surface.[260] The specific molecular mechanism of such events is yet to be elucidated. Together, these studies demonstrate that in skeletal muscle, adipose tissue and the ventricles, there is significantly less GLUT4 on the membrane for glucose uptake during diabetes. However,

the role and regulation of GLUT4 in the atria during healthy and diabetic conditions warrants further investigations.

#### 2.3.3.b Glucose Transporter 8 (GLUT8)

Glucose transporter 8 is a novel class III glucose transporter that has been shown to be present in substantial quantities in the cardiac tissue and may play a significant role in glucose uptake in the heart.[237] GLUT8 has the highest mRNA level after GLUT4 in the myocardium.[27;237] Unlike the well understood class I transporter GLUT4, the subcellular localization, regulation and physiological role of GLUT8 in mammalian tissue is not well understood. So far GLUT8 has been reported in the skeletal muscle, adipose tissue, liver, spleen, testes, kidney, brain and the cardiac tissue. It has been reported that GLUT8 is a dual specific glucose and fructose transporter and is intimately linked to glucose homeostasis.[30;261]

The structure of GLUT8 is yet to be well characterized; however, the amino acid sequence of GLUT8 exhibits all elements and motifs that are characteristic of sugar transporters or GLUTs (i.e. 12 membrane spanning helices, glutamate and arginine residues in loops 4 and 10) with a molecular weight of 50 kDa.[29] It has been reported that GLUT8 is predominantly located in intracellular compartments due to the presence of di-leucine motif in the amino terminus domain.[27;30;31;262] However, the site of intracellular localization of GLUT8 has been widely debated. In the brain hippocampus, GLUT8 is reported to be associated with membrane containing intracellular compartments of neuronal bodies. The authors reported that upon peripheral insulin stimulation GLUT8 translocates from the smooth ER to the rough ER in the rat hippocampus. The insulin

sensitivity of GLUT8 in other tissue, specifically the cardiac tissue is yet to be investigated.

Whether or not GLUT8 is insulin responsive in the mammalian tissue has not been well characterized.[263;264] Mutation of the di-leucine residues to alanine in the GLUT8 structure potentially targets the transporter to the plasma membrane. A similar motif is present in GLUT4, which is located in the intracellular compartments and translocation to the cell surface upon insulin stimulation. Therefore, it can be speculated that GLUT8 has similar translocation potential.[29] However, the extracellular stimulus that may cause GLUT8 translocation to the plasma membrane remains to be identified. Insulin-stimulated translocation of GLUT8 to the plasma membrane of blastocysts has been reported; however, this mechanism has not been observed in adipocytes. Indeed, Lisinski et al. reported that GLUT8 is not insulin responsive in adipocytes. Interestingly, the GLUT8 gene expression was down-regulated during glucose deprivation and hypoxia in 3T3-L1 adipocytes.[30] These data suggest that GLUT8 may play a major role in glucose homeostasis.

During diabetes, liver GLUT8 mRNA levels were correlated with the circulating insulin. In a study conducted by Gorovits et al. significant decrease of GLUT8 mRNA was observed during insulinopenia/hyperglycemia, whereas significant increase of GLUT8 mRNA was observed in hyperinsulinemia/hyperglycemia, suggesting a regulatory role of insulin in GLUT8 mRNA expression.[30] In addition, it was reported that STZ-induced diabetes induced significant decrease in GLUT8 mRNA expression in hepatocytes with a paradoxical increase in GLUT8 protein levels.[30] Piroli *et al*. reported that hyperglycemic and insulinopenic rats (following STZ injection) expressed lower

levels of GLUT8 and ER GLUT8 trafficking in the hippocampus.[263] DeBosch *et al*. suggested that GLUT8 caused deleterious metabolic effects during high fructose diet. Their studies reported that, GLUT8 deficient mice were protected against high-fructose-diet-induced fructose intolerance, high-fat-diet-induced obesity, insulin resistance, and hypoinsulinemia compared to wild type controls.[261;265-267] Together, these studies suggest that GLUT8 plays an important role in metabolic and homeostatic functions. Although the heart is one of the main organs to utilize glucose, to our knowledge there is no study that investigates the functional role of GLUT8 in the cardiac tissue, specifically in the atria during both heathy and diabetic conditions.

### 2.3.4. Insulin Signaling Pathway

During the post prandial absorptive state, as the blood glucose level increases, the pancreatic beta cells release insulin into the blood stream. Insulin modulates cellular glucose uptake by binding to the insulin receptor[4]. The binding of insulin to its receptor initiates an intracellular signaling cascade, which translocate the insulin sensitive glucose transporters (GLUTs) from the cytosol to the plasma membrane. As the glucose transporters translocate to the plasma membrane (active site), glucose molecules from the blood stream enter the cells via the GLUTs.[242] The intracellular signal transduction initiated by ligand binding of insulin is known as the insulin dependent pathway or the PI3 kinase (phosphoinositide 3 kinase) pathway.

#### 2.3.4.a. Signal Transduction Pathway:

The insulin receptor on the cell surface is a member of the large transmembrane tyrosine kinase receptor family (RTKs). It is a heterotrimeric structure with two

extracellular (α) and two intracellular (β) subunits.[268] Insulin binds to the extracellular domain of the insulin receptor. This causes a conformational change in the receptor's intracellular domain, followed by auto phosphorylation at the intracellular β subunits of the tyrosine kinase domain.[242] Cytosolic scaffolding substrate protein IRS-1 binds to the phosphorylated domain of the receptor. This IRS-1 substrate serves as a binding and activation site for the effector protein PI3 kinase.[268] The PI3 kinase takes part in numerous cellular responses, such as anti-apoptosis, protein synthesis and glycogen synthesis. Apart from insulin, PI3K is attributed as the most important enzyme for the insulin stimulated glucose transport.[269] As PI3 kinase binds to the phosphorylated site of the IRS, it phosphorylates phosphatidyinositol (4,5) bisphosphate or PIP2 to phosphatidyinisitol (3,4,5) trisphosphate or PIP3. PIP3 binds to the PH domain of the 3-phosphoinositide dependent protein kinase-1 (PDK-1) and protein kinase B (also known as Akt), localizing them to the cell surface.[268] At the membrane, PDK-1 phosphorylates Akt at threonine 308 (Th 308) residues, which is located in the kinase domain of the Akt activation loop. The full activation of Akt requires a further phosphorylation of serine 473 (s473) residues by mTOR2 (rapamycin insensitive companion of mTOR).[268;269] Activated Akt phosphorylates a downstream protein AS160 (Akt substrate protein of 160 kDA) which is essential for the exocytosis of GLUT4 to the cell membrane. AS160 is the most distal protein in the insulin-mediated GLUT trafficking pathway and plays a major regulatory role. Under basal conditions, AS160 retains the GLUT vesicles at their intracellular location by its GTPase activating protein (GAP domain of the members of Rab G protein family). Unstimulated Rab is maintained in its inactive GDP form, while phosphorylation of AS160 (at 6 different phospho-Akt-substrate (PAS) sites) inhibits its

GAP activity and elevates the GTP form of Rab. It has been suggested that GLUT vesicular translocation to the plasma membrane requires the active GTP bound Rab.[23;249;268;269] Figure 2.1 briefly illustrates the different events of the insulin-stimulated PI3 kinase pathway. It is important to note that, the initiation of the signaling cascade by the activation of the insulin receptor not only increases GLUT4 exocytosis at the membrane, it also attenuates GLUT4 endocytosis causing an enhanced re-distribution of GLUT4 protein at the plasma membrane, facilitating cellular glucose uptake.[270] Although the role of the downstream insulin signaling pathway regarding GLUT4 trafficking has been extensively studied in the skeletal muscle and adipose tissue, it not well defined in the cardiac tissue., [242;249;268-271] In addition the role of the downstream insulin signaling pathway on the novel GLUT8 isoform is yet to be determined.

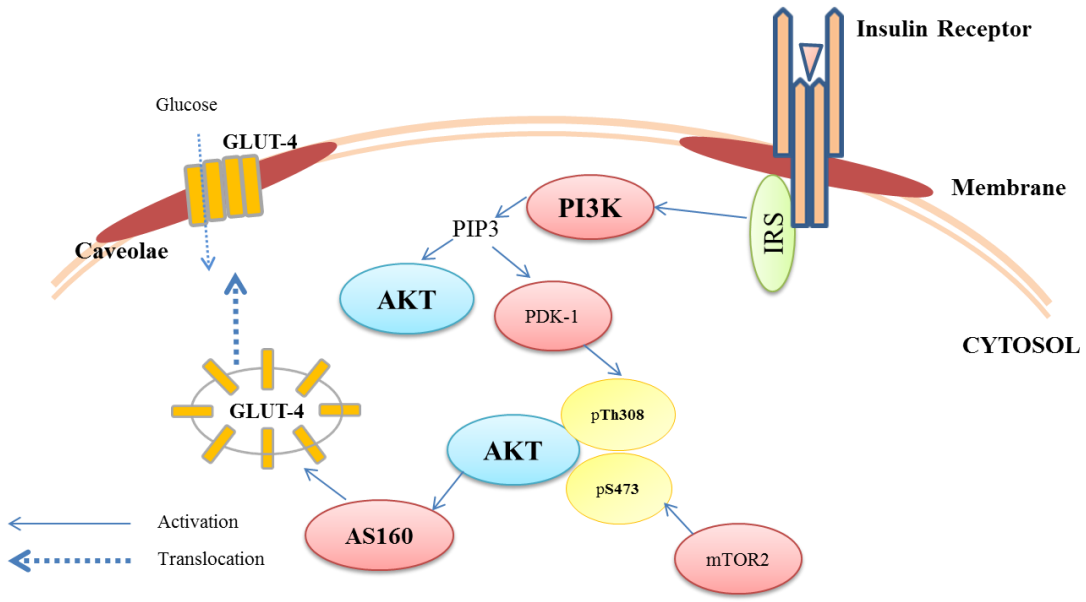


**Figure 2.1: Insulin stimulated PI3K pathway and GLUT-4 translocation.** The figure illustrates the activation of the PI3K pathway by the ligand binding of the insulin receptor, followed by the phosphorylation of Akt at Th308 and s473 leading to the translocation of the glucose transporter (GLUT-4) to the membrane.

### 2.3.4.b Protein Kinase B AKT

Serine/threonine kinase Akt, also known as the protein kinase B (PKB), is a central player in multiple cellular processes, such as glucose metabolism, cell survival, cell proliferation and cell cycle progression.[272]The Akt subfamily consists of 3 isoforms (Akt1, Akt2 and Akt3) each with three functional domains: an N terminal pleckstrin homology (PH) domain, a protein kinase domain and a C- terminal hydrophobic phosphorylation site.[272] Among the three isoforms, Akt 2 is highly expressed in the striated muscle, adipose tissue and the brain. Akt activation is dependent on the PI3K signaling pathway and subsequent phosphorylation at Th308 and s473 residues.[273] Phosphorylated Akt regulates the function of many cellular proteins involved in metabolism, survival/apoptosis and angiogenesis.[273]

Akt is a key regulator of glucose uptake by stimulating the translocation of GLUT4 and the survival and proliferation of insulin secreting pancreatic beta cells. Over expression of phosphorylated Akt has been reported to significantly increase overall pancreatic beta cell mass, by increasing both cell size and number; thereby enhancing the insulin secretory capacity.[274] Conversely, a reduction in Akt phosphorylation has been reported in insulin resistant individuals. Akt also participates in the activation of NF-κB by tumor necrosis factor-α (TNFα) by phosphorylating inhibitory protein IκBα.[275;276]

Due to the major role of Akt in the insulin signaling pathway, Akt has been widely studied during both insulin deficient type 1 diabetes (T1Dx) and insulin resistant type 2 diabetes (T2Dx). According to current literature, during T1Dx insulin signaling is normal or slightly affected due to the hyperglycemia. It has been reported by

Gerhardinger *et al*. that low dose insulin stimulation rescues Akt phosphorylation in the retina of STZ-induced rats.[277] Laviola et al. reported that Akt expression was unchanged in the myocardium of STZ-induced diabetic rats.[278] However, in another study by Dobryzynski et al. it was reported that insulin-deficient STZ diabetic rats demonstrated reduction in baseline Akt activity in the cardiac tissue.[279]

During T2Dx, Krook *et al*. first reported impairment in Akt phosphorylation in the skeletal muscle tissue of insulin resistant rats and biopsies from T2Dx patients.[280;281] Similarly, there was significant impairment in Akt phosphorylation (at s473 site) in the skeletal muscle and adipose tissue of db/db mice, although its total protein expression remained unchanged.[282] Karlsson *et al*. reported that an attenuation of insulin induced AS160 phosphorylation in human skeletal muscle during hyperinsulinemia, the hallmark of T2Dx. This impairment of insulin action on AS160 was associated with reduced phosphorylation of Th308.[258] Similarly, Akt activation was impaired in adipocytes of obese human T2Dx patients and T2Dx rodents.[283;284] Defects in GLUT4 translocation was also reported to be associated with defective Akt phosphorylation.[285;286] Cho *et al*. used an Akt2 knockout mouse model to investigate the role of Akt during insulin resistance, and reported that the Akt2 null mice displayed hyperglycemia, impaired glucose tolerance and hyperinsulinemia.[287] Together all of the above studies strongly suggest the crucial role of Akt in the insulin signaling pathway and GLUT trafficking. Despite the crucial role of Akt in GLUT trafficking and glucose uptake, there have been very few studies that investigate its functional role in the cardiac tissue during both T1Dx and T2Dx.

# CHAPTER III

# DIABETES ALTERS THE REGULATION AND TRANSLOCATION OF THE INSULIN SENSITIVE GLUCOSE TRANSPORTER 4 AND 8 IN THE ATRIA

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*Published Dec 31, 2015, PLoS ON*

**ABSTRACT**

Although diabetes has been identified as a major risk factor for atrial fibrillation, little is known about glucose metabolism in the healthy and diabetic atria. Glucose transport into the cell, the rate-limiting step of glucose utilization, is regulated by the Glucose Transporters (GLUTs). Although GLUT4 is the major isoform in the heart, GLUT8 has recently emerged as a novel cardiac isoform. We hypothesized that GLUT-4 and -8 translocation to the atrial cell surface will be regulated by insulin and impaired during insulin-dependent diabetes. GLUT protein content was measured by Western blotting in healthy cardiac myocytes and type 1 (streptozotocin-induced, T1Dx) diabetic rodents. Active cell surface GLUT content was measured using a biotinylated photolabeled assay in the perfused heart. In the healthy atria, insulin stimulation increased both GLUT-4 and -8 translocation to the cell surface (by 100 % and 240 %, respectively, $P<0.05$). Upon insulin stimulation, we reported an increase in Akt (Th308 and s473 sites) and AS160 phosphorylation, which was positively ($P<0.05$) correlated with GLUT4 protein content in the healthy atria. During diabetes, active cell surface GLUT-4 and -8 content was downregulated in the atria (by 70 % and 90 %, respectively, $P<0.05$). Akt and AS160 phosphorylation was not impaired in the diabetic atria, suggesting the presence of an intact insulin signaling pathway. This was confirmed by the rescued translocation of GLUT-4 and -8 to the atrial cell surface upon insulin stimulation in the atria of type 1 diabetic subjects. In conclusion, our data suggest that: 1) both GLUT-4 and -8 are insulin-sensitive in the healthy atria through an Akt/AS160 dependent pathway; 2) GLUT-4 and -8 trafficking is impaired in the diabetic atria and rescued by insulin treatment. Alterations in atrial glucose transport may induce perturbations in energy production, which may provide a metabolic substrate for atrial fibrillation during diabetes.

# INTRODUCTION

Diabetes mellitus is a serious metabolic disorder affecting 387 million people worldwide [2;60]. Diabetes has now reached epidemic levels and has been identified as the 7th leading cause of death in the USA [2;3]. Hyperglycemia, the hallmark of diabetes, results from an impaired glucose uptake due to a lack of insulin production by pancreatic beta cell (type 1) or lack of insulin action (type 2). Diabetes results in multiple organ dysfunction including cardiomyopathy, coronary artery disease and atrial fibrillation. [4;5;14;288]

Glucose is a major energy substrate for the heart, which generates ~30 % of its total energy from glucose oxidation during physiological condition.[21] Therefore, cardiac glucose uptake and utilization is crucial for proper cardiac function. This is germane to the fact that the atria, which is the pacemaker of the heart, significantly contributes to the overall cardiac function. Although the rate of glucose utilization in the heart is greater than in other tissue, little is known about glucose metabolism in the atria during both healthy and disease states.[20] Glucose transport into the cell is the rate limiting step of glucose utilization and is regulated by a family of membrane proteins known as Glucose Transporters (GLUTs).[289] Although GLUT4 (from the class I of GLUTs) is the main cardiac isoform (approximately 70 % of the total cardiac GLUTs), recent evidence suggests that GLUT8, one of the most recently discovered isoforms in the class III, is also expressed in the heart [27;29-31]. The GLUT8 mRNA expression is reported to be the highest (i.e., ~7% of total GLUTs) in the murine left ventricle, following GLUT4 and GLUT1. In addition, it has been reported that there was a significant upregulation of GLUT8 protein expression in the left ventricle of GLUT4 knock out mouse.[237] However,

there is no study that relatively quantifies the abundance of GLUT8 protein expression in the heart. Although GLUT8 has been reported to be an insulin-dependent isoform in blastocysts [31], its functional role in the myocardium is yet to be determined. Whereas some other isoforms have been referred to as basal GLUTs located primarily at the cell surface (i.e., GLUT1, GLUT12), the translocation of the main GLUT protein, GLUT4, from an intracellular sequestration inactive site to the plasma membrane (active site) is largely regulated by insulin-dependent processes, although other factors can alter myocardial glucose transport [24;290]. Importantly, GLUT4 trafficking has been shown to precede glucose transport in the insulin-sensitive tissues [291-293]. In skeletal muscle, following insulin stimulation, activation of IRS-1 protein induces the activation of several kinases, which in turn recruit the pivotal serine/threonine protein kinase, namely, Akt. The activated Akt phosphorylates a downstream protein AS160 (an Akt substrate protein of 160 kDa) which is essential for the exocytosis of GLUT4 to the plasma membrane [271]. Although the downstream insulin signaling pathway has been studied in the skeletal muscle, it is yet to be characterized in the heart, especially in the atria. Better understanding of the role and regulation of glucose transport in the healthy and diabetic atria will give novel insights in understanding the pathophysiology of diabetes.

It has been well documented that there is a regional heterogeneity between the atria and ventricles regarding their structure and function. Therefore, one could hypothesize that the differences in contraction and flow distribution pattern between atria vs. ventricle may also contribute to the regional metabolic heterogeneity [33;294]. However, the majority of the studies that have investigated cardiac energetics have studied global or left ventricular changes [37;295]. A recent study from our group suggested an impairment of

glucose transport in the atria of a canine model of heart failure [294]. However, in this model, heart failure was induced by rapid ventricular pacing, which may have had a profound effect on metabolism. Therefore, the role of glucose uptake and utilization in the healthy and diseased atria has received little attention. In the current study, we hypothesized that 1) the major cardiac GLUT isoform, GLUT4, and the novel GLUT isoform, GLUT8, will be regulated by insulin in the atria and 2) GLUT translocation will be impaired during diabetes. The enclosed study provides novel insights into the expression and regulation of insulin-sensitive GLUTs in the atria, which could lead to a better understanding of the uptake of glucose, a major metabolic substrate for the heart.

## MATERIALS AND METHODS

### Animals

Healthy and insulin-deficient type 1 diabetic (T1Dx) FVB/N mice were used, as previously described [37;295-297]. Briefly, type 1 diabetes was induced at 10–12 weeks of age by 3 consecutive doses of freshly prepared steptozotocin (STZ, 65-95 mg/kg IP every 48 hours, diluted in citrate buffer), while the control group received placebo injection (citrate buffer). All mice were fed a standard diet to maintain body weight (10 % kcal from fat) for the duration of the study. Diabetes was confirmed by measuring venous blood glucose concentration (facial vein) at baseline and every week for both groups, using a glucometer (Bayer Contour, Tarrytown, NY) on mice fasted overnight for 8 hours. Fasted plasma serum insulin was measured in duplicate using an ELISA kit (Millipore). Eight weeks after the induction of diabetes, animals were deeply anesthetized and the heart was rapidly removed and underwent a retrograde perfusion using a Langendorff apparatus.

All the procedures of this study were approved by the Oklahoma State University Institutional Animal Care and Use Committee (#VM-12-3).

**Myocyte isolation**

In order to measure protein expression of GLUTs and the insulin signaling pathway in atrial myocytes, isolation of myocytes from healthy adult rats was performed by retrograde perfusion of the heart using a Langendorff apparatus, as previously described [37;295-298]. Atrial and ventricular myocytes were obtained by enzymatic perfusion of collagenase (Worthington labs, NJ), where a minimum yield of 70% live cells were considered acceptable. Both atrial and ventricular myocytes were incubated with or without (basal) insulin (0.01µM, for 1 hour at room temperature).

**Protein extraction**

Total and crude extracts of membrane-enriched protein lysates of atrial and ventricular myocardium were prepared as previously described [37;295-297]. Briefly, total protein lysates from fresh atrial and ventricular myocytes/tissue were collected by incubating the myocytes/tissue with lysis buffer (RIPA, Thermo fisher Scientific). Samples were centrifuged at 3000 *g* for 25 min, and the supernatant was stored at − 80 °C until further analysis. Crude membrane protein extracts were collected from frozen tissue samples that were homogenized in buffer containing (mM): sucrose 210, NaCl 40, EDTA 2, HEPES 30, and protease inhibitor (Sigma, St. Louis, MO). The homogenate was incubated with sodium pyrophosphate 58 mM and KCl 1.17 mM. Crude membranes were then recovered by centrifugation at 100,000 *g* for 90 min at 4°C. Pellets were re-suspended with a cell lysis buffer (RIPA, Thermofisher Scientific). Samples were

centrifuged at 3000 *g* for 25 min, and the supernatant was stored at − 80 °C until further analysis.

**Western immunoblotting**

Equal amounts of protein (5-20 μg) were resolved in an 8-12 % SDS-polyacrylamide gel and electrophoretically transferred (BioRad) to a polyvinyl-idine fluoride membrane (Biorad), as previously described [37;294-297;299;300]. After blocking (1-5 % non-fat dry milk or 2 % goat serum albumin), membranes were incubated with optimally diluted primary antibodies overnight (polyclonal rabbit anti-human GLUT4, 1:750, AbD Serotec 4670-1704; polyclonal rabbit anti-human GLUT8, 1:500, Bioss bs-4241R; monoclonal rabbit anti-mouse total Akt, 1:1000, Cell Signaling 4061; monoclonal rabbit anti-human phosphorylated Akt s473, 1:1000, Cell Signaling 4060; monoclonal rabbit anti-mouse phosphorylated Akt Th308, 1:1000, Cell Signaling 2965; monoclonal rabbit anti-human total AS160, 1:1000, Cell Signaling 2670 and polyclonal rabbit anti-human phosphorylated AS160, 1:1000, Cell Signaling 9611) followed by a 1 hour incubation of appropriate secondary antibodies conjugated to horseradish peroxidase (for total and phosphorylated Akt and AS160, Cell Signaling 7074, 1:2000, polyclonal goat anti-rabbit; for others, GE Healthcare NA934V, polyclonal donkey anti-rabbit). Primary antibodies were chosen based on their 100 % sequence homology with the protein of interest in rodents, and validated against a positive control (i.e., tissue, peptide). Antibody-bound transporter proteins were quantified by enhanced chemiluminescence reaction (KPL) and autoradiography. Band density and molecular weight were quantified using GelPro Analyzer (Media Cybernetics). The data was expressed relative to appropriate controls. Equal protein loading was confirmed by reprobing each membrane

with Calsequestrin monoclonal IgG (Thermo-Scientific PA1-903, 1:2500, polyclonal rabbit anti-dog).

**Quantification of GLUT translocation to the cell surface**

Following a 1 hour Langendorff perfusion (with and without 0.7 nm insulin), both healthy and T1Dx atria and ventricles were photolabeled with the cell surface impermeant biotinylated bis-glucose photolabeling reagent (bio-LC-ATB-BGPA, 300 μM, Toronto Research Chemicals, ON, Canada), of which the hexose group interacts specifically with the extracellular binding site of GLUT. The photolabeled reagent was infused into the intact heart through the aorta before cross-linkage to cell surface GLUTs using a Rayonet photochemical reactor (340 nm, Southern New England UV), as previously described [37;295;301]. Protein extraction was immediately followed with homogenization and ultracentrifugation (227,000 g, 50 min at 4°C). Recovery of photolabeled (cell surface) GLUTs from total cardiac membranes (200 μg) was achieved using streptavidin isolation (bound to 6% agarose beads) to facilitate separation of non-cell surface GLUTs ("unlabeled" or intracellular fraction that remains in the supernatant) from cell surface GLUTs ("labeled" or sarcolemmal fraction). The labeled GLUTs were then dissociated from the streptavidin by boiling in Laemmli buffer for 30 min prior to SDS-PAGE and subsequent immunoblotting with GLUT antibody. Proteins from the labeled fraction were quantified by densitometry relative to the positive control, as previously described [37;295;299;300].

### Statistical analysis

Normality and homogeneity of data were tested using Shapiro-wilk and Levene test, respectively. Differences between means were assessed using Student's t-tests, one or two-way analysis of variance (treatment and/or tissue) for *in vitro* measurements, as appropriate. Repeated measured 2 way ANOVA was performed with Student Newman Keuls post-hoc test for the *in vivo* measurements. If not normally distributed, the data was analyzed with a Mann-Whitney or Friedman test. Correlations were analyzed by linear regression. Statistical significance was defined as $P<0.05$. Data are presented as mean ± SE**.**

## RESULTS

### Regional heterogeneity of GLUT in the healthy myocardium

Since glucose transport has not been well characterized in the atria, we first quantified total GLUT-4 and -8 protein content in the healthy myocardium. Our results indicated a significant regional heterogeneity of both the GLUTs in the atria vs. the ventricle. Total GLUT4 protein content was significantly greater in the isolated rat atrial myocytes compared to the ventricle (Fig 1A). In contrast, there was significantly greater GLUT8 content in the healthy ventricle compared to the atria (Fig 1B). In order to investigate the insulin sensitivity of the GLUTs in the healthy atria, we then measured protein content of GLUT-4 and -8 upon insulin stimulation in isolated rat atrial myocytes. Our results indicated an increase in total GLUT-4 and -8 total protein content (by 44 % and 60 % respectively, $P<0.05$) upon insulin stimulation (Fig 1C and 1D). In order to measure GLUT translocation to the cell surface, the rate limiting step in glucose uptake, we used

the biotinylation photolabeled assay in the intact perfused mouse heart to reliably quantify the proportion of active cell surface GLUTs at the atrial and ventricular cell surface (Fig 1E and 1F). Our results confirmed the regional heterogeneity of GLUT4 and GLUT8 in the atrial and ventricular cell surface, showing significantly greater cell surface GLUT4 content in the atria and greater cell surface GLUT8 content in the ventricle respectively (Fig 1E and 1F). To further determine whether GLUT-4 and -8 are insulin-sensitive, the biotinylation photolabeled assay was performed in the heart perfused with or without (basal) physiological concentration of insulin. Upon insulin stimulation, there was significant increase in cell surface GLUT-4 and -8 content in both the atrial (by 100 % and 240 % vs. basal condition, respectively, $P<0.05$) and ventricular cell surface (by 97 % and 40 % vs. basal condition, respectively, $P<0.05$), indicating that both GLUT-4 and -8 are insulin-sensitive in the atria and ventricle. Interestingly, the response to insulin was greater in the atria compared to the ventricle, with an increase in GLUT8 trafficking by 240 % vs. 40 % (compared to basal condition), respectively ($P<0.05$).

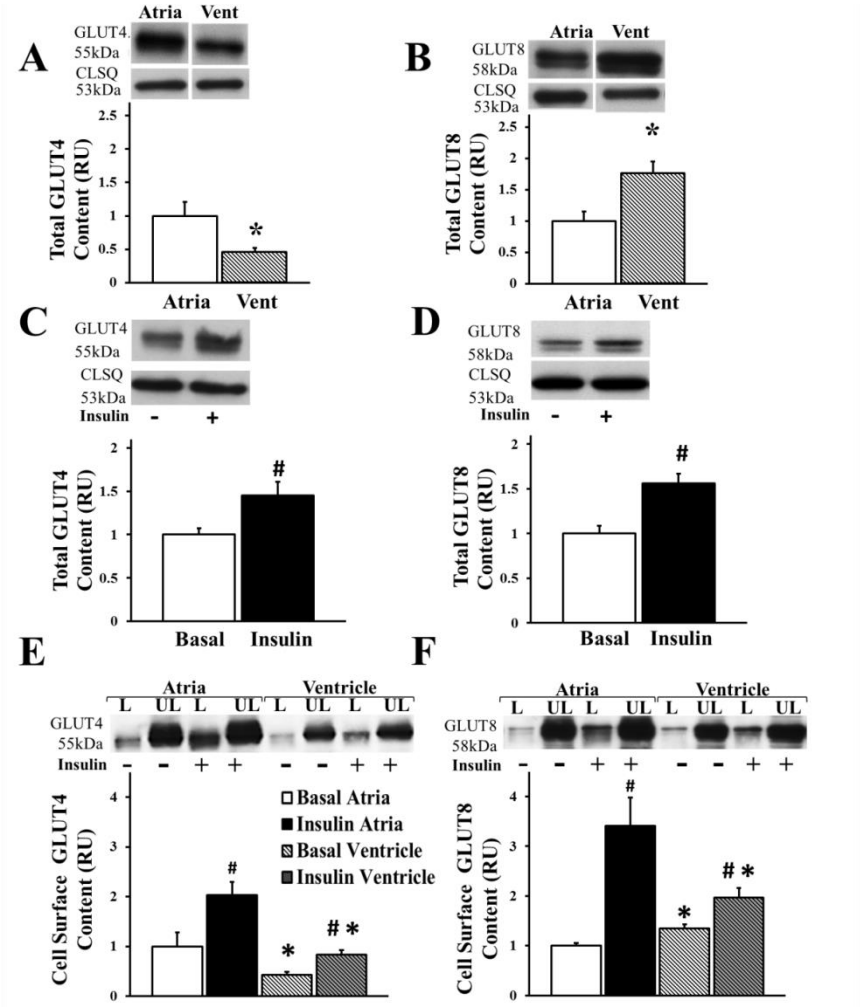


**Fig 1: Regional heterogeneity of the insulin-sensitive GLUT4 and GLUT8 in the healthy myocardium.**

**Higher total protein expression of A) GLUT4 and lower B) GLUT8 content in the healthy atrial compared to ventricular myocytes.** Top panels: representative Western blot from total lysate of isolated rat myocytes; calsequestrin (CLSQ) was used as a loading control; representative bands were obtained from the same membrane. Bottom panels: Mean ± SE of total GLUT protein content (values expressed relative to atria; n=6/group); * P<0.05 vs. atria. GLUT: glucose transporters. **Insulin stimulation increases C) GLUT4 and D) GLUT8 protein content** in the healthy atrial myocytes. Top panels: representative Western blot from total lysate of isolated rat myocytes, calsequestrin (CLSQ) was used as a loading control. Bottom panels: Mean ± SE of total GLUT protein content (values expressed relative to basal atria; n=8-11/group); # P<0.05 vs. basal. **Insulin stimulates E) GLUT4 and F) GLUT8 trafficking to the atrial cell and ventricular cell surface.** Top panels: representative Western blot. Bottom panels: Mean ± SE of cell surface GLUT protein content (values expressed relative to labeled basal atria; n=3-4/group); # P<0.05 vs. basal; *P<0.05 vs. atria. Methods: Cell surface GLUT measured using biotinylated photolabeling technique in the intact perfused mouse heart. L: Labeled (cell surface fraction); UL: Unlabeled (intracellular fraction).

**Analysis of downstream insulin signaling pathway in the healthy atria**

In order to investigate the mechanisms regulating the translocation of GLUT-4 and -8 to the atrial cell surface, we then explored the downstream insulin signaling pathway, by incubating rat atrial myocytes with and without (basal) insulin. We reported a significant increase in the phosphorylation of Akt (at s473 and Th308 sites) and AS160 upon insulin stimulation (Fig 2A and 2C). In addition, we reported a significant positive linear correlation between total GLUT4 protein content (Fig 1C) and phosphorylation of Akt (Fig 2B), as well as between total GLUT protein content (Fig 1C and 1D) and AS160 activation (Fig 2D). However, no significant change was observed when phospho Akt (data not shown) and phospho AS160 (Fig 2C) were compared to total Akt and AS160, respectively. Collectively these data suggested that Akt and/or AS160 phosphorylation regulates GLUT-4 and -8 trafficking in the atria upon insulin stimulation.

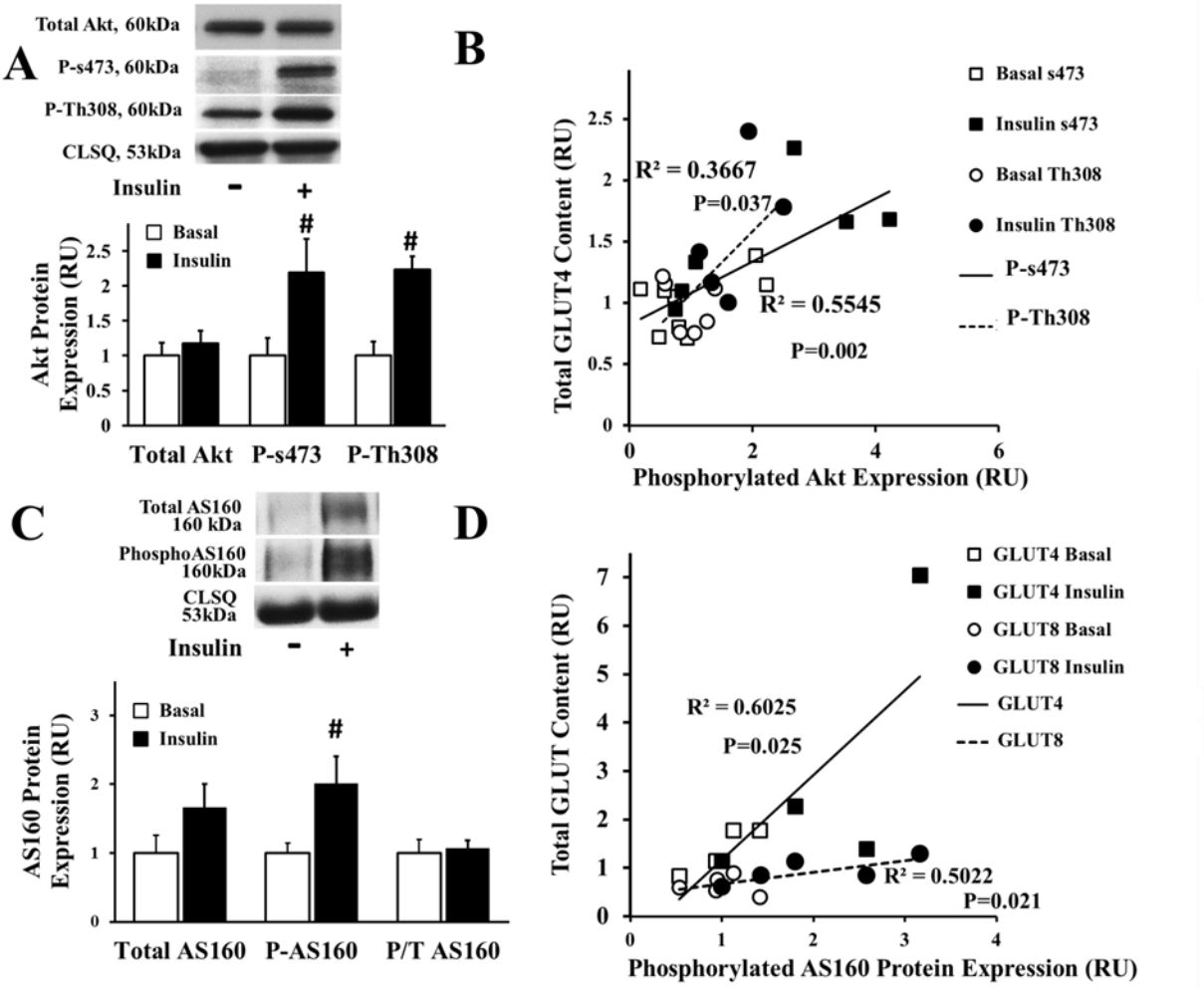


**Fig 2: Analysis of the downstream insulin signaling pathways in the healthy atria.**

**A) Insulin stimulates phosphorylation of Akt at s473 and Th308 site** in atrial myocytes. Top panels: representative Western blot from total lysate of isolated rat atrial myocytes incubated with (0.01µM) and without (basal) insulin; calsequestrin (CLSQ) was used as a loading control. Bottom panels: Mean ± SE of protein expression (values expressed relative to basal; n=5/group); # P<0.05 vs. basal. **B) Significant linear positive linear correlation between Akt phosphorylation (at s473 and Th308 site) and GLUT4 expression** in the healthy atria. **C) Insulin stimulates phosphorylation of AS160** in atrial myocytes. Top panels: representative Western blot from total lysate of isolated rat atrial myocytes; calsequestrin (CLSQ) was used as a loading control. Bottom panels: Mean ± SE of protein expression (values expressed relative to basal; n=6-8/group); # P<0.05 vs. basal. **D) Significant linear correlation between AS160 phosphorylation and GLUT-4 and -8 expression** in the healthy atria.

**Alteration in GLUT expression and trafficking during type 1 diabetes**

In order to investigate alterations in glucose transport during diabetic conditions, we used an insulin-deficient diabetic animal model (T1Dx). As expected, within 1 week after injection, STZ-treated animals developed hyperglycemia (i.e., [glucose]>200 mg/dl), which persisted throughout the experimental period; while the control group remained euglycemic (Fig 3A). There was no difference in body weight between groups, although both control and diabetic mice weighted more ($P<0.05$) at the end of the experimental period (Fig 3B). In addition, serum insulin level was significantly lower in diabetic compared to control mice, confirming that STZ administration destroyed the beta cells of the pancreas (Fig 3C).

We then quantified GLUT-4 and -8 total protein expressions in a crude membrane enriched extract of the mouse atria (Fig 4A and 4B). Our results indicated a significant decrease in GLUT-4 and -8 total protein content in the type 1 diabetic group compared to healthy controls. To accurately differentiate plasma membrane-associated GLUT4 from intracellular membrane-associated GLUT4, we used the biotinylated assay in the intact perfused atria. We observed a down-regulation of atrial cell surface GLUT-4 and -8 by 70 % and 88 %, respectively, in diabetic animals compared to healthy controls ($P<0.05$, Fig 4C and 4D). Furthermore, we quantified the proportion of GLUT located at the cell surface compared to the intracellular pool for both GLUT-4 and -8 (Fig 4E and 4F). As expected, under basal conditions, GLUT4 cell surface fraction was 26 % and 17 % of the intracellular fraction during healthy and diabetic condition, respectively ($P<0.05$). Similarly, under basal conditions, GLUT8 cell surface fraction was 25 % and 5 % of the intracellular fraction during healthy and diabetic condition respectively ($P<0.05$, Fig 4E and 4F). Therefore, our results demonstrated a

significant down-regulation of total protein expression and trafficking to the atrial cell surface of GLUT-4 and -8 during type 1 diabetic condition.

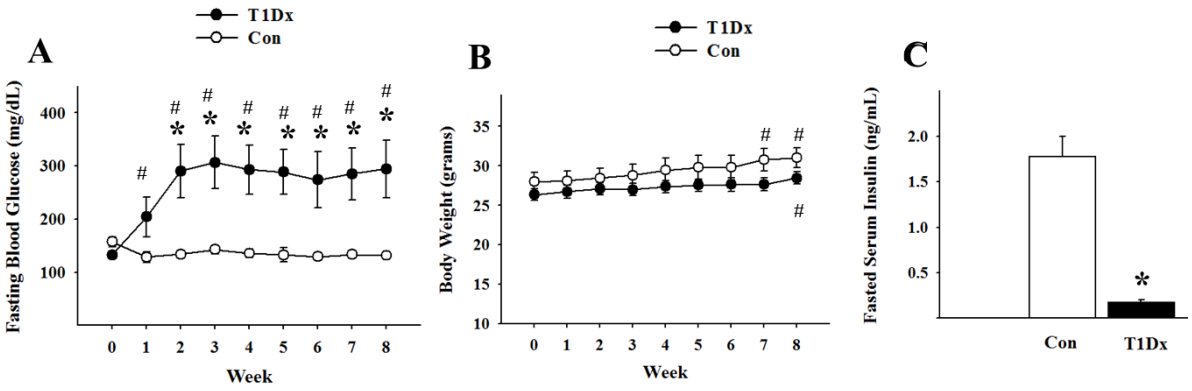


**Fig 3: Validation of the insulin-deficient (type 1) diabetic animal model.**

**A) Mean ± SE venous blood glucose concentration** obtained at baseline and up to 8 weeks in type 1 diabetic (T1Dx) and age-matched control (Con) mice (n=9-11/group). **B) Mean ± SE body weight** obtained at baseline and up to 8 weeks after induction of type 1 diabetes (n=9-11/group). **C) Mean ± SE serum insulin concentration** obtained at 8 weeks after induction of type 1 diabetes (n=6-8/group). T1Dx: type 1 diabetic; Con: control; *P<0.05 vs. control; # P<0.05 vs. baseline.

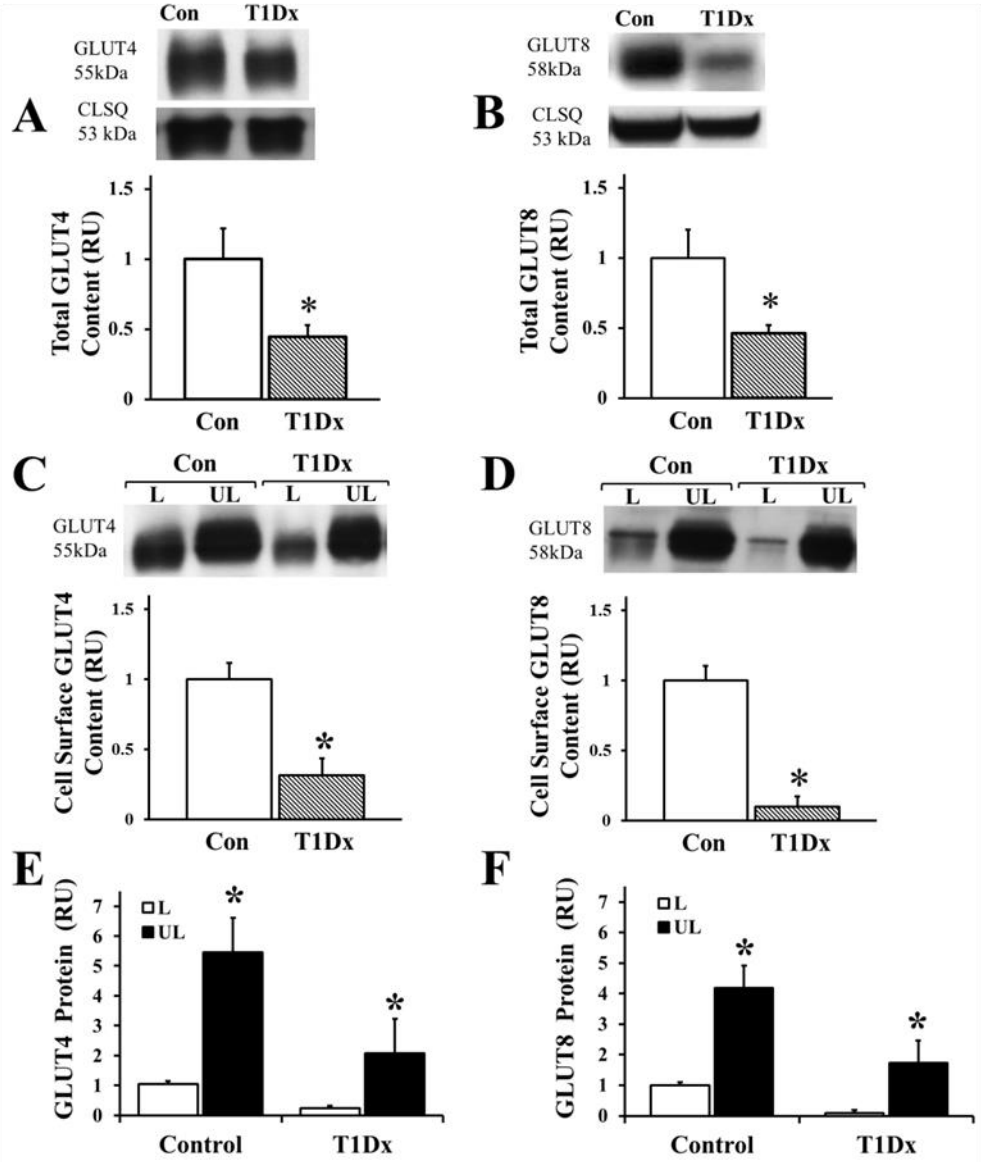


**Fig. 4: Alteration of the trafficking of the insulin-sensitive GLUTs in the diabetic atria.**

**Decreased atrial A) GLUT4 and B) GLUT8 content during type 1 diabetes** (T1Dx). Top panels: representative Western blot from crude membrane extract of perfused mouse atria, calsequestrin (CLSQ) was used as a loading control. Bottom panels: Mean ± SE of GLUT protein content (values expressed relative to control; n=7-8/group). **Type 1 diabetes decreased C) GLUT4 and D) GLUT8 trafficking to the atrial cell surface.** Top panels: representative Western blot. Bottom panels: Mean ± SE of cell surface GLUT protein content (values expressed relative to control; n=4-5/group). **Majority of E) GLUT4 and F) GLUT8 is intracellular under basal conditions** (mean ± SE, values expressed relative to control labeled, n=5/group). Methods: Intact perfused mouse hearts were photolabeled with bio-LC-ATB-BGPA to determine the amount of cell surface (L: labeled fraction) and intracellular (UL: unlabeled fraction) content. T1Dx: type 1 diabetic; Con: control; * $P<0.05$ vs. control.

**Role of insulin in regulating GLUT trafficking to the atrial cell surface during type 1 diabetes**

Despite intensive research during the last few decades (primarily in skeletal muscle and adipose tissue), the pathogenic cause of altered glucose transport during diabetes remains elusive. Therefore, we further analyzed the possible alteration in the downstream insulin signaling pathway under diabetic conditions, to understand whether or not the impairment in GLUT trafficking can be attributed to the defective insulin signaling pathway. Interestingly, our results indicated no alteration in the phosphorylation of either Akt or AS160 in diabetic subjects (Fig 5A and 5B) compared to the controls. Using the biotinylation assay in the intact perfused mouse atria, we further observed a rescued trafficking of both GLUT-4 and -8 to the atrial cell surface upon *in vitro* insulin stimulation in the diabetic atria (Fig 5C and 5D). Collectively, the data suggested an intact insulin signaling pathway in the T1Dx atria.

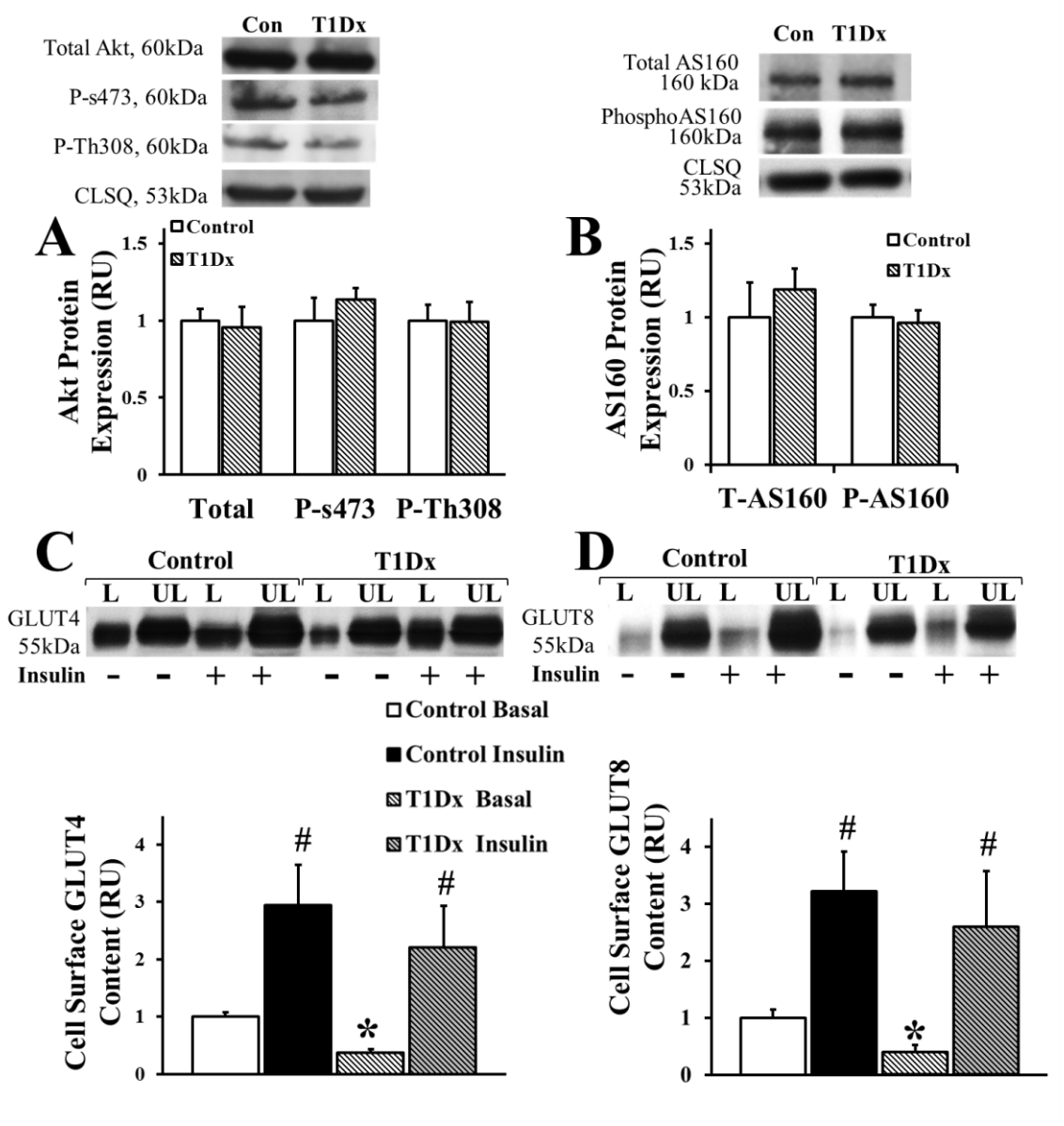


**Fig. 5: Intact insulin signaling pathway in the atria of insulin-deficient diabetic animals.**

**Type 1 diabetes (T1 Dx) did not alter A) Akt or B) AS160 phosphorylation in the atria**. Top panels: representative Western blot from total lysate of mouse atria; calsequestrin (CLSQ) was used as a loading control. Bottom panels: Mean ± SE of protein expression (values expressed relative to control; n=4-5/group). **Insulin stimulates C) GLUT4 and D) GLUT8 trafficking to the atrial cell surface in type 1 diabetic (T1 Dx) subjects**. Top panels: representative Western blot. Bottom panels: Mean ± SE of cell surface GLUT protein content (values expressed relative to control basal labeled; n=4-6/group). Methods: Intact mouse hearts were perfused with and without insulin, and photolabeled with bio-LC-ATB-BGPA to determine the amount of cell surface (L: labeled fraction) and intracellular (UL: unlabeled fraction) content. T1Dx: type 1 diabetic; * $P<0.05$ vs. control; # $P<0.05$ vs. basal.

## DISCUSSION

Although the heart is one of the main organs to utilize glucose as a metabolic substrate, very little is known about atrial glucose metabolism in both healthy and diseased conditions. Our data demonstrates that 1) a regional heterogeneity exists between GLUT4 and 8 expression in atria vs. ventricle; 2) both GLUT-4 and -8 are insulin-sensitive in the healthy atria; 3) diabetes impaired GLUT-4 and -8 trafficking to the atrial cell surface; and 4) insulin stimulation rescued GLUT translocation to the atrial cell surface during type 1 diabetes.

In order to sustain its high energy demand, the rate of glucose utilization in the heart is greater than in skeletal muscle, adipose tissue and lung, despite the ability of the myocardium to use other substrates (i.e., fatty acids, lactate, ketone bodies and amino acids) [20]. Glucose entry from the blood stream into the cell is the rate-limiting step in glucose utilization and occurs primarily via a group of facilitative glucose transporters (GLUT) [289]. Despite the crucial role of the atria as a pacemaker of the heart, studies on the role of glucose transport and utilization in the atria have been scarce. The majority of the studies that have investigated cardiac energetics have studied the global or left ventricular changes [32-37]. Due to the relatively small size of atria, specifically in rodent models (i.e., <30 mg), it is difficult to study membrane trafficking of GLUTs and, to our knowledge, we are the first to apply the biotinylation assay technique to the study of GLUTs in the atria. Please note that we previously reported that GLUT trafficking upon insulin stimulation is comparable between isolated rat ventricular myocytes and intact mouse ventricle [295]. Using the photolabeled technique in the intact perfused mouse heart, we demonstrated that GLUT4 translocated to the atrial cell surface upon insulin

stimulation, similar to findings reported in the ventricle [37;295]. We further observed in the current study a greater active cell surface GLUT4 content in the healthy atria compared to the ventricle under basal and insulin stimulated conditions. The finding of this study is concurrent with Ware *et al.* [294], which demonstrated regional pattern of total GLUT4 content in the chambers of the healthy myocardium. The mechanisms underlying this regional heterogeneity are not well elucidated. In order to sustain the faster contraction rate, atrial myocytes possess unique electrophysiological properties, including faster calcium release from the sarcoplasmic reticulum (SR), greater inositol 1,4,5-trisphosphate (IP3) content and greater calcium buffering abilities [302]. Since SR calcium handling regulates GLUT4 trafficking to cell surface in the myocardium,[295] one could speculate that these well-known differences in calcium homeostasis could explain the greater cell surface GLUT4 expression in the atria compared to the ventricle. This regional heterogeneity further underscores the importance to specifically study glucose metabolism in the tissue of interest and the fact that one cannot extrapolate findings from the ventricle to the atria.

Class I transporter GLUT4, the most extensively studied insulin-sensitive GLUT, is predominantly expressed in the adult heart (~70% of total myocardium GLUTs) and is thought to be responsible for insulin-stimulated glucose uptake [24]. However, in a study conducted by Katz *et al*., it has been reported that GLUT4 null mice do not develop hyperglycemia, suggesting that other GLUT isoforms could be involved in the regulation of whole-body glucose homeostasis [25]. GLUT8, one of the most recently discovered GLUT isoforms and member of the lesser known Class III, has been shown to be present in substantial quantities in the cardiac tissue and thus, may play a significant role in

glucose uptake [237]. However, whether or not GLUT8 is primarily located at the cell surface or in intracellular compartment is unclear. Although GLUT8 has been reported to be an insulin-dependent isoform in blastocysts and hepatocytes [30;31], its role in the heart has not been determined. Similar to GLUT4, our data demonstrated that GLUT8 translocates from an intracellular pool to the cell surface of the healthy myocardium upon insulin stimulation using the biotinylation assay. In addition, our findings also demonstrate that similar to GLUT4, GLUT8 is mostly intracellular under basal condition. This is in agreement with previous studies that reported that GLUT8 carries a $NH_2$-terminal di-leucine motif that directs the protein to an intracellular localization and has the potential to translocate to the plasma membrane [237]. Collectively, these data suggest that GLUT8 is a novel insulin-sensitive GLUT in the heart. In addition, in the present study, insulin stimulation increased GLUT8 translocation to the cell surface in the atria to a greater extent than in the ventricle, suggesting that GLUT8 primarily contributes to insulin-stimulated glucose uptake in the atria. Due to the diversity of the insulin-sensitive GLUTs, characterization of the relative abundance and role of these novel GLUT isoforms (primarily from the Class III) in the heart is an important step towards understanding how these GLUTs contribute towards maintaining myocardial glucose transport.

In all the insulin-sensitive tissues (i.e., striated muscle and adipose tissue), insulin signaling via PI3K/Akt pathways plays a key role in cardiac glucose uptake. As insulin binds to its receptor, the downstream insulin signaling pathway is activated, resulting in the phosphorylation of protein kinase Akt at threonine 308 (Th308) residues, which is located in the kinase domain of the Akt activation loop. The full activation of Akt

requires a further phosphorylation of serine 473 (s473) residues [268;269]. It is well documented that the phosphorylation of both sites (s473 and Th308) are crucial for GLUT trafficking. The activated Akt in turn phosphorylates AS160, which is the most distal signaling protein that has been implicated in insulin mediated GLUT translocation and has emerged as a key regulator of GLUT trafficking [271]. Under basal conditions, AS160 retains GLUT vesicles at the intracellular inactive pool [269]. The phosphorylation of AS160 allows the activation of RabGTP, which initiates the signal transduction pathway that is necessary for the docking and diffusion of GLUT vesicles on the plasma membrane [271]. It is important to note that, the initiation of the signaling cascade by the activation of the insulin receptor not only increases GLUT4 exocytosis at the membrane, it also attenuates GLUT4 endocytosis causing an enhanced re-distribution of GLUT4 protein at the plasma membrane, facilitating cellular glucose uptake [270]. Although this pathway has been well investigated in skeletal muscle and adipose tissue, the downstream signaling pathway that regulates glucose transport in the atria remains to be characterized. Our findings indicated that insulin stimulation increased the phosphorylation of Akt (at both s473 and Th308 sites) and AS160 in cardiac myocytes. We also demonstrated that the activation of downstream insulin signaling pathway was significantly correlated to the increased GLUT-4 and -8 protein expression in the atria. Due to the small size of the rodent atria, both membrane trafficking experiments and quantification of cytosolic proteins were not technically possible to perform in the same sample. Nonetheless, findings from this study provide novel insights in understanding the regulation of the insulin-sensitive GLUT isoform in the atria. In addition to Akt activating GLUT trafficking, there is an increasing evidence that Rac1 activation is

essential for GLUT4 trafficking to the cell surface and insulin-stimulated glucose uptake in skeletal muscle [303-307]. In addition, Akt-independent signaling pathway through TUG (tether containing a UBX domain for GLUT4) has been reported to be essential in maintaining protein stability and trapping the GLUT4 containing storage vesicles in the Golgi matrix in skeletal muscle and adipose tissue [308-311]. Although cardiac GLUT trafficking may be regulated by similar Akt-dependent and Akt-independent pathways, additional studies are required to fully characterize the regulation of GLUT trafficking in the myocardium, including in the atria.

STZ administration destroys the beta cell of the pancreas and induces a hyperglycemic state. As such, it is a well-established experimental model of insulin-deficient (type 1) diabetes [37;295;296]. Although alterations of metabolic efficiency have been described in the ventricle of this animal model [37;251;295;296], whether diabetes induces alterations in glucose metabolism in the diabetic atria is unknown. Our data demonstrated a decrease in total GLUT-4 and -8 protein content in the atria of STZ-induced diabetic animals. Further, using the biotinylated photolabeled assay, our data demonstrated a 70% and 88% decrease in cell surface GLUT-4 and -8 protein content in the diabetic atria, respectively. This finding is consistent with the previous studies that reported impaired GLUT4 translocation in the ventricle of type 1 diabetic rats [251]. In addition, the role and regulation of GLUT8 during insulin-deficient diabetic conditions have received scarce attention. Gorovits *et al*. reported that short-term STZ-induced diabetes significantly decreased GLUT8 mRNA levels in the liver and suggested that GLUT8 contributes to glucose homeostasis [30]. Similar quantification of GLUT mRNA expression in the atria could be an avenue for future investigations. In another study, Piroli *et al*. reported the

alteration of protein expression and disruption of subcellular distribution of GLUT8 in the hippocampus of the type 1 diabetic rat [263;264]. However, to our knowledge, there is no study that has investigated the regulation of GLUT8 in the cardiac tissue, more specifically in the atria. Our findings demonstrate that diabetes induces an impairment in GLUT8 trafficking to the cell surface of the atria.

We further investigated whether the down-regulation of cell surface GLUT-4 and -8 protein expressions was attributed to a defective insulin signaling pathway and/or due to a lack of insulin production in the atria of type 1 diabetic subjects. We reported no significant impairment in the phosphorylation of Akt (both s473 and Th308) and AS160, suggesting that the downstream insulin signaling pathway is not altered. This finding is consistent with a study by Laviola and colleagues, who reported that there was no significant decrease in the phosphorylation of Akt at s473 and Th308 sites in the ventricle of STZ induced diabetic rats compared to controls [278]. To further confirm these findings, we stimulated the diabetic atria with and without insulin (at physiological concentration), and our results demonstrated that insulin stimulation rescued cell surface GLUT4 and 8 protein expression in the diabetic atria, suggesting that the down-regulation of both insulin-sensitive GLUTs was mostly attributed to the lack of insulin production (following beta cell destruction) rather than an existing defect in the insulin signaling pathway in the atria of type 1 diabetic animals.

## CONCLUSIONS

Using a cell surface biotinylation assay, we demonstrated that the major GLUT isoform, GLUT4, and the novel GLUT8 isoform are both insulin-sensitive transporters. Our data

further suggested that GLUT translocation to the cell surface is modulated by the downstream insulin signaling pathway via Akt and/or AS160 phosphorylation. We further demonstrated that diabetes impairs the trafficking of both GLUT-4 and -8, which was rescued by insulin stimulation in the diabetic atria. These alterations in atrial glucose transport may induce perturbations in energy production and could provide a metabolic substrate for atrial fibrillation, the most common abnormal heart rhythm in the world. Therefore, better understanding of the regulation of glucose transport may lead to the discovery of novel therapeutic targets for the treatment of cardiovascular complications associated with diabetes, including atrial fibrillation.

**ACKNOWLEDGMENTS**

We would like to thank Dr. Emilie Martinez and Jill Murray for their excellent technical assistance and animal care.

# CHAPTER IV

# THE REGULATION OF THE INSULIN-SENSITIVE GLUCOSE TRANSPORTERS ARE ALTERED DURING INSULIN RESISTANCE-INDUCED ATRIAL FIBRILLATION

## ABSTRACT

Diabetes and obesity have been identified as major risk factors for atrial fibrillation (AF). However, whether a metabolic substrate underlies AF is unknown. Glucose transport into the cell via Glucose Transporters (GLUTs) is the rate-limiting step of glucose utilization. Although GLUT4 is the major isoform in the heart, GLUT8 has recently emerged as a novel cardiac isoform. However, its role in the heart is not well known. We hypothesized that GLUT-4 and -8 translocation to the atrial cell surface will be impaired during insulin- resistance-induced AF. AF was induced by transesophageal atrial pacing in healthy and long-term high-fat-diet (HFD)-induced insulin-resistant (IR) mice. Expression of GLUTs and key proteins involved in the insulin signaling pathway was measured by Western blot in atrial tissue. Active cell surface GLUT content was measured using the state-of-the-art biotinylated photolabeled assay in the perfused heart. After 6 months on a HFD, mice were obese and hyperglycemic, and developed insulin resistance compared to mice on a control diet. IR animals demonstrated an increased susceptibility and propensity for AF. In the IR atria, active cell surface and total GLUT4 content was down-regulated (by 66% and 40%, respectively, $P<0.05$) under basal condition. Long-term HFD-induced IR resulted in impairment in Akt and AS160 phosphorylation, which was significantly correlated with GLUT4 protein content in the atria. These data suggest an impairment of the insulin signaling pathway, which was further confirmed by altered trafficking of both GLUT-4 and -8 to the cell surface upon insulin stimulation in the IR atria. In conclusion, our data suggest that: 1) IR increases the vulnerability to AF; 2) GLUT-4 and -8 trafficking is altered in the IR atria due to impairments in the insulin signaling pathway. Therefore, alterations in atrial glucose

transport may induce perturbations in energy production and could provide a metabolic substrate for atrial fibrillation during insulin-resistance and obesity.

## INTRODUCTION

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia in the world, affecting 2% of the entire world population, and has been identified as an increasing public health risk as well.[7] AF considerably increases the risk factors for heart failure, ischemic heart disease and stroke.[7-10] Although AF is thought to be multifactorial and its pathogenesis remains elusive, recent epidemiological studies have identified type 2 diabetes and obesity as major and independent risk factors of AF.[15;19;185;186;189;190;312-315] Meta-analysis studies have reported that diabetic and obese populations have increased risk of AF by 40% and 49% respectively.[184;316]

Diabetes mellitus is a global epidemic health problem currently affecting approximately 30 million people in the US.[1] Non-insulin-dependent type 2 diabetes affects approximately 90% of the total diabetic population. Type 2 diabetes significantly increases the risk factors for various cardiac complications including myocardial infarction and AF. Importantly, it has been reported that cardiac complications occur even in the pre-diabetic state (marked by glucose intolerance, normal glycemia to mild hyperglycemia and insulin resistance), which poses a significant medical and economic burden on the society.[317-320] According to the American Diabetes Association, 37% of American adults over the age of 20 suffer from pre-diabetes.[321] In addition, reports from prospective clinical studies state that each year approximately 11% of the pre-diabetic population progress to type 2 diabetes.[321] Results from meta-analysis studies suggest that pre-diabetic patients have 1.5 folds increased risk of cardiovascular diseases compared to

2-4 folds increased in risk in diabetes patients.[322;323] Although the risk factor of a diabetic patient to develop AF is 1.4-2.2 compared to a healthy non-diabetic person,[185;189;190;312;318] the underlying pathophysiologic mechanisms linking diabetes and AF are not well understood. Insulin resistance and hyperglycemia are the hallmark of type 2 diabetes, caused by a lack of insulin action in the insulin-sensitive tissues (skeletal muscle, adipose tissue and the heart) resulting in dysfunctional glucose uptake.[4;113;324;325] Interestingly, insulin resistance has also been reported to be more prominent in patients with AF compared to those with sinus rhythm.[19] Insulin resistance coupled with inadequate glycemic control during diabetes has often been associated with AF episodes.[5;13;17;19] In addition, AF attacks in non-insulin-dependent type 2 diabetes patient have been reported to be preceded by hyperglycemic states.[16] Together the results of all these studies suggest a strong correlation of insulin resistance and an inadequate glycemic control with AF. Since the rate of glucose uptake and utilization in the heart is higher than any other tissue, dysfunctional glucose uptake (i.e., lack of energy substrate) in the cardiac tissue, specifically the atria (containing the pacemaker of the heart) could be a major pathophysiologic factor contributing to cardiac arrhythmia during insulin resistance.[14;18;19]

Glucose transport from the blood stream into the cell is conducted by a facilitative diffusion process involving a specialized family of Glucose Transporters (GLUTs) and is the rate limiting step of glucose utilization.[289] Although they are many isoforms, GLUT4 and GLUT8 have been identified as the major insulin sensitive isoforms in healthy cardiac tissue.[27;29-31;326] However, the expression and regulation of these isoforms in the heart during insulin resistant type 2 diabetes remain unclear. In adipocytes, it has been

reported that there is a substantial down regulation of GLUT4 protein and mRNA levels during type 2 diabetes and obesity.[252;253] In the skeletal muscle, no change in the total level of GLUT4 has been reported between type 2 diabetic and non-diabetic patients.[252;256;257;260;327;328] Although the level of GLUT4 was unchanged in skeletal muscle, impairment in GLUT4 vesicular trafficking has been reported in these tissues.[243;260] In contrast to the extensive research carried out on GLUT4, the role of GLUT8 in many tissues still needs to be investigated. Gorovits *et al.* reported that GLUT8 mRNA decreased during hypoinsulinemia and increased during hyperinsulinemia in the liver, suggesting a regulatory role of insulin in GLUT8 mRNA expression.[30] To the best of our knowledge, there has been no study that investigates the regulation and expression of these GLUT isoforms in the atria and in the context of insulin resistance and AF.

Although insulin-resistant type 2 diabetic patients have an increased risk to develop AF, no causal link has been established between these 2 insidious conditions. Therefore, in this study, we hypothesized that 1) mice fed a high-fat-diet will be more vulnerable to AF induction with higher propensity to AF and 2) GLUT trafficking and its downstream insulin signaling pathway will be impaired in the atria of insulin resistant mice. Our goal is to determine whether alterations in atrial glucose transport could constitute a novel metabolic substrate that underlies insulin resistance-induced AF.

# MATERIALS AND METHOD

## Insulin-Resistant Animals

Five-week-old C57BL/6 male mice (Charles River Laboratories) were used. After a one-week acclimatization period, mice were fed either a control diet (9.4% kcal from fat - soybean oil, 14.7% kcal protein and 75.9% kcal from carbohydrate; AIN-93M) or a high-fat diet (60% kcal from fat (soybean oil and lard), 20% kcal protein and 20% kcal from carbohydrate; Research Diets, Inc.; D12492) for 24 weeks. Both diets had comparable amounts of vitamin and minerals. Body weights and blood glucose levels were recorded every 4 weeks throughout the experimental period. At 24 weeks, the mice were anesthetized using 3% isoflurane and euthanized by excision of the heart followed by either flash freezing the heart in liquid $N_2$ or Langendorff perfusion. Due to experimental constraints, few of the control animals were of age matched FVB/N strain. All procedures were approved by the Oklahoma State University Institutional Animal Care and Use Committee.

## Blood Chemistry

After 8 hours of fasting, blood glucose was measured using a handheld glucometer (Bayer Contour, Tarrytown, NY). Venous blood was collected by lancing the facial vein. At the end of the study period (24 weeks) fasting serum insulin was measured using commercially available sandwich ELISAs (Serum Insulin ELISA kit, Millipore, MO). All samples were analyzed in duplicate.

**Intraperitoneal Glucose Tolerance Test**

At 24 weeks, an intraperitoneal glucose tolerance test was performed. Following a 8 hour fast, non-sedated mice received 1.5 g/kg glucose bolus intraperitoneally and blood glucose concentrations were measured at time points from 0, 15, 30, 45, 60 and 120 min. Changes in blood glucose was determined by calculating the area under the curve (AUC). Lower AUC values reflect more efficient glucose clearance.

**Intraperitoneal Insulin Tolerance Test**

At 24 weeks, an intraperitoneal insulin tolerance test was performed. Following a 8 hour fast, non-sedated mice received 0.75 U/kg insulin (Novolin, Novonordisk) bolus intraperitoneally and blood glucose concentrations were measured at time points from 0, 15, 30, 45, 60 and 120 min. Changes in blood glucose was determined by calculating the area under the curve (AUC). Lower AUC values reflect more efficient glucose clearance.

**Atrial Fibrillation Induction**

All of the mice were fully electrophysiologically examined by closed chest transesophageal recording and atrial stimulation under anesthesia (ketamine/xylazine IP injection). A 2.2 Fr 6-polar catheter was inserted through the esophagus and positioned near the left atrium. Positioning of the appropriate bipolar pair of electrodes adjacent to the left atrium was accomplished by sequentially connecting each bipolar pair to an iPhone in the AliveCor application mode. An iPhone with the AliveCor application was also used to record the body surface ECG by inserting two metal pins under the mouse's skin. Heart rate and body temperature was recorded during the entire procedure for all of the animals. The current, in milliamperes, was determined by connecting the terminals of

the predetermined pairs of electrodes to the battery powered (DC) stimulator (each pulse duration = 1millisecond), which provided pace capture of the atrium. This threshold was recorded and increased by 2X to ensure constant atrial pacing at a rate faster than the baseline rate. By switching the same terminals to the AC-powered stimulator the atrial pacing threshold (in volts) was determined. This voltage threshold was recorded and at 2X threshold constant atrial pacing was achieved and noted. In order to induce atrial fibrillation (AF), the same site was subjected to burst pacing for 10 seconds at 2 different frequencies - 20 Hz (1200/min) and 40 Hz (2400/min) - twice. The same protocol was applied for both the control and the IR animals.

Duration (in seconds), frequency (number of incidences per animal) of atrial fibrillation (defined as an absence of a defined P-waves and presence of irregularly irregular R-R interval), atrial tachycardia (increased heart rate without the defined P wave) and sick sinus syndrome (intermittent tachycardia and sinus bradycardia) were recorded after each burst pacing from the surface ECG traces. The total duration and frequencies of the atrial arrhythmias were recorded.

**Protein Extraction**

Total and crude extracts of membrane-enriched protein lysates of atrial myocardium were prepared as previously described.[37;295-297] Briefly, total protein lysates from fresh atrial tissue were collected by incubating the tissue with lysis buffer (RIPA, Thermo fisher Scientific). Samples were centrifuged at 3000 g for 25 min, and the supernatant was stored at − 80 °C until further analysis. Crude membrane protein extracts were collected from frozen tissue samples that were homogenized in buffer containing

(mM): sucrose 210, NaCl 40, EDTA 2, HEPES 30, and protease inhibitor (Sigma, St. Louis, MO). The homogenate was incubated with sodium pyrophosphate 58 mM and KCl 1.17 mM. Crude membranes were then recovered by centrifugation at 100,000 g for 90 min at 4°C. Pellets were re-suspended with a cell lysis buffer (RIPA, Thermofisher Scientific). Samples were centrifuged at 3000 g for 25 min, and the supernatant was stored at − 80 °C until further analysis.

**Western Immunoblotting**

Equal amounts of protein (5-20 μg) were resolved in an 8-12% SDS-polyacrylamide gel and electrophoretically transferred (BioRad) to a polyvinyl-idine fluoride membrane (Biorad), as previously described.[37;294-297;299;300] After blocking (1-5% non-fat dry milk or 2% goat serum albumin), membranes were incubated with optimally diluted primary antibodies overnight (polyclonal rabbit anti-human GLUT4, 1:750, AbD Serotec 4670-1704; polyclonal rabbit anti-human GLUT8, 1:500, Bioss bs-4241R; monoclonal rabbit anti-mouse total Akt, 1:1000, Cell Signaling 4061; monoclonal rabbit anti-human phosphorylated Akt s473, 1:1000, Cell Signaling 4060; monoclonal rabbit anti-mouse phosphorylated Akt Th308, 1:1000, Cell Signaling 2965; monoclonal rabbit anti-human total AS160, 1:1000, Cell Signaling 2670 and polyclonal rabbit anti-human phosphorylated AS160, 1:1000, Cell Signaling 9611) followed by a 1 hour incubation of appropriate secondary antibodies conjugated to horseradish peroxidase (for total and phosphorylated Akt and AS160, Cell Signaling 7074, 1:2000, polyclonal goat anti-rabbit; for others, GE Healthcare NA934V, polyclonal donkey anti-rabbit). Primary antibodies were chosen based on their 100% sequence homology with the protein of interest in rodents, and validated against a positive control (i.e., tissue, peptide). Antibody-bound

transporter proteins were quantified by enhanced chemiluminescence reaction (KPL) and autoradiography. Band density and molecular weight were quantified using Gel Pro Analyzer (Media Cybernetics). The data was expressed relative to appropriate controls. Equal protein loading was confirmed by reprobing each membrane with Calsequestrin monoclonal IgG (Thermo-Scientific PA1-903, 1:2500, polyclonal rabbit anti-dog).

**Quantification of GLUT Translocation to the Cell Surface**

Following a 1 hour Langendorff perfusion (with and without 0.7 nm insulin), both healthy and insulin-resistant (IR) atrium were photolabeled with the cell surface impermeant biotinylated bis-glucose photolabeling reagent (bio-LC-ATB-BGPA, 300 μM, Toronto Research Chemicals), of which the hexose group interacts specifically with the extracellular binding site of GLUTs. The photolabeled reagent was infused into the intact heart through the aorta before cross-linkage to cell surface GLUTs using a Rayonet photochemical reactor (340 nm, Southern New England UV), as previously described.[37;295;301] Protein extraction was immediately followed with homogenization and ultracentrifugation (227,000 g, 50 min at 4°C). Recovery of photolabeled (cell surface) GLUTs from total cardiac membranes (200 μg) was achieved using streptavidin isolation (bound to 6% agarose beads) to facilitate separation of non-cell surface GLUTs ("unlabeled" or intracellular fraction that remains in the supernatant) from cell surface GLUTs ("labeled" or sarcolemmal fraction). The labeled GLUTs were then dissociated from the streptavidin by boiling in Laemmli buffer for 30 min prior to SDS-PAGE and subsequent immunoblotting with GLUT antibody. Proteins from the labeled fraction were quantified by densitometry relative to the positive control, as previously described.[42;43;46;47]

## Statistical Analysis

Number of animals: Power calculation for minimum sample size required was conducted using a statistical power of 0.5 and an alpha level of 0.1 to detect a 10% difference between groups with SigmaStat software 3.5 (Systat).

Data Analysis: Normality and homogeneity of data were tested using Shapiro-wilk and Levene test, respectively. Differences between means were assessed using Student's t-tests, one or two-way analysis of variance (treatment and tissue) for *in vitro* measurements, as appropriate. Repeated measured 2-way ANOVA was performed for the *in vivo* measurements, with Student Newman Keuls post-hoc test. If not normally distributed, the data was analyzed with a Mann-Whitney or Friedman test. Linear correlations were performed using linear regression. Statistical significance was defined as $P<0.05$. Data are presented as mean ± SE.

# RESULTS

## Validation of the Animal Model

We first confirmed obesity, hyperglycemia, hyperinsulinemia and impaired glucose tolerance in the high-fat diet-induced insulin-resistant (IR) animals. The animals that were fed a high fat diet quickly gained weight compared to the control animals (Fig 1A) and the obesity persisted through the end of the study (24 weeks). At the end of the 24 week period, the high-fat diet-fed mice gained 150% of their initial body weight compared to control mice which gained only 40% ($P < 0.05$, Fig 1A). Within 12 weeks, the IR animals developed mild hyperglycemia in contrast to their control counterparts ($P < 0.05$, Fig 1B). At 24 weeks, glucose and insulin tolerance tests (IPGTT and IPITT

respectively) were performed on animals following fasting for 8h. All the mice fed the high-fat diet developed impaired glucose and insulin tolerance ($P < 0.05$, Fig 1 C/D, IPGTT and 1 E/F, IPITT) compared to their normal-diet-fed control counterparts. In addition, the high-fat-diet-fed animals had approximately fivefold higher fasting serum insulin level compared to the control animals ($P < 0.05$, Fig 1G). Therefore, by the end of the 24 week study period, the high-fat diet-fed animals were obese, mildly hyperglycemic (blood glucose level of 217±52 mg/dL), hyperinsulinemic and developed impaired glucose tolerance. With these findings we confirmed insulin resistance in our high-fat-diet fed animals.

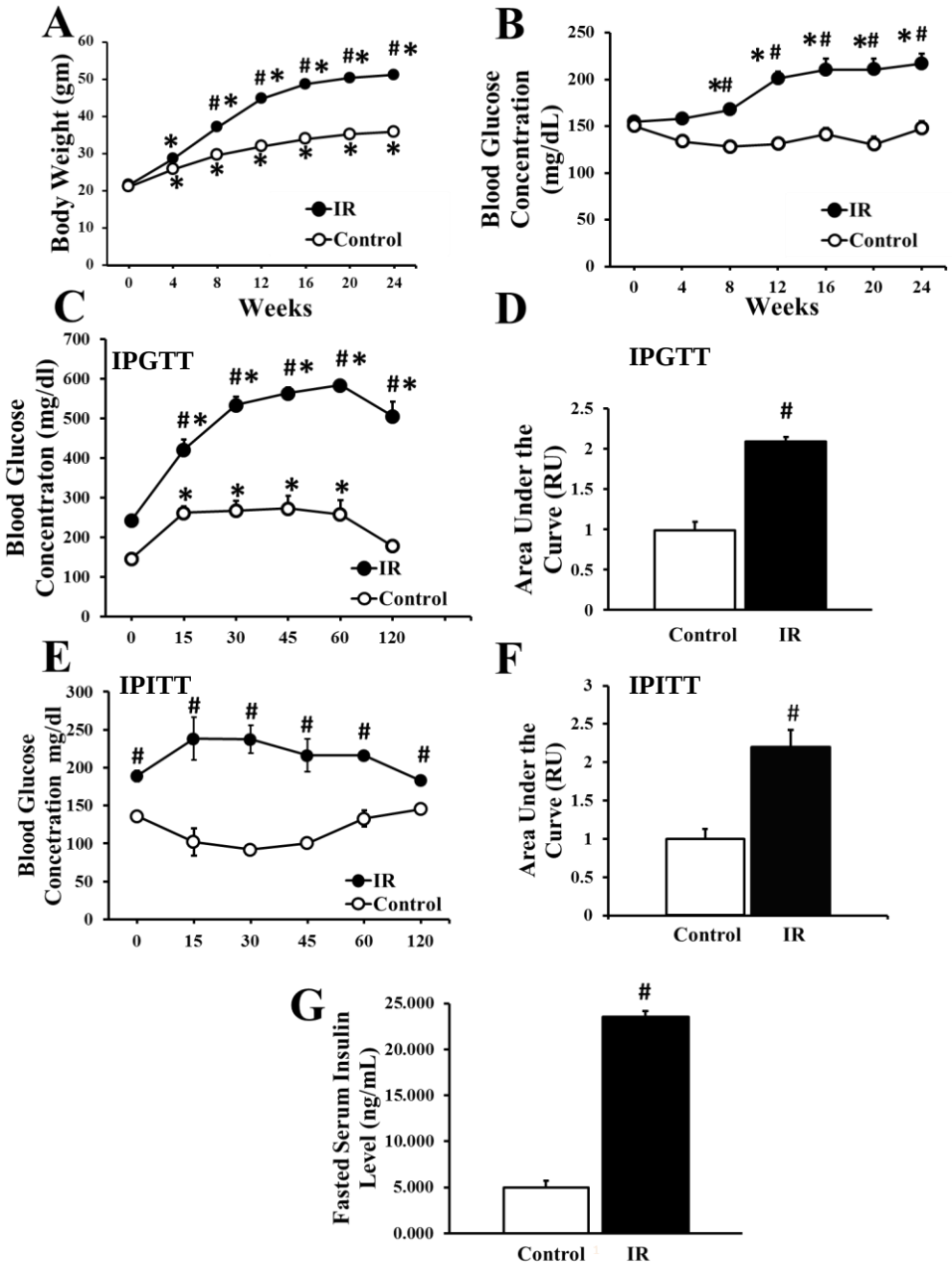


**Figure 1: Validation of the insulin-resistant animal model**

**Higher A) Mean ± SE Body Weight** and **B) Mean ± SE venous blood glucose concentration** obtained at baseline and up to 24 weeks after induction of insulin-resistance (IR) (n=10-15 mice/group); # P<0.05 vs. Control; * P<0.05 vs. Baseline (0 weeks) **C) Impaired Intraperitoneal glucose tolerance (IPGTT) of IR animals** (n=15-16 mice/group); # P<0.05 vs. Control; * P<0.05 vs. Baseline (0 weeks) **D)** AUC = area under the curve for [glucose] measured during IPGTT. (n=15-16 mice/group); # P<0.05 vs. Control **F) Impaired Intraperitoneal Insulin tolerance (IPITT) of IR animals** (n=7 mice/group); # P<0.05 vs. Control; * P<0.05 vs. Baseline (0 weeks) **D)** AUC = area under the curve for [glucose] measured during IPITT. (n=7 mice/group); # P<0.05 vs. Control **G) Hyperinsulinemia** observed in IR animals. Fasted serum insulin levels measured at 24 weeks of high-fat diet. (n=8-10 mice/group); # P<0.05 vs. Control.

**Increased vulnerability of insulin resistant subjects towards AF induction**

In order to investigate the vulnerability of our animal model to the induction of AF, we paced the atria with a total of 4 consecutive 10 second bursts of 20Hz (1200/min) (2 bursts) and 40Hz (2400/min) (2 bursts). Our results indicated longer duration and greater frequency of atrial arrhythmia in all the insulin-resistant (IR) animals ($P < 0.05$, Fig 2). We first quantified the average duration (Fig 2A) and frequency (Fig 2B) of all the observed atrial tachy-arrhythmias, including atrial fibrillation, atrial tachycardia and sick sinus syndrome. Our results indicated an 8 times greater average duration and 6 times greater frequency of atrial arrhythmia in the IR animals compared to the controls. It is important to note that, all of the diabetic atria were susceptible to some or all forms of atrial tachyarrhythmia (Fig 2C/D). Importantly, atrial fibrillation was observed in all of the IR animals (7 out of 7) with the total duration ranging from 9-43 seconds (Fig 2C/D). These findings strongly indicate higher AF inducibility and propensity in the IR animals.

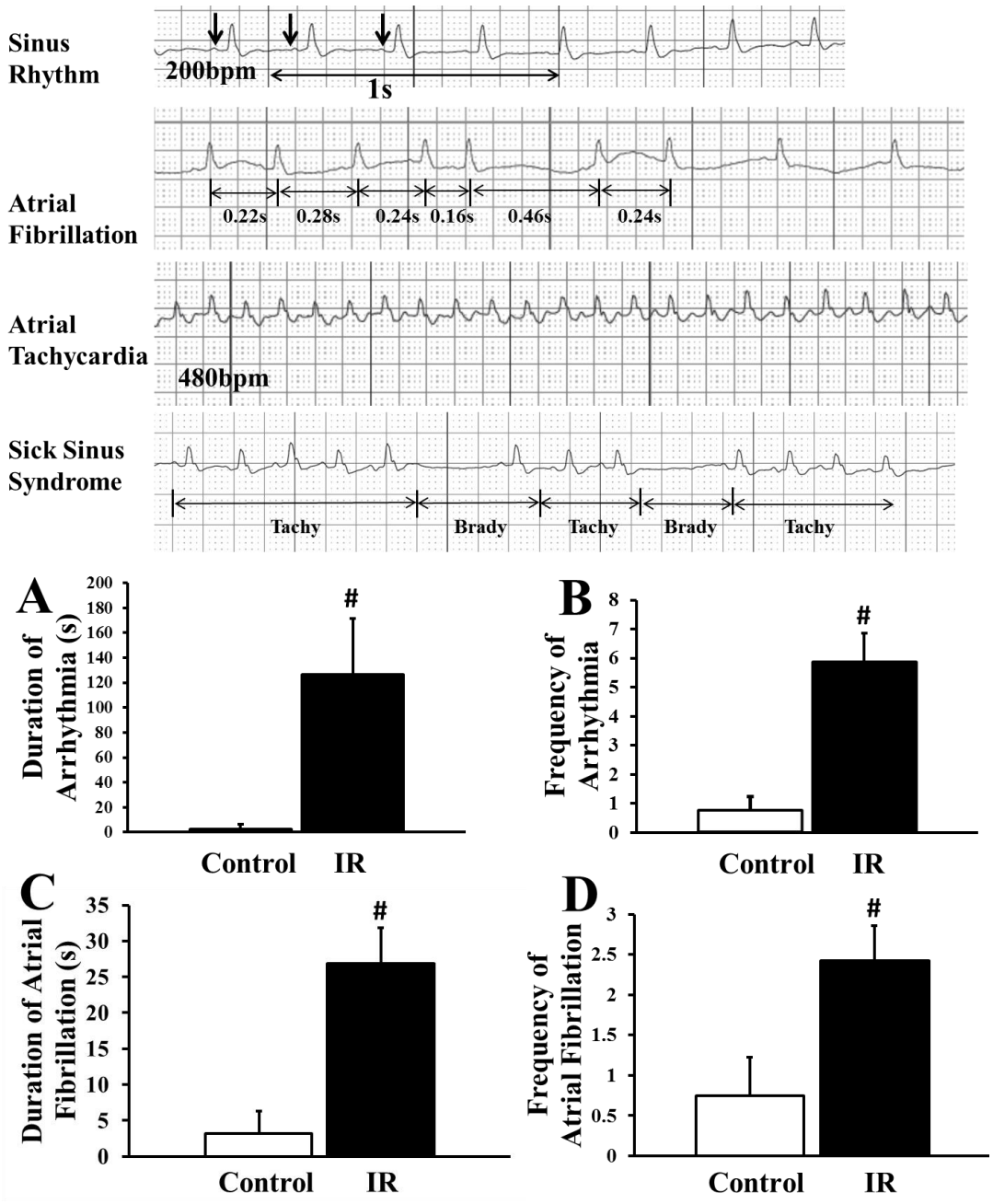


**Figure 2: Longer duration and greater frequency of atrial tachy-arrhythmia and atrial fibrillation in the insulin-resistant animals**

**(A) Longer duration and (B) greater frequency of combined atrial arrhythmia in the insulin-resistant (IR) animals** (n=4-7 mice/group); # P<0.05 vs. Control; Top panels: Representative ECG Traces. **(C) Longer duration and (D) greater frequency of atrial fibrillation in the IR animals** (n=4-7 mice/group); # P<0.05 vs. Control. Atrial arrhythmia: Includes atrial fibrillation, atrial tachycardia and sick sinus syndrome. Frequency: Average of the total incidence of atrial arrhythmia/atrial fibrillation. Methods: Transesophageal atrial pacing.

**Impaired GLUT trafficking in the atria of the insulin-resistant animals**

After we had established the increased vulnerability to AF in the insulin-resistant (IR) animals, we further investigated the alterations in the atrial glucose transport by quantifying the expression and regulation of the major GLUT isoforms (GLUT-4 and -8). We first measured the total GLUT-4 and -8 protein expressions in the atria of healthy and IR mice (Fig 3 A/B). Our results indicated a significant decrease by 38% in total GLUT4 protein expression in the atrial tissue during IR compared to control ($P < 0.05$, Fig 3A). However, there were no significant changes in GLUT8 total protein expression (Fig 4B). To accurately differentiate plasma membrane-associated GLUTs (labeled fraction) from intracellular GLUT (unlabeled fraction), we used the biotinylated assay in the intact perfused atria of control and IR animals (Fig 3 C/D/E/F). Under basal conditions, we observed a significant down-regulation of atrial cell surface GLUT4 by 66%, in the IR atria compared to healthy controls ($P < 0.05$, Fig 3C/E). However, our results did not indicate any alteration in cell surface GLUT8 expression in the IR atria (Fig 3D/F). We further compared the cell surface fraction of both GLUTs (4 and 8) to the intracellular fractions in both the healthy and IR animals (Fig 3C/D). As expected both the isoforms were mostly intracellular under basal conditions. Cell surface fraction of GLUT4 was 27% and 13% of the intracellular fraction in the healthy and IR animals, respectively. Similarly, under basal conditions, GLUT8 cell surface fraction was 7% and 6.5% in the healthy and IR animals respectively (Fig 3C/D).

In order to investigate alterations in GLUT trafficking during IR, cell surface GLUT trafficking was measured using the biotinylated assay in the intact perfused atria after *in vitro* insulin stimulation (Fig 3 E/F). We have previously described that both

GLUT-4 and -8 are insulin sensitive in the healthy atria.[326] Therefore, as expected in the healthy animals, we observed a significant increase in cell surface GLUT-4 and -8 expressions (by 250% and 500% respectively) upon insulin stimulation. In contrast, insulin –induced GLUT (-4 and -8) translocation to the atrial cell surface was impaired in the atria of IR mice. Although we did not observe any alterations in total GLUT8 expression in the IR animals, our results indicated an impairment in GLUT8 trafficking to the atrial cell surface. Therefore, we speculated that cardiac insulin resistance caused impairment in the insulin signaling pathway during IR.

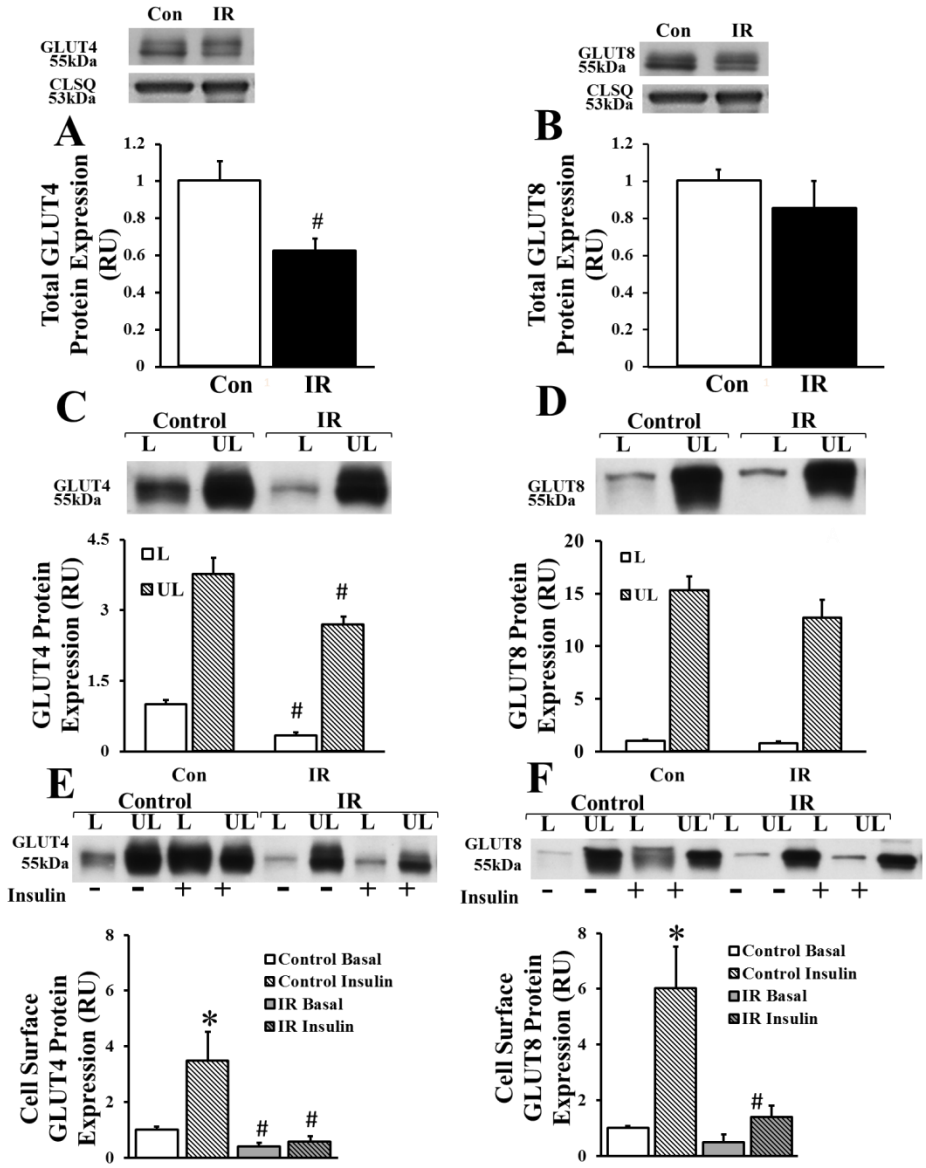


**Figure 3: Alteration in the trafficking of the insulin-sensitive GLUTs in the atria of the insulin-resistant animals**

**Total expression of A) GLUT4 and B) GLUT8** during insulin-resistance (IR) vs. healthy (Control). Top panels: representative Western blot. Calsequestrin (CLSQ) was used as a loading control. Bottom Panels: Mean ± SE of Total GLUT protein content, normalized to control (n=6/group). **Majority of C) GLUT4 and D) GLUT8 is intracellular under basal conditions** (n=4/group). Methods: Cell surface GLUT measured using biotinylated photolabeling technique in intact perfused mouse heart. L: Labeled (cell surface fraction); UL: Unlabeled (intracellular fraction). # $P<0.05$ vs. control**. Insulin stimulates E) GLUT4 and F) GLUT8 trafficking to the healthy but not IR atrial cell surface** in the intact perfused mouse heart. Top panels: representative Western blot. Bottom Panels: Mean ± SE of cell surface GLUT protein content; values normalized to basal (n=4/group); # $P<0.05$ vs. basal; * $P<0.05$ vs. Control; Methods: Cell surface GLUT measured using biotinylated photolabeling technique. L: Labeled (cell surface fraction); UL: Unlabeled (intracellular fraction).

**Impaired activation of insulin signaling pathway during insulin-resistance**

Our next aim was to investigate whether or not the alterations in GLUT (-4 and -8) trafficking was due to impairment in the activation of the downstream insulin signaling pathway. To this end, we measured (total and phosphorylated) Akt and AS160 protein expression, key regulators of GLUT vesicular trafficking, in the healthy and insulin-resistant (IR) atria by Western blotting (Fig 4). Our results indicated a significant decrease in phosphorylated Akt expression, at both serine 473 and threonine 308 phosphorylation sites in the IR atria ($P<0.05$, Fig 4A). In addition, there was decreased phosphorylation of AS160, the most downstream proteins in the insulin signaling pathway regulating GLUT trafficking ($P<0.05$, 4B). Moreover, we reported a positive linear correlation observed between the decreased phosphorylation of Akt and AS160 and total GLUT4 expression in both the healthy and IR atria ($P<0.05$, Fig 4 C/D). These results suggest that Akt and AS160 regulate GLUT expression and/or trafficking in the atrial tissue of healthy and IR subjects. In contrast, we did not observe any significant linear correlation between GLUT8 and phosphorylated Akt or AS160 (Fig 4 E/F), which suggests that, in addition to the downstream insulin signaling pathway, other insulin-independent pathways regulate GLUT8 expression and/or trafficking as well.

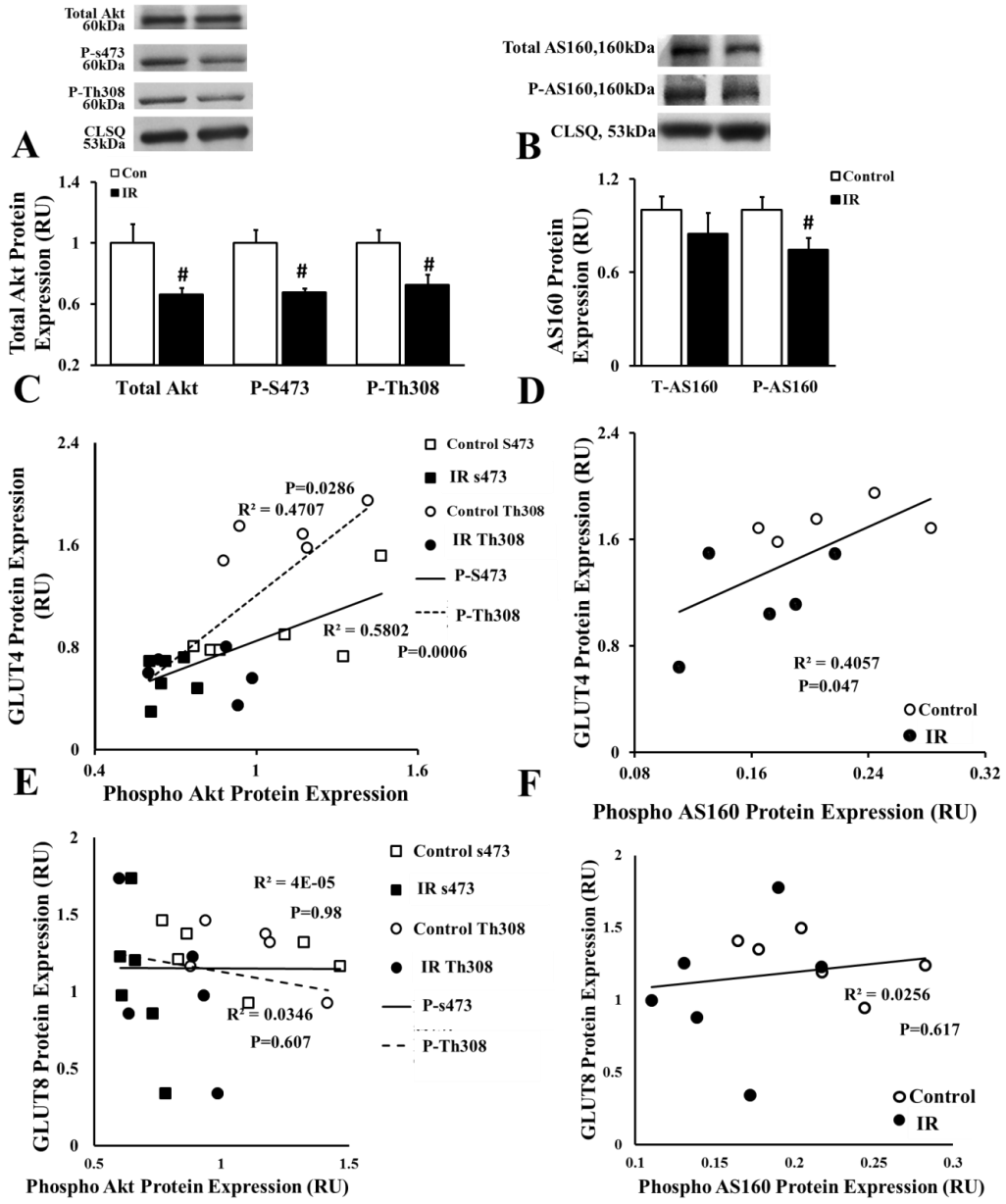


**Figure 4: Impaired activation of insulin signaling pathway during insulin resistance**

**Insulin-resistance (IR) decreased A) Akt and B) AS160 phosphorylation** in the atria. Top panels: representative Western blot of total lysate from atrial tissue; calsequestrin (CLSQ) was used as a loading control. Bottom Panels: Mean ± SE; values normalized to control (n=5-6/group). # P<0.05 vs. Control. **C) Significant positive linear correlation** between Akt phosphorylation and total GLUT4 expression in healthy and IR atria. Mean ± SE; values normalized to control (n=5-6/group). **D) Significant linear correlation between AS160 phosphorylation** and total GLUT4 and expression in the healthy atria. Linear Correlation between **E) Akt and F) AS160 phosphorylation** and total GLUT8 protein expression. Mean ± SE; values normalized to control (n=5-6/group).

**Spontaneous Atrial Fibrillation in the insulin-resistant animals**

In order to further investigate whether the insulin-resistant (IR) animals develop spontaneous AF, we monitored the animals for an additional 8 weeks (totaling 32 weeks on high fat diet). Our results indicated 6 folds longer duration (Fig 5A) and 8 folds greater frequency (Fig 5B) of AF in the IR animals. However, because of some individual variations, we only reported a statistical trend. In addition, the cumulative duration and observations of AF was significantly higher in the IR compared to the control groups (74.9 s vs 21.6 s and 16 vs. 2, respectively; $P<0.001$, Fig 5C and D). These findings clearly indicated the higher arrhythmogenicity in the IR atria.

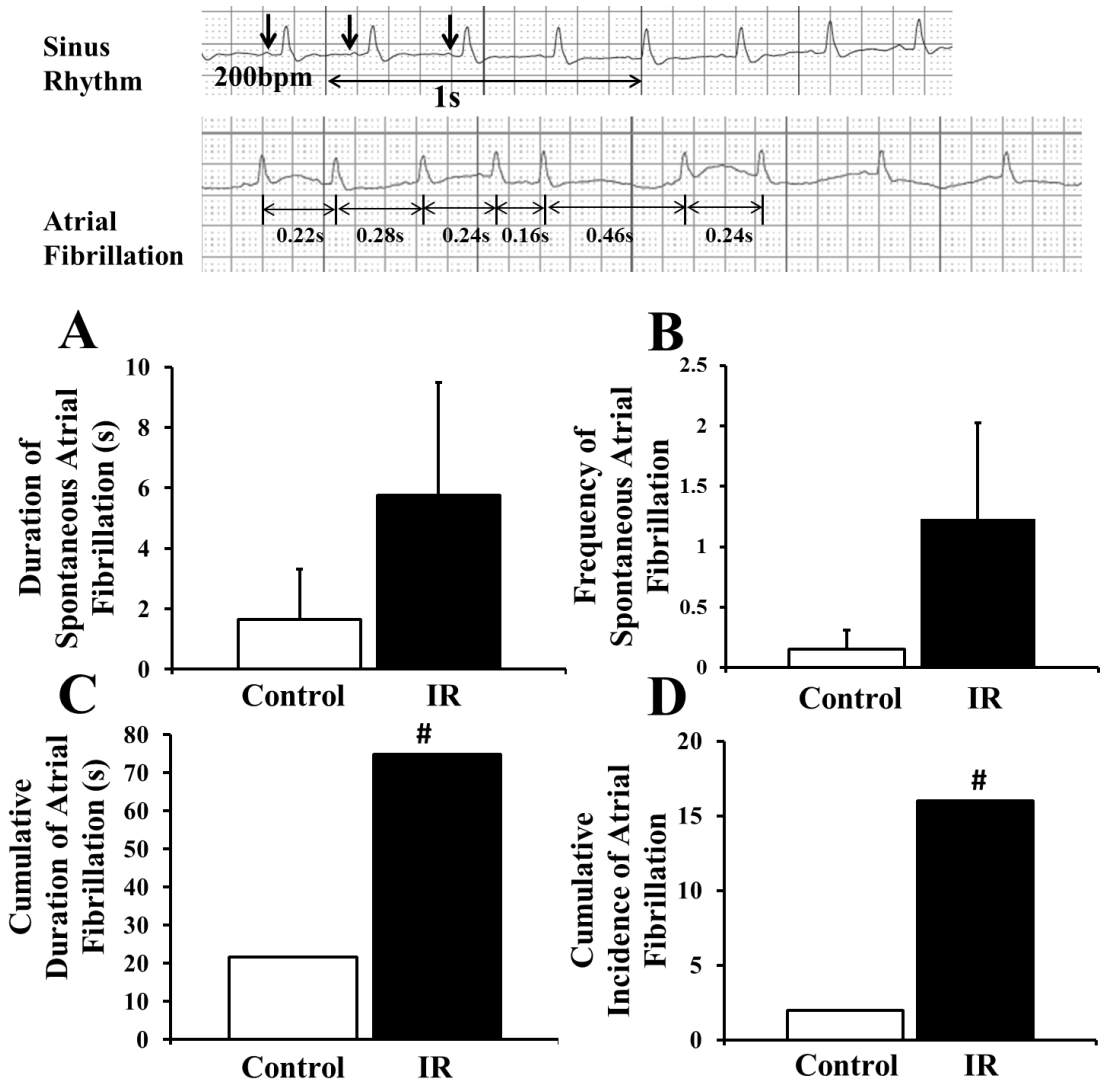


**Figure 5: Spontaneous atrial fibrillation in the insulin-resistant animals**

**(A) Mean duration and (B) Frequency of Spontaneous Atrial Fibrillation in the insulin-resistant (IR) animals** (n=13/group); **(C) Cumulative Duration and (D) Cumulative Incident of Atrial Fibrillation in the IR animals.** (n=13/group); #, P<0.001 vs. Control; Frequency: Average of the total incidence of atrial fibrillation. Methods: Transesophageal atrial pacing.

## DISCUSSION

Although type 2 diabetes and insulin resistance have been identified as an independent risk factors of atrial fibrillation, to the best of our knowledge, no study has established a causal link between insulin-resistance and atrial fibrillation. In this study, we demonstrated that 1) the insulin-resistant atria had an increased vulnerability and propensity to AF induction and 2) the insulin-resistant atria had impairment in trafficking of major cardiac isoform GLUT4 and novel isoform GLUT8 to the atrial cell surface, along with decreased activation of downstream insulin signaling pathway.

Insulin-resistant type 2 diabetes has been reported to account for 90% of the entire diabetic population and, similar to AF, is more prevalent among adults. In the current study we have used long term (6 months) high-fat diet-fed mice as an insulin-resistant (IR) animal model, which is characterized by obesity, glucose intolerance, hyperinsulinemia and mild hyperglycemia.[329-333] With the exception of mild hyperglycemia, these animals demonstrated the characteristics of type 2 diabetes and therefore can be considered to be in a 'pre-diabetic' state. However, since pre-diabetes has been recognized as an independent risk factor for diabetic cardiomyopathy, the IR animal model developed in the current study can be considered appropriate to investigate the underlying mechanisms of diabetes induced AF.[317-320]

Due to the absence of an animal model of spontaneous AF, it has been difficult to characterize the underlying pathophysiological link between these two insidious diseases. The majority of the research in this area has been therefore conducted by inducing short term AF with transesophageal or trans-venous atrial pacing.[154;334-337] The advantages of

using transesophageal stimulation is that it is minimally invasive, allows multiple examinations of the same animal with different experimental conditions and that it is free from the mechanical alterations of the atrial tissue which are associated with the more invasive open chest stimulations or catheterization.[334] Due to the small size of the mouse atria, the propensity to generate re-entry circuits (which is crucial for the initiation of AF) in the mouse atrium is much less probable compared to a large animal model. However, by placing the lead directly adjacent to the left atrium, this technique allows successful atrial pacing in a small animal model. Therefore, in the current study we have used transesophageal atrial pacing to test the vulnerability of our animal model to AF induction. Our results indicated that hyperinsulinemic mice had greater frequency and longer duration of atrial tachy-arrhythmia, including atrial fibrillation, atrial tachycardia and sick sinus syndrome. Similarly Takahashi *et al.* reported increased atrial tachycardia in short term high-fat diet-fed mice during transvenous (jugular vein) atrial pacing, although they did not report the incidence of AF itself.[335] In the current study, the duration and frequency of AF in the hyperinsulinemic animals was significantly longer compared to the healthy control animals. In a large animal model of long term high-fat diet, Abed *et al.* reported higher susceptibility to AF during open chest atrial pacing. However, none of these studies investigated the metabolic state of the animals and its relation to AF.[312] Although Kato *et al*. reported higher arrhythmogenic factors in the excised perfused rat atria of genetic type 2 diabetic rat model, arrhythmia was induced ex vivo (intact perfused heart) by subjecting the left atrial appendage with to an extrastimulus.[14] Finally, the occurrence of spontaneous AF in animal models with metabolic diseases has not been reported. This is germane to the fact that "AF begets

AF", [122] meaning initial sporadic AF cause spontaneous series of structural and electrical remodeling that promote maintenance of persistent AF.[118;122] In order to investigate whether our animal model will also develop spontaneous AF (in addition to atrial pacing-induced AF), we recorded baseline ECGs after feeding a long term (i.e., 32 weeks) high fat diet. Interestingly, we observed significantly higher cumulative duration of AF in the IR animals compared to the control. In conclusion, we are the first to report a causal link between (induced and spontaneous) AF in an animal model of insulin resistance.

Cellular insulin resistance in the insulin-sensitive tissue (skeletal muscle, adipose tissue and the heart) is the hallmark of type 2 diabetes. Interestingly, insulin resistance has been associated with atrial fibrillation. Ostgren *et al*., identified that a combination of insulin resistance and impaired glycemic control was more prominent in patients with AF compared to those with sinus rhythm.[19] In another study, it was reported that high fasting blood glucose and insulin levels in diabetic patients were correlated with the risk factor for the development of AF. This risk factor increased with the duration and severity of diabetes.[15] In addition, Dublin *et al*. reported that the risk of developing AF of a diabetic patient increases approximately 3% for each additional year of diabetes duration.[17] Another study by Rigalleau *et al*. reported that major hyperglycemic episodes precede AF attack in insulin-resistant type 2 diabetic patients.[16] Together the results of all these studies[5] suggest a strong correlation of insulin resistance and an irregular glycemic state with AF. In agreement with the above studies[17;19;185;189-192] our findings also demonstrated a higher AF inducibility in animals with hyperinsulinemia and insulin resistance.

In a recent study, we reported that the major cardiac isoform GLUT4 and novel isoform GLUT8 are insulin sensitive in the healthy atria.[326] Therefore, in the current study we speculated that alterations in the expression and regulation of these major insulin-sensitive GLUT isoforms in the insulin resistant model could play a major role in the pathogenesis in the induction of atrial fibrillation. Although GLUT4 is the major insulin-sensitive glucose transporter in the heart, there have been very few studies that have investigated cardiac-specific alterations of GLUT4 during IR. Our results indicated a significant decrease in both total and active cell surface GLUT4 protein expression in the atria of IR compared to the control group under basal conditions. In contrast to GLUT4, the total protein and active cell surface expression of GLUT8 under basal conditions remained unchanged. However, when stimulated with insulin, there was a significantly impairment in trafficking of both insulin-sensitive GLUT8 and GLUT4 to the atrial cell surface, with indicated atrial insulin resistance. Similarly, decreased GLUT4 protein and mRNA levels have been reported in adipocytes during type 2 diabetes.[253;293;338] In skeletal muscle, no change in the total level of GLUT4 has been reported between insulin-resistant, diabetic and healthy patients. [252;256;257;327;328] However, significant impairment of insulin stimulated GLUT4 vesicular trafficking has been reported in patients with type 2 diabetes.[258-260] It has been documented that, in skeletal muscle, insulin resistance alters sub-cellular localization of GLUT4 storage vesicles to dense membrane compartments from which insulin is unable to recruit GLUT to the cell surface.[260] However, the regulation of glucose transport in the IR atria remains elusive. In contrast to GLUT4, there is a gap of knowledge regarding the molecular mechanisms regulating GLUT8 expression and translocation during insulin-resistance. Gorovits *et al.*

suggested a regulatory role of insulin on GLUT8 mRNA expression by showing a positive linear correlation between GLUT8 mRNA expression and circulating levels of plasma insulin in the liver.[30] Similarly, previous findings from our laboratory reported significantly lower active GLUT8 expression in the atria of insulin-deficient diabetic animals.[326] Adastra *et al*. suggested the involvement of GLUT8 in insulin resistance and obesity by reporting that, GLUT8 knock out mice are resistant to dietary-fat-induced weight gain and insulin resistance.[339] In a similar study of a high fructose diet, DeBosch *et al.* suggested GLUT8 mediated deleterious metabolic effects such as decreased insulin sensitivity and glucose tolerance.[265] However, the underlying mechanisms are still under debate. The results of the current study demonstrated the impaired trafficking of both insulin-sensitive GLUT-4 and -8 during IR, indicative of atrial insulin resistance.

In order to investigate the mechanisms underlying the impaired trafficking of GLUT4 and GLUT8 to the atrial cell surface of the IR animals, we investigated the activation of downstream insulin signaling pathway by quantifying the phosphorylation of two key proteins Akt and AS160 by Western Blotting. It has been previously reported that in skeletal muscle and adipose tissue phosphorylation of Akt at th308 and s473 sites along with AS160 is essential in the vesicular trafficking of the intracellular GLUT4 molecules.[242;243;245;247;249;268-271] For instance, decreased Akt phosphorylation has been reported in skeletal muscle and adipose tissue of insulin resistant diabetic rats, genetically obese mice and biopsies of type 2 diabetic patients.[258;280-282;284;285] In addition, Karlsson *et al*. found that impairment of insulin action on AS160 activation was associated with reduced phosphorylation of th308 in skeletal muscle of hyperinsulinemic subjects.[258] Alteration in GLUT4 translocation in the adipose tissue was also reported to be

associated with defective Akt phosphorylation.[285;286] Similar to these findings, in the present study, we observed a significant decrease in phosphorylation of both Akt sites (th308 and s473) and AS160 in the atria of hyperinsulinemic animals compared to the healthy control group. In addition, we observed a significant linear positive correlation between Akt/AS160 activation and GLUT4 protein expression. One of the limitations of the current study is that due to the small size of the atria, analysis of downstream insulin signaling pathway and GLUT trafficking upon insulin stimulation was not quantifiable in the same atrial tissue (Fig 4). With the downregulation of Akt/AS160 activation under basal condition and impairment of insulin stimulated GLUT4/GLUT8 trafficking, the findings from the current study strongly indicate atrial/cardiac insulin resistance.

In contrast to GLUT4, we did not observe any significant correlation between decreased Akt/AS160 activation with GLUT8 protein expression. Although in our previous study we reported a significant correlation with insulin stimulated AS160 phosphorylation and increased GLUT8 expression in healthy isolated atrial myocytes.[326] Together, with these findings, it can be speculated that, in addition to the downstream insulin signaling pathway, other pathways regulate GLUT8 trafficking as well. Indeed, we previously reported that GLUT8 protein expression was positively correlated with pro-inflammatory cytokines IL-6 and SOCS3 in the ventricular tissue of mice fed a long-term high-fat diet, suggesting that cardiac inflammation could be a modulator of the novel GLUT8 isoform.[340] In addition, DeBosch *et al*. suggested that GLUT8 caused deleterious metabolic effects during high fructose diet.[265] Their studies reported that GLUT8 deficient mice were protected against high-fructose-diet-induced fructose intolerance, insulin resistance, in addition to demonstrating decreased plasma insulin and cholesterol

levels.[261;265-267] However, further studies are required to elucidate the role and regulation of GLUT8 during diabetes.

## CONCLUSIONS

The heart is one of the main organs to utilize glucose as an energy substrate. Therefore, proper glucose uptake in the cardiac tissue is pivotal to proper cardiac function. Since the atria contains the pacemaker of the heart (the sinoatrial node), the availability of sufficient energy substrate (i.e., glucose) in the atria is essential. With the use of high-fat diet-induced insulin resistance and the state-of-the-art biotinylated photolabeled assay, we demonstrated an impairment in the trafficking of insulin-sensitive GLUT4 and GLUT8 (i.e., potential for dysfunctional glucose uptake) in the IR atria along with an impairment in the activation of downstream insulin signaling pathway, indicative of cardiac insulin resistance. In addition, we demonstrated that the insulin-resistant animals were highly vulnerable to AF and other atrial tachy-arrhythmias. Together, these findings suggest that alteration in the glucose transport in the insulin-resistant atria could be a novel metabolic substrate that underlies insulin-resistance-induced atrial fibrillation.

# CHAPTER V

# INSULIN DYSREGULATION CONTRIBUTES TO ATRIAL FIBRILLATION VULNERABILITY

## ABSTRACT

Diabetes has been identified as major independent risk factor for atrial fibrillation (AF). However, the underlying pathophysiologic mechanisms of diabetes-induced AF are not well understood. Although recent studies suggest atrial fibrosis and advanced glycation end products (AGEs) as precursors, whether a metabolic substrate underlies AF remains unknown. It is possible that, in addition to structural remodeling, glucose and insulin disturbances during diabetes directly affect the function of the atrium, potentially leading to AF. We hypothesized that impaired glucose metabolism resulting from insulin dysregulation provides a metabolic substrate for the development and maintenance of atrial fibrillation. Transesophageal atrial pacing was used to induce AF in healthy, streptozotocin (STZ)-induced insulin-deficient (ID) and long-term high-fat-diet (HFD)-induced insulin-resistant (IR) mice. The expression of pro-fibrotic markers Transforming Growth Factor β-1 (TGFβ-1) and Matrix Metallo-Proteinase-9 (MMP-9) were measured in the healthy and diseased atrium using Western blotting. Atrial fibrosis, advanced glycation end-products (AGE) and glycogen accumulation were measured using histological analysis. The insulin-deficient (ID) and insulin-resistant (IR) subjects demonstrated an enhanced vulnerability and propensity to AF induction compared to the age-matched paired controls ($P<0.05$, respectively). In addition, we reported the absence of atrial fibrosis, glycogen and AGE accumulation in both groups with insulin dysregulation. Collectively, our data suggest that, in the absence of structural remodeling and atrial fibrosis, disturbances in the insulin and glycemic states were sufficient to increase the susceptibility towards AF. Therefore, these data suggested that insulin

dysregulation leads to perturbations in energy production in the atria, which enhances its vulnerability to AF.

## INTRODUCTION

Diabetes, a global epidemic health condition, currently affects 30 million people in the United States with approximately 1.5 million new cases every year. In the year 2010, diabetes had been identified as 7$^{th}$ leading cause of death in the US. Diabetes, characterized by chronic hyperglycemia, is either caused by lack of insulin production (type 1 diabetes) or the lack of insulin action/cellular insulin resistance (type 2 diabetes). It is well known that, diabetes significantly increases the risk factors for cardiovascular disease. The most common cardiac complications associated with diabetes are coronary artery disease, ischemic heart disease, diabetic cardiomyopathy and atrial fibrillation.[2] Atrial fibrillation (AF), the most common cardiac arrhythmia in the world, has been identified as an increasing public health problem as well.[5] Approximately, 2% of the world's population suffer from AF, and it is predicted that 16 million Americans will be affected by this insidious disease by the year 2050. In addition to worsening patient quality of life, AF is associated with stroke, heart failure and increased mortality. Recent epidemiological studies have identified diabetes as an independent risk factor for AF. The risk factor of a diabetic patient to develop AF has been reported to be 1.4-2.2 compared to a healthy non-diabetic person.[6;11-13;19] However, the underlying pathophysiological mechanism involved in the development and progression of diabetes-induced AF is not well understood.

The development of AF is thought to be multifactorial, including structural and electrical remodeling, atrial fibrosis and dilation.[11;130] It has been suggested that atrial fibrosis and AF occur in a concurrent manner, however whether or not there exists a direct cause and effect relationship remains elusive.[156] The increased space between the cardiac myocytes due to the fibrotic deposit and expansion of extra cellular matrix can cause conduction delays and allow alternate pathways of conduction, thereby increasing the propensity of AF.[156;158] Atrial fibrosis is difficult to reverse and therefore has been considered a major contributor in the progression from paroxysmal to persistent and eventually to permanent AF.[157]

In addition to structural-electrical remodeling, glucose and insulin disturbances may also directly affect the function of the atrium potentially leading to AF.[13] It has been reported that blood glucose level is significantly elevated during an AF attack.[5 15;16] It has also been reported that the risk of developing AF increases approximately 3% for each additional year of the duration of diabetes.[17] In addition, increased accumulation of glycogen in the atrial tissue has been reported following 48 hours of rapid atrial pacing in a goat model, which eventually resulted in permanent AF. It was further reported that a greater glycogen accumulation occurred at the intercalated disc and at the myocyte junction in the left atrial appendage.[179;180] Since greater glycogen accumulation has been reported in the diabetic myocardium of both insulin-deficient and insulin-resistant rodents,[206;207;341-347] one could speculate that glycogen accumulation could be a major player in the perpetuation of AF by disrupting intracellular communication. However, glycogen accumulation leads to AF warrants further investigation.[179;180]

Chronic hyperglycemia during diabetes accelerates the reaction between glucose and proteins, leading to the formation of advanced glycation end products (AGEs). AGEs form irreversible cross-links throughout the lifetime of many large proteins (such as collagen and hemoglobin), covalently modifying their structure and function, starting at 8-12 weeks after the onset.[348] During diabetes, and to a lesser extent during aging, AGEs accumulate at an accelerated rate in various cell types and produce multiple organ dysfunctions.[39;44] However, the role of AGE accumulation in the development of AF in diabetic patients is controversial. Increased plasma levels of AGE and sRAGE (soluble cellular receptor of AGE) have been reported in patients with AF.[50;51] In contrast, a recent large population based study reported no association between AGEs and the incidence and development of AF.[198] Therefore, whether AGEs accumulation during diabetes is the cause of or simply correlates with the incidence of AF needs to be further investigated.

Though diabetes has been identified as an independent risk factor for atrial fibrillation, the underlying pathophysiological mechanism of diabetes-induced atrial fibrillation remains elusive. Atrial fibrosis, AGE and glycogen accumulation have been suggested as biomarkers of AF; however, whether a metabolic substrate that underlies AF remains to be investigated. In the current study, we hypothesized that the impaired glucose metabolism resulting from insulin dysregulation induces an increased propensity to atrial fibrillation.

## MATERIALS AND METHODS

### Insulin-Deficient Animals

Healthy and insulin-deficient (ID) diabetic FVB/N mice were used, as previously described.[37;295-297] Insulin deficiency (ID) was induced at 10 – 12 weeks of age by 3 consecutive doses of freshly prepared steptozotocin (STZ, 65-95 mg/kg IP every 48 hours, diluted in citrate buffer), while the control group (Con) received placebo injection (citrate buffer). All mice were fed a standard diet to maintain body weight (10 % kcal from fat) for the duration of the study. Venous blood glucose concentration (facial vein) was measured at baseline and every week for both groups, using a glucometer (Bayer Contour, Tarrytown, NY) on mice fasted overnight for 8 hours. At the end of the study period (8 weeks), fasting serum insulin was measured using commercially available sandwich ELISAs (Serum Insulin ELISA kit, Millipore, MO). At 8 weeks, the mice were anesthetized using 3% isoflurane and euthanized by excision of the heart followed by either flash freezing the heart in liquid $N_2$ or preserving in 10% formalin for histology/immunohistochemistry. All of the procedures of this study were approved by the Oklahoma State University Institutional Animal Care and Use Committee.

### Insulin-Resistant Animals

Five-week-old C57BL/6 male mice (Charles River Laboratories) were used. After a one-week acclimatization period, mice were fed either a control diet (Con) (9.4% kcal from fat- soybean oil, 14.7% kcal protein and 75.9% kcal from carbohydrate; AIN-93M) or a high-fat diet (60% kcal from fat (soybean oil and lard), 20% kcal protein and 20% kcal from carbohydrate; Research Diets, Inc.; D12492) for 24 weeks. Both diets had

comparable amounts of vitamins and minerals. Body weights and blood glucose levels were recorded every 4 weeks throughout the experimental period. At the end of the study period (24 weeks), fasting serum insulin was measured using commercially available sandwich ELISAs (Serum Insulin ELISA kit, Millipore, MO). At 24 weeks, the mice were anesthetized using 3% isoflurane and euthanized by excision of the heart followed by either flash freezing the heart in liquid $N_2$ or preserved in 10% formalin for histology/immunohistochemistry. All of the procedures of this study were approved by the Oklahoma State University Institutional Animal Care and Use Committee.

**Atrial Fibrillation Induction**

All of the study animals were fully electrophysiologically examined by closed chest transesophageal recording and atrial stimulation under anesthesia (ketamine/xylazine IP injection). Body surface ECG was recorded via dual subcutaneous ECG leads with telemetric data communication to the AliveCor iPhone application. A 2.2 Fr 6-polar catheter was inserted through the esophagus and positioned near the left atrium. Positioning of the appropriate bipolar pair of electrodes adjacent to the left atrium was accomplished by sequentially connecting each bipolar pair to the AliveCor application. Heart rate and body temperature were recorded continuously during the entire procedure for all of the animals. The current, in milliamperes, was determined by connecting the terminals of the predetermined pairs of electrodes to the battery powered (DC) stimulator (each pulse duration = 1 millisecond), which provided pace capture of the atrium. This threshold was recorded and increased by 2X to ensure constant atrial pacing at a rate faster than the baseline rate. By switching the same terminals to the AC-powered stimulator the atrial pacing threshold (in volts) was determined. This voltage threshold

was recorded and at 2X threshold constant atrial pacing was achieved and noted. In order to induce atrial fibrillation (AF), the same site was subjected to burst pacing for 10 seconds at 2 different frequencies - 20 Hz (1200/min) and 40 Hz (2400/min) (Fig 1A) twice, for a total of four bursts per animal. The same protocol was applied for all 4 groups (i.e., ID, IR groups and their aged-matched control groups).

Duration of atrial fibrillation (in seconds), frequency (number of incidences per animal) (defined as an absence of a defined P-waves and presence of irregularly irregular R-R interval) and atrial tachycardia (increased heart rate without the defined P wave) were recorded after each burst pacing from the surface ECG traces. The total duration and frequencies of the atrial tachy-arrhythmias were recorded.

**Protein extraction**

Briefly, total protein lysates from fresh atrial tissue were collected by incubating the tissue with lysis buffer (RIPA, Thermo fisher Scientific), as previously described.[37;295-297]

**Western immunoblotting**

Equal amounts of protein (5-20 μg) were resolved in an 8-12% SDS-polyacrylamide gel and electrophoretically transferred (BioRad) to a polyvinyl-idine fluoride membrane (BioRad), as previously described.[37;294-297;299;300] After blocking (1-5% non-fat dry milk or 2% goat serum albumin), membranes were incubated with optimally diluted primary antibodies overnight (polyclonal rabbit anti-MMP9, 1:1000, EMD Millipore; polyclonal rabbit anti-TGFβ-1, 1:300, Abcam Antibodies) followed by a 1 hour incubation of appropriate secondary antibodies conjugated to horseradish

peroxidase (polyclonal goat anti-rabbit; 1:2500, GE Healthcare NA934V). Primary antibodies were chosen based on their 100% sequence homology with the protein of interest in rodents, and validated against a positive control (i.e., tissue, peptide). Antibody-bound transporter proteins were quantified by enhanced chemiluminescence reaction (KPL) and autoradiography. Band density and molecular weight were quantified using GelPro Analyzer (Media Cybernetics). The data was expressed relative to appropriate controls. Equal protein loading was confirmed by reprobing each membrane with Calsequestrin monoclonal IgG (Thermo-Scientific PA1-903, 1:2500, polyclonal rabbit anti-dog).

**Histochemical Procedures**

Atrial tissues were fixed in 10% formalin for 24 h at room temperature. Upon dehydration, the tissue specimens were processed for paraffin impregnation. Five μm thick sections were cut and mounted on clean glass slides. These sections were processed for Periodic acid-Schiff (PAS) staining with (Fig 1C) and without (Fig 1B) diastase digestion to assess the amount of glycogen, or for Masson Trichrome staining to assess the amount of collagen, in accordance with standard procedures for morphologic analysis. Images of the stained sections were captured with Axio Vision software (Axio Vision 4.1 Zeiss, Germany) using a CCD camera attached to an inverted light microscope (Carl Zeiss, Germany). Images of all tissues were acquired at the same time with a constant set of imaging parameters on the microscope and imaging software.

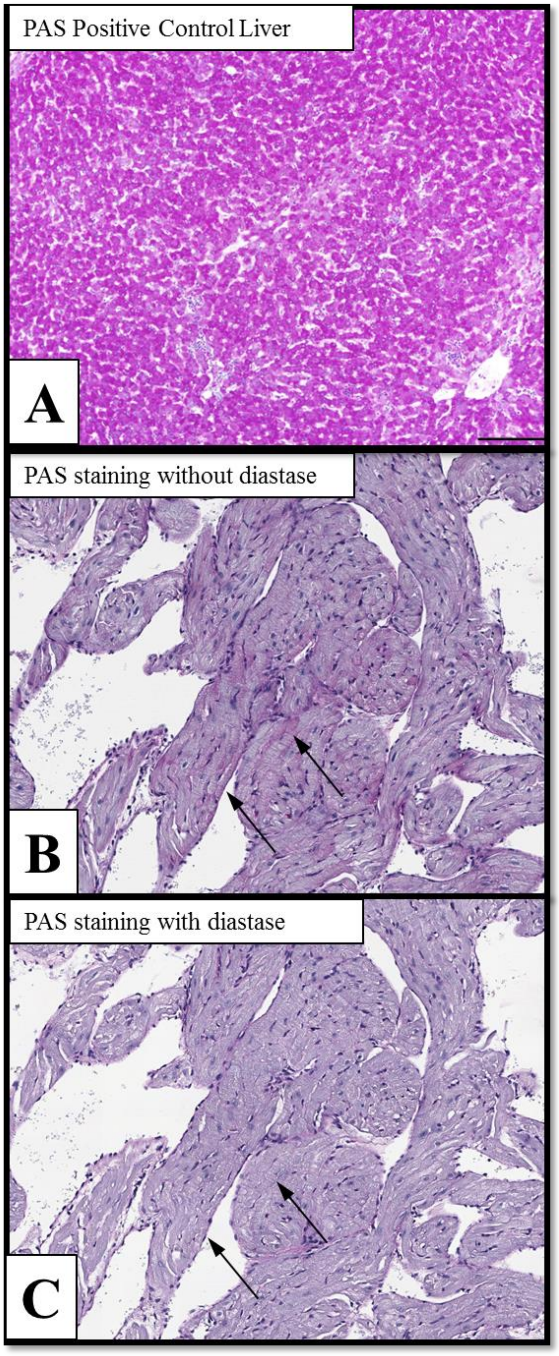


**Figure 1: Periodic Acid Schiff Staining**

**A) Positive control for PAS staining.** Liver tissue was used to confirm PAS staining of glycogen (in purple) **B) Glycogen Staining of the atrial tissue.** Arrowheads point towards glycogen stores **C) Diastase digestion of glycogen.** Diastase digestion of glycogen confirmed positive glycogen staining in panel B.

*Carboxymethyl-Lysine-AGE staining:*

To characterize AGE deposition in the atrial tissue, immunohistochemistry for carboxymethyl-Lysine was performed using automated immunostainer (Leica, Bond-III, Leica, Buffalo Grove, IL) on 10% formalin fixed paraffin embedded sections. The slides were overlaid with mouse monoclonal anti-carboxymethyl-lysine (CML-AGE) (1:100, Abcam, primary antibody) and normal goat serum (1:25) in PBS and incubated overnight at $40^0$C. This was followed by an overlay with Fluorescein isothiocyanate (FITC) conjugate Rat anti-Mouse IgG1 antibody (1:20, Thermo Fisher Scientific, secondary antibody) (Fig 2A). In order to confirm the absence of non-specific binding 10% formalin fixed paraffin embedded section were incubated only with Fluorescein isothiocyanate (FITC) conjugate Rat anti-Mouse IgG1 antibody (1:20, Thermo Fisher Scientific, secondary antibody) (Fig 2B).

Olympus DP25 digital photomicroscpe was used to capture digital images of the CML-stained sections. Randomly, color photomicrographs were obtained from all regions of atrial tissue samples (4-5 animals/group). The digital images were then converted to NIH Image J software and the total area of the image calculated ($\mu m^2$) by setting the measurement parameters to the scale bar acquired with the image. The color threshold was set to identify CML-AGE only (brown stain). The images were then converted to black and white images in order to conduct binary measurements. To confirm scope intensity of CML-AGE staining, the binary images were regularly compared with the original image acquired using the photomicroscope. CML-AGE staining was reported as the area ($\mu m^2$) in the binary image and then compared to the total area of the original image.

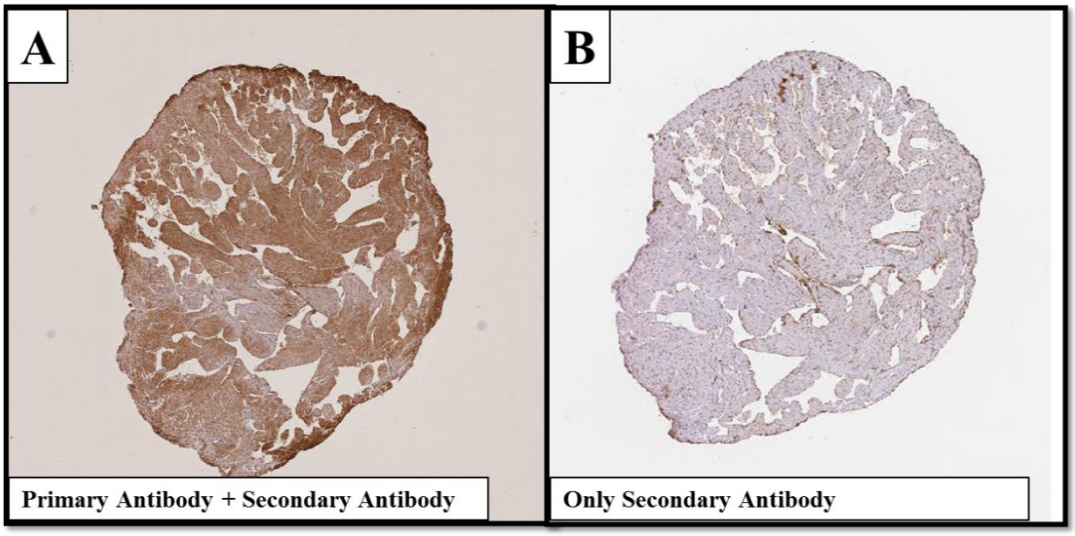


**Figure 2: Carboxymethyl-Lysine-AGE staining:**

**A) Atrial tissue incubated with primary and secondary antibody.** CML-AGE is stained in brown. **B) Atrial tissue incubated with only secondary antibody.** The absence of brown in panel B is the confirmation of no non-specific binding.

## Statistical Analysis

Number of animals: Power calculation for minimum sample size required was conducted using a statistical power of 0.5 and an alpha level of 0.1 to detect a 10% difference between groups with Sigma Stat software 3.5.

Data Analysis: Normality and homogeneity of data were tested using Shapiro-wilk and Levene test, respectively. Differences between means were assessed using Student's t-tests, one or two-way analysis of variance (treatment and tissue) for *in vitro* measurements, as appropriate. Repeated measured 2-way ANOVA was performed for the *in vivo* measurements with Student Newman Keuls post-hoc test. If not normally distributed, the data was analyzed with a Mann-Whitney or Friedman test. Linear correlations were performed using linear regression. Statistical significance was defined as $P<0.05$. Data are presented as mean ± SE**.**

# RESULTS

## Validation of the insulin-deficient and insulin-resistant animal models

In order to investigate the vulnerability of the diabetic animal to AF induction, we used an insulin-deficient animal model (ID) and a high-fat-diet-fed insulin-resistant model (IR). As expected, within 1 week after injection STZ-treated animals developed hyperglycemia (i.e., [glucose]>200 mg/dl), which persisted throughout the experimental period. The paired control group remained euglycemic (Fig 3A). There was no difference in body weight between groups (Fig 3B). The STZ-treated animals were hypoinsulinemic at the end of 8 weeks (Fig 3C).

Within 2 weeks the blood glucose levels of the high-fat-diet fed animals started to elevate and mild fasting hyperglycemia was maintained until the end of the 24 week study period. The blood glucose concentration of the high-fat group was significantly higher than that of the euglycemic control group (Fig 3D). Which was also significantly higher compared to the control animals until the end of the 24 week study period. These animals also quickly became obese with approximately 2.5 times their initial body weight and were significantly higher compared to the control group at the end of the study period (Fig 3E). The fasted serum insulin level of the high-fat diet animals were significantly higher at the end of 24 weeks compared to the paired control animals confirming hyperinsulinemia (Fig 3F).

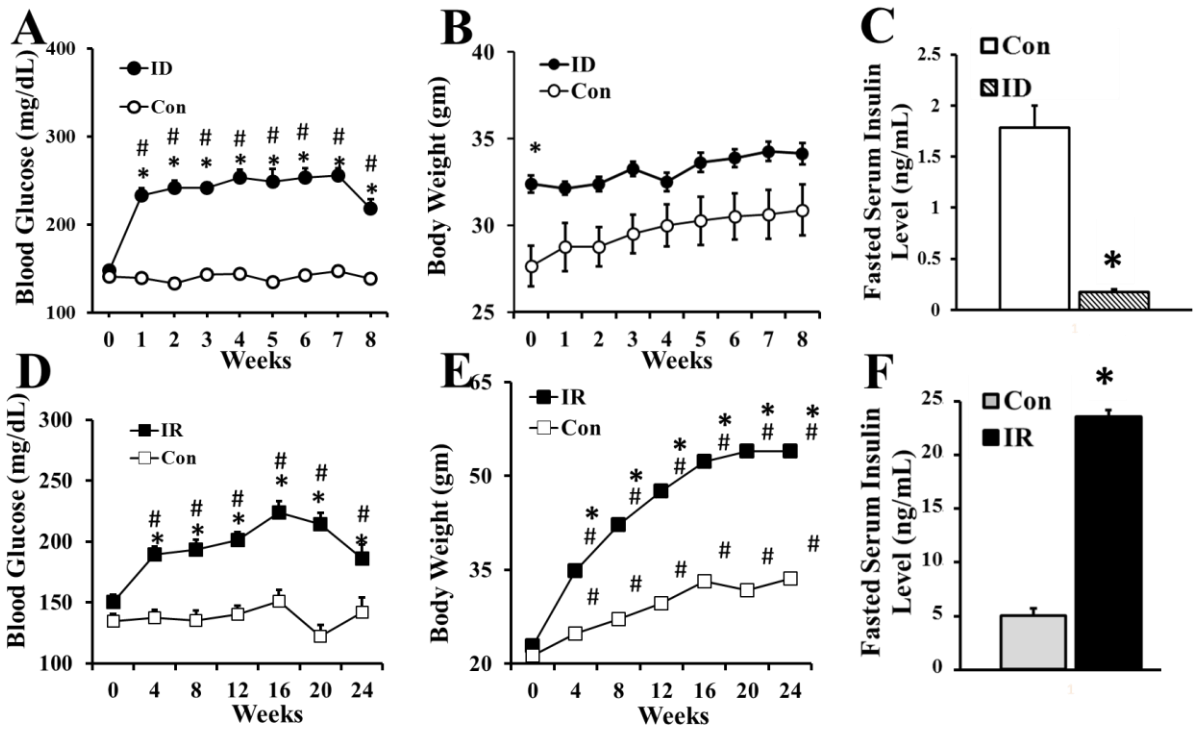


**Figure 3: Validation of the insulin-deficient and insulin-resistant animal models**

**Validation of the insulin-deficient (ID) diabetic animal model. A) Mean ± SE venous blood glucose concentration** obtained at baseline and up to 8 weeks in STZ-treated (ID) and age-matched control (Con) mice (n=8-9/group). **B) Mean ± SE body weight** obtained at baseline and up to 8 weeks in STZ-treated (ID) and age-matched control (Con) mice (n=8-9/group). **C) Mean ± SE serum insulin concentration** obtained at 8weeks from STZ-treated (ID) and age-matched control (Con) mice (n=4-6/group).**Validation of the insulin-resistant (IR) animal model. D) Mean ± SE venous blood glucose concentration** obtained at baseline and up to 24 weeks in high-fat-diet-fed (IR) and age-matched control (Con) mice (n=20/group). **E) Mean ± SE body weight** obtained at baseline and up to 24 weeks in high-fat-diet-fed (IR) and age-matched control (Con) mice (n=20/group). **F) Mean ± SE serum insulin concentration** obtained at 24 weeks from high-fat-diet-fed (IR) and age-matched control (Con) mice (n=20/group). ID: Insulin-deficient; IR: Insulin-resistant; Con: control animals paired with age matched ID or IR animals as appropriate; *P<0.05 vs. control; # P<0.05 vs. baseline.

**Vulnerability of the insulin-deficient and insulin-resistant animals to atrial fibrillation induction**

In order to investigate the vulnerability of our animal models to the induction of AF, the atria of the healthy, insulin-deficient (ID) and insulin-resistant (IR) animals were paced with a total of 4 consecutive 10 second bursts of 20Hz (1200/min) (2 bursts) and 40Hz (2400/min) (2 bursts). Our results indicated longer duration and greater frequency of atrial arrhythmia in insulin-deficient and insulin-resistant animals ($P<0.05$, Fig 2A-D). We first quantified the duration (Fig 4A/C) and frequency (Fig 4B/D) of all observed atrial tachy-arrhythmias, including atrial fibrillation, atrial tachycardia and sick sinus syndrome in both ID and IR animals. Our results indicated a 6 and 8 times greater average duration of arrhythmia in the ID and IR, respectively, compared to their paired control groups. Similarly, the ID and IR animals displayed 4 and 6 times greater frequency of atrial tachy-arrhythmia compared to the healthy controls. In addition, 85% of the ID (6 out of 7) and 100% of the IR (7 out of 7) subjects were susceptible to some or all forms of atrial tachy-arrhythmia. Next, we specifically quantified the duration and frequency of atrial fibrillation in the ID and IR animals. Our results indicated significantly greater frequency of AF in both ID ($P<0.05$, Fig 4F) and IR ($P<0.05$, Fig 4H) animals compared to their age-matched control groups. These findings strongly indicate higher AF inducibility and propensity in the ID and IR animals.

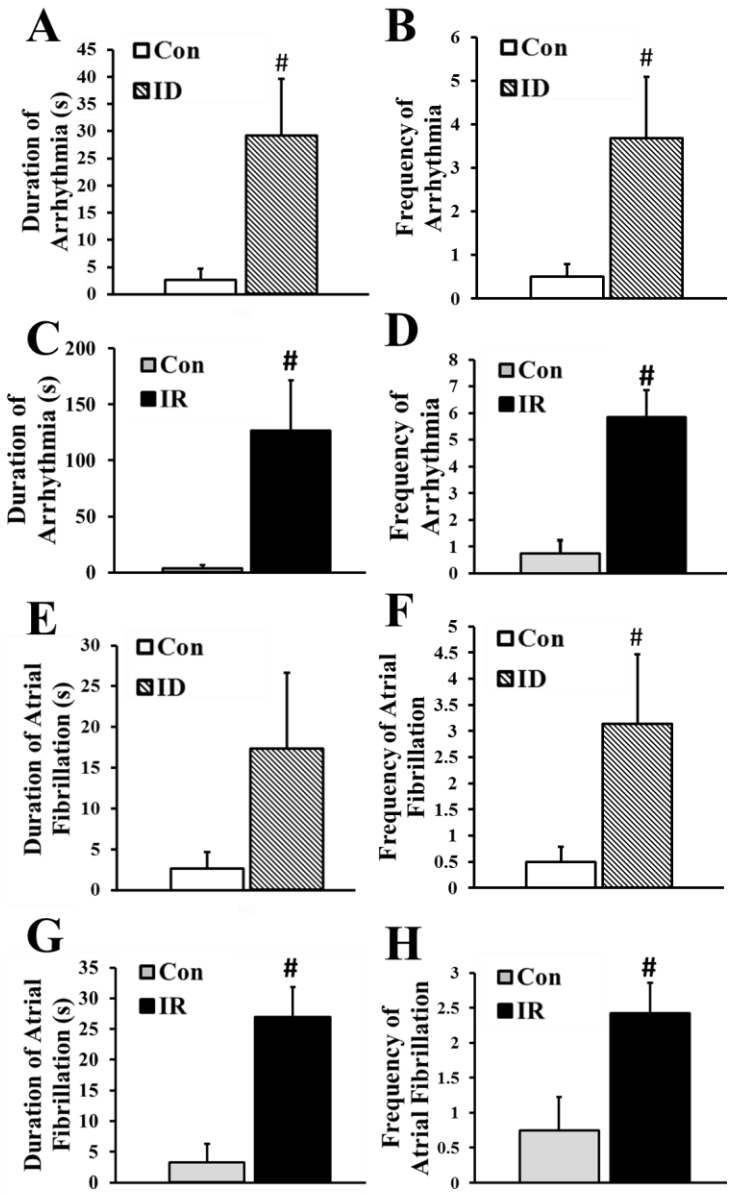


**Figure 4: Vulnerability of the insulin-deficient and insulin-resistant animals to atrial fibrillation induction**

**(A) Longer duration and (B) greater frequency of combined atrial arrhythmia in the insulin deficient (ID) animals** (n=4-7 mice/group); # P<0.05 vs. Con; **(C) Longer duration and (D) greater frequency of combined atrial arrhythmia in the insulin resistant (IR) animals** (n=4-7 mice/group); # P<0.05 vs. Con; **(E) Longer duration and (F) greater frequency of atrial fibrillation in the ID animals** (n=4-7 mice/group); # P<0.05 vs. Con. **(G) Longer duration and (H) greater frequency of atrial fibrillation in the IR animals** (n=4-7 mice/group); # P<0.05 vs. Con. Atrial arrhythmia: Includes atrial fibrillation, atrial tachycardia and sick sinus syndrome. Frequency: Average of the total incidence of atrial arrhythmia/atrial fibrillation. ID: Insulin-deficient; IR: Insulin-resistant; Con: control animals paired with age matched ID or IR animals as appropriate; *P<0.05 vs. control; # P<0.05 vs. baseline.

**Glycogen accumulation in the atria of insulin-deficient and insulin-resistant subjects**

In order to investigate alterations in atrial metabolism, we used Periodic acid-Schiff (PAS) staining to measure potential glycogen accumulation (Fig 5 A-H) in the insulin-deficient (ID) and insulin-resistant (IR) atria. In both control groups, the presence of glycogen was minimal in both the right and left atria (Fig 5). Furthermore, we did not identify any differences in glycogen accumulation between the diseased groups vs. paired control or the right vs left atria. Due to the low intensity of glycogen staining, quantitative analyses were not possible.

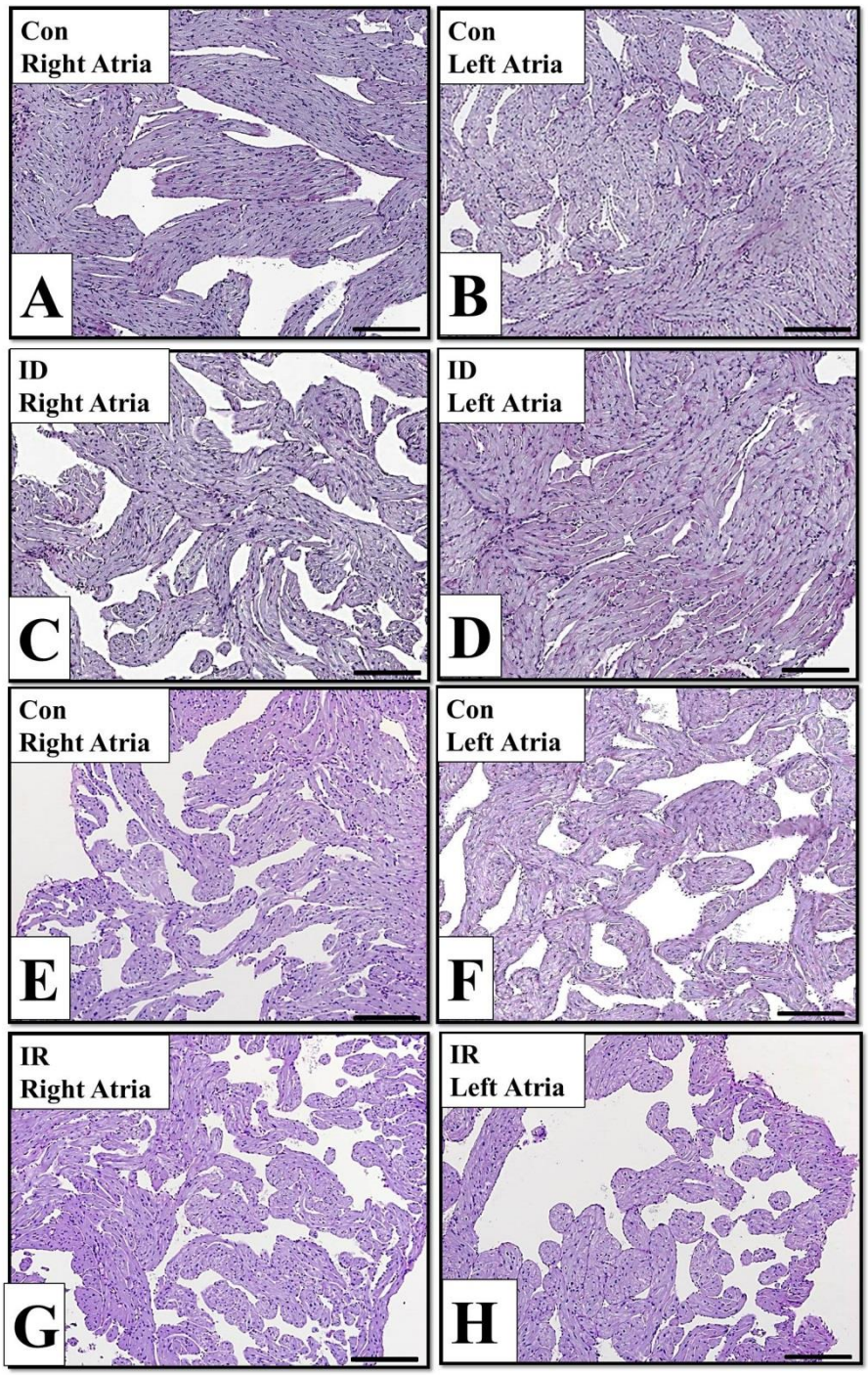


**Figure 5: Glycogen accumulation in the atria of insulin-deficient and insulin-resistant subjects**

**Representative images demonstrating no alteration of glycogen deposition in the control (Con), insulin-deficient (ID) and insulin-resistant (IR) atria.** n=5/group; Methods: Periodic-acid Schiff (PAS) Stain (Purple). Scale Bar: 200µm. ID: Insulin-deficient; IR: Insulin-resistant; Con: control animals paired with age matched ID or IR animals as appropriate.

**Accumulation of advanced glycation end product in the atria of insulin-deficient and insulin-resistant mice**

Immunohistochemistry was used to measure the deposition of AGEs in atria of the control, insulin-deficient (ID) and insulin-resistant (IR) mice. AGE deposition was compared between the control vs diseased groups, as well as the right vs left atria. Quantification of positive staining for carboxymethyl lysine, a major AGE (in brown) indicated that there was no difference of AGE deposition in the atria of ID and IR groups vs. their paired control groups (Fig 6).

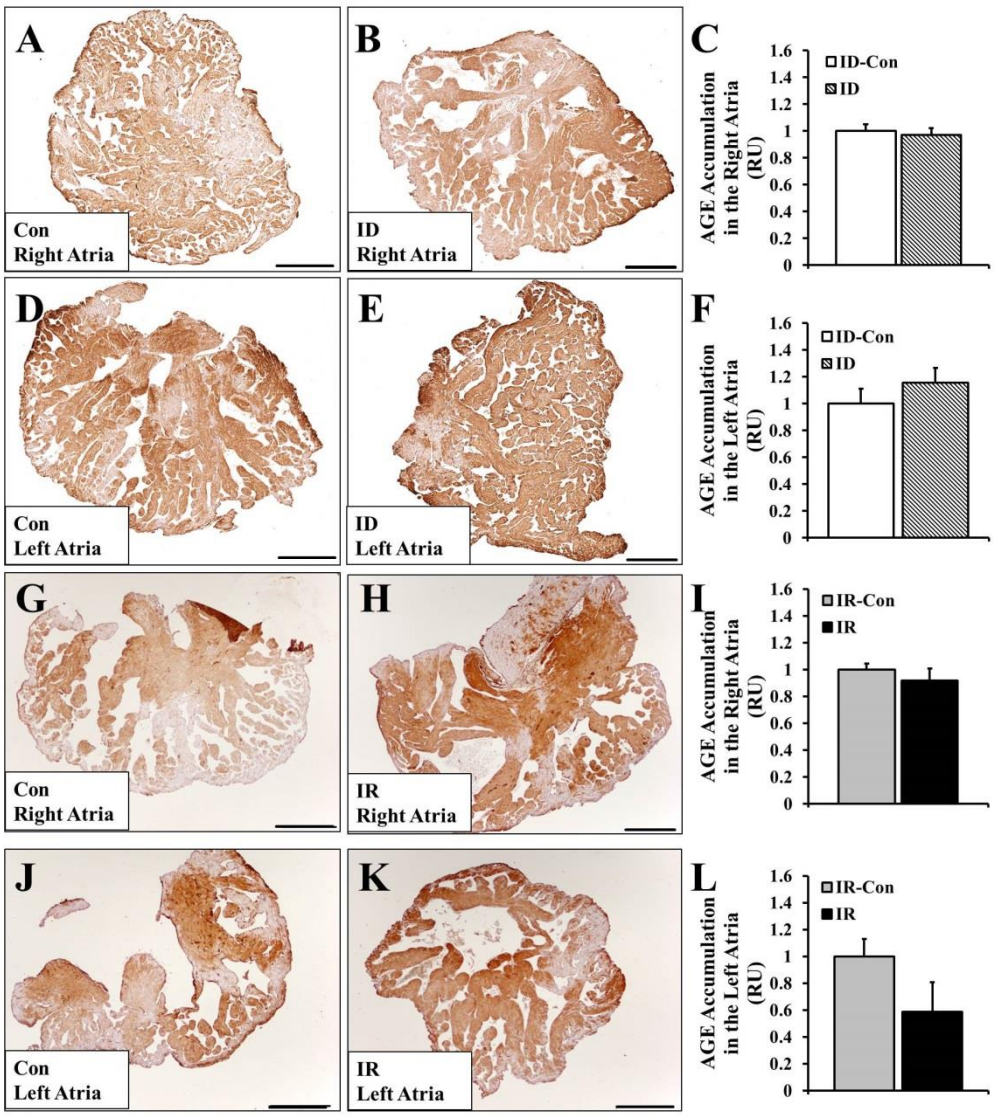


**Figure 6 : Accumulation of advanced glycation end products in the atria of insulin-deficient and insulin-resistant mice**

**No alteration in CML-AGE deposition in the Control, insulin-deficient (ID) and insulin-resistant (IR) atria. A/B)** Representative IHC images of the Con and ID right atria. **C)** Mean ± SEM of CML-AGE deposition in the Con vs ID right atria; n=5-6/group; **D/E)** Representative IHC images of the Con and ID left atria. **F)** Mean ± SEM of CML-AGE deposition in the Con vs ID left atria; n=5-6/group. **G/H)** Representative IHC images of the Con and IR right atria. **I)** Mean ± SEM of CML-AGE deposition in the Con vs IR right atria; n=2-4/group; **J/K)** Representative IHC images of the Con and IR left atria. **L)** Mean ± SEM of CML-AGE deposition in the Con vs IR left atria; n=3-4/group. Methods: Immunohistochemistry; Anti Carboxymethyl Lysine (Brown); Scale Bar: 500µm. ID: Insulin-deficient; IR: Insulin-resistant; Con: control animals paired with age matched ID or IR animals as appropriate. IHC: Immunohistochemistry.

**Atrial fibrosis in the insulin-deficient and insulin-resistant atria**

Since atrial fibrosis and atrial remodeling has been established as a pre-cursor to AF,[130;135;155;160;162] we determined whether atrial fibrosis was present in the atria of ID and IR animals. We first quantified the protein expression of the pro- (latent) and active- (active form of the protein; activation is achieved from proteolytic cleavage of the latent form followed by dimerization)[349] TGFβ-1, a pro-fibrotic marker by Western blotting. Our results demonstrated a significant increase in the expression of the active TGFβ-1 in the atria of both ID ($P<0.05$, Fig 7A) and IR ($P<0.05$, Fig 7B) subjects compared to their respective healthy controls. We also quantified the protein expression of MMP-9, which has been identified as an independent risk factor for AF and an activator of TGFβ-1.[145;162;350] Our results indicated up-regulation of active-MMP-9 in the ID ($P<0.05$, Fig 7C) and IR ($P<0.05$, Fig 7D) atria. These findings suggested that the atria of the animals with insulin dysregulation are susceptible to fibrosis. We then performed Masson Trichrome Staining to assess the presence of (Fig 8) of atrial fibrosis in the healthy and diseased animals. Similar to the control groups, we did not observe any fibrotic deposits in the atria of the ID or IR group. Therefore, these findings are indicative of the fact that at this stage the ID and IR subjects, although vulnerable to AF induction, do not demonstrate atrial fibrosis.

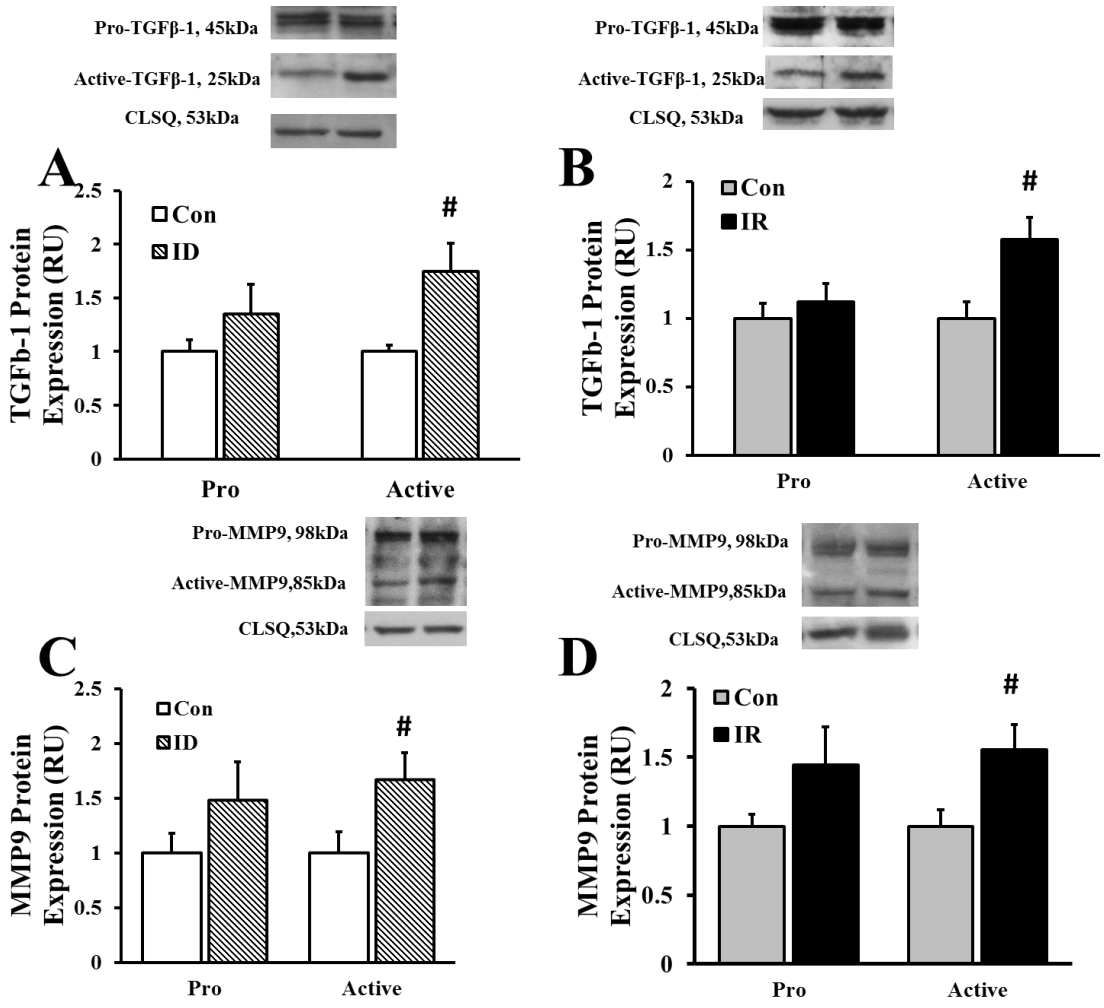


**Figure 7: Increased expression of fibrotic markers in the atria of the insulin-deficient and insulin-resistant animals**

**Increased protein expression of Active Transforming Growth Factor β-1 (TGFβ-1) in A)** ID atria and **B)** IR atria. n=5/group; (#, P<0.05 vs Con); **Increased protein expression of Active Matrix Metallo-Proteinase-9 (MMP-9) in C)** ID atria and **D)** IR atria. n=5/group; (#, P<0.05 vs Con); Values normalized to respective controls; Methods: Western blotting. ID: Insulin-deficient; IR: Insulin-resistant; Con: control animals paired with age matched ID or IR animals as appropriate.

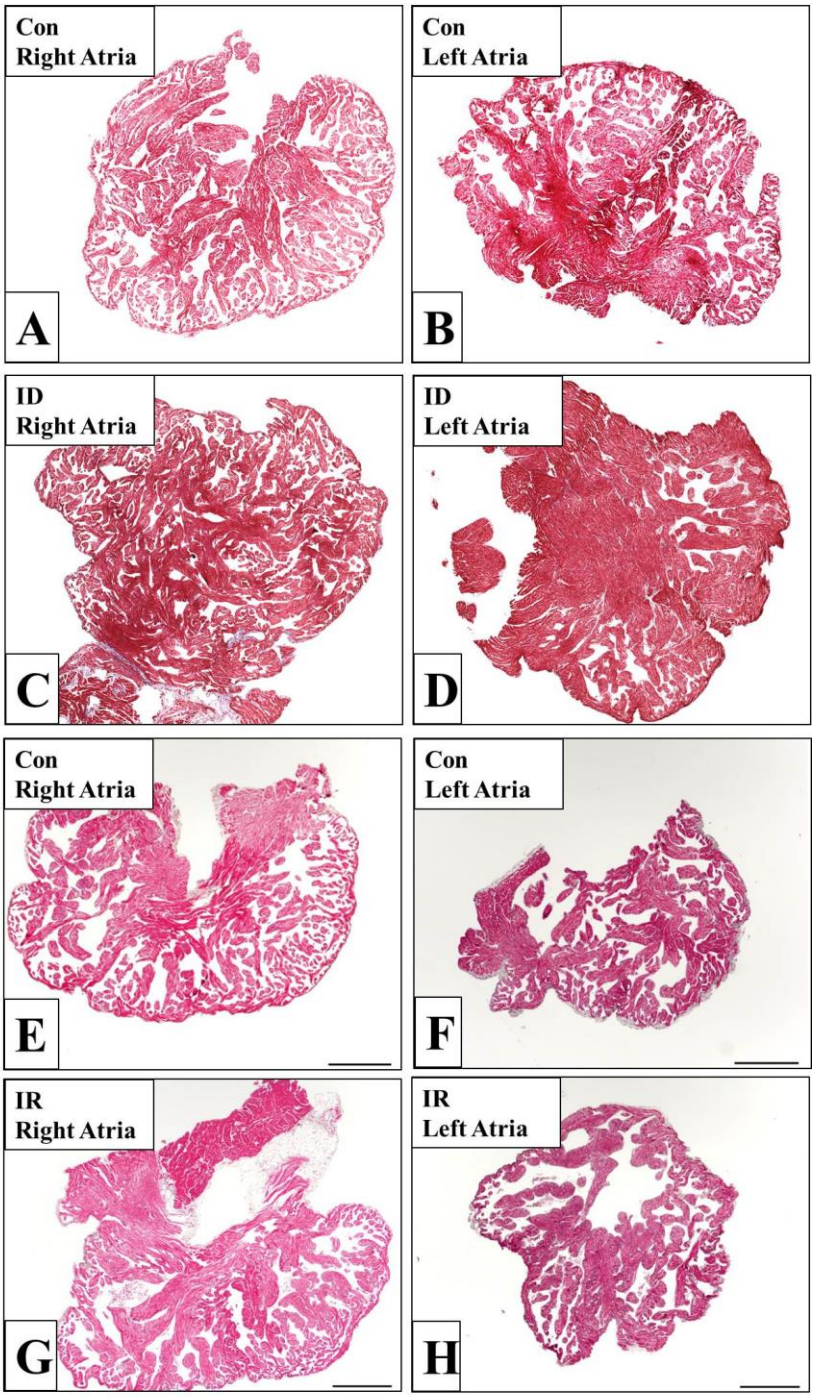


**Figure 8: Atrial fibrosis in the atria of the insulin-deficient and insulin-resistant animals**

**Representative images demonstrating the absence of fibrotic deposits in the Control (Con), insulin-deficient (ID) and insulin-resistant (IR) atria.** n=4-5/group; Methods: Masson Trichrome Stain (Blue staining collagen). Scale Bar: 500µm; ID: Insulin-deficient; IR: Insulin-resistant; Con: control animals paired with age matched ID or IR animals as appropriate;

## DISCUSSION

The goal of this study was to investigate the possible underlying substrates that render the diabetic atria vulnerable to AF induction. Our results indicated that 1) both the insulin-deficient and insulin-resistant mice have increased vulnerability and propensity to AF induction; 2) there was no significant accumulation of glycogen, AGE or fibrotic deposits in the atria of subjects with insulin dysregulation.

Recent epidemiologic studies have identified diabetes as an independent risk factor for AF.[15;17;19;185;189-192] A large population-based study reported that 7.6% of the diabetic subjects displayed baseline AF.[186] It has also been suggested that combination of insulin-resistance, and fluctuations in the glycemic state is one of the mechanisms of diabetes-induced AF.[19] In a similar study, it was reported that high fasting blood glucose and insulin levels were correlated with the risk factor for the development of AF in the diabetic patients.[15;184] The risk factor of AF is also suggested to increase with the duration of diabetes and level of glycemic control.[15] Additionally, AF attacks in insulin-dependent type 1 diabetic patients have been associated with periods of hypoglycemia.[187;188] Another study by Rigalleau *et al.* reported that major hyperglycemic episodes precede AF attack in insulin-resistant type 2 diabetes patient.[16] Together, the results of these studies suggest a strong correlation of insulin disturbance and an inadequate glycemic control with AF.[15;17;19;185;189-192] In agreement with the above studies[15;17;19;185;189-192] our findings demonstrated a higher AF inducibility in animals with insulin-deficiency (ID) and insulin-resistance (IR).

In the absence of a spontaneous model of AF, it has been difficult to investigate the pathophysiological link between diabetes and AF. Therefore, in order to investigate the mechanisms of AF development and to emulate the pathological conditions during AF, transvenous or transesophageal rapid atrial pacing has been used. [154;334-337] In the current study we have used transesophageal atrial pacing, which is minimally invasive, does not require mechanical alterations of the atrial tissue (invasive open chest stimulation or catheterization) and allows the animal an immediate recovery. The results from the transesophageal atrial pacing of the insulin-deficient (ID) animals indicated that the ID mice had greater frequency and longer duration of atrial tachy-arrhythmias, including atrial fibrillation and atrial tachycardia. Eighty five percent of the ID animals displayed some or all forms of tachy-arrhythmias. Similarly, IR mice had greater frequency and longer duration of atrial tachy-arrhythmia. The IR group demonstrated 100% vulnerability to AF induction whereas only 50% in the control animals displayed mild AF. The overall duration of AF was approximately 6 times longer in both of the groups with insulin dysregulation compared to their respective controls. Together, these data strongly suggest that insulin dysregulation significantly increases the arrhythmogenicity of the atria.

We further investigated the possible underlying mechanisms underlying increased arrhythmogenicity of the atria. One of the predominant changes that have been reported in the atria at the onset of AF is glycogen accumulation.[176-180] Increased glycogen accumulation in the left atrial appendage has been reported at the onset of AF in large animal models, where persistent AF was induced by atrial pacing in healthy goats.[179;180] These studies reported that glycogen accumulated at the intercalated discs and at the myocyte junctions, inducing lateral and longitudinal conduction blockage in the electrical

signal transduction pathway. This phenomenon facilitated the formation of the re-entry circuits and thereby promoted AF induction.[179;180] In similar studies, Ausma *et al.* reported that there was significant electrical (shortening of the atrial refractory period) and structural remodeling (including glycogen accumulation, loss of contractile apparatus function and two fold increase in myocyte cell size) that occurred due to the induction of AF by rapid atrial pacing in healthy goat hearts. [176;177;181] In contrast to these studies, Kondrat've *et al.* reported that AF induction in healthy Wistar rats (5-10 mins for 8-10 times) depleted the atrial glycogen storage by activating the glycogen phosphorylase system. They speculated that this was caused by the increase in need of energy consumption for the inefficient and frequent contractions of the fibrillating myocardium.[219] However, the animal models used in these studies were healthy at the beginning of the study and not diabetic.[176-181] We recently reported impairment in regulation of the insulin-sensitive glucose transporter isoforms GLUT4 and GLUT8 in the atria of ID and the IR subjects (using the same animal models as the current study). In light of these recent findings, we investigated potential alterations in glycogen accumulation in the atria of ID and IR mice. In contrast with previous studies that reported increased glycogen accumulation in the myocardium of diabetic patients and rodents,[206;207;341-347] our results did not indicate any distinct location or distribution of glycogen in the ID or the IR groups compared to their respective controls. Possible interpretations of these findings include the fact that: 1) the mouse atria, being extremely small in size, do not possess the capacity for glycogen accumulation; 2) due to the extremely high baseline heart rate, the mouse atria utilize most of the glycogen stores under physiological conditions (AF and glycogen accumulation are reported in large animal models).

Advanced glycation end products (AGEs) are formed during chronic hyperglycemia by a glycosylation reaction (Millard's reaction) between the large proteins with slow turnover rate and the glucose present in the blood. AGEs, once formed, are irreversible and have been deemed responsible for many of the cardiac complications associated with diabetes. The role of AGEs in the perpetuation and progression of AF have been debated in recent studies. In a study conducted by Begieneman *et al.,* increased expression of major AGEs ($N^{\varepsilon}$- carboxymethyl lysine) was reported in the left atrial appendage of AF patients.[50] Raposeiras-Roubin *et al.* reported increased levels of plasma AGEs and sRAGE (soluble receptor for AGE) in patients with AF.[51] In contrast, Schneider *et al.* reported that, in a large community based study, the incidence of AF was unrelated to the level of sRAGE. [198] Therefore, whether AGEs play a direct role in the development of AF remains elusive. In the current study, our results do not indicate any difference in AGE deposition between the highly vulnerable (to AF) ID and IR atria and the less susceptible control atria. Therefore, in the current study our findings do not demonstrate any association between AGE deposition and AF during insulin dysregulation.

The relationship between atrial fibrosis and AF has been the subject of intense investigations in the past 25 years. Atrial fibrosis is difficult to reverse and therefore has been considered as major contributor in the progression from paroxysmal to persistent and eventually, permanent AF.[157] It has been suggested that fibrotic deposits in the atria can cause conduction blockage and thereby create a disorganized conduction wave, facilitating the development of AF. Interestingly, it has been reported that the atrium is more vulnerable to fibrosis compared to the ventricle.[159] Venteclef *et al.* reported

increased atrial fibrosis constituting collagen I, III and VI in the atrial biopsies of human AF patients.[164] In another study, Frustaci *et al*. reported the existence of patchy fibrosis in endo-myocardial biopsies from the right atrium of human patients with paroxysmal lone AF (AF in the absence of other cardiac complications).[36;60] In a diabetic small animal model, Kato *et al.* reported fibrotic deposits in the atria that demonstrated increased atrial arrhythmogenicity.[14] Therefore, in the current study we investigated atrial fibrosis in the ID and IR animals, which demonstrate high susceptibility to AF.. To this end, we measured pro-fibrotic marker TGFβ-1, MMP-9 and collagen deposition in the atrial tissue.

Transforming growth factor 1 (TGFβ-1), secreted by both myocytes and fibroblasts, is one of the well-established pro-fibrotic molecules. It has been suggested that cardiac overexpression of active TGFβ-1 causes atrial fibrosis, disruption in atrial conductivity, and promotes AF.[162;163] Verheule *et al*. reported that selective overexpression of TGFβ-1 in the atrium was sufficient to increase AF inducibility during rapid atrial pacing in a transgenic mouse model.[162] In a large animal model, Polejaeva *et al*. reported that overexpression of TGFβ-1 in the heart resulted in atrial fibrosis which in turn increased inducibility of AF in transgenic goats.[165] Together, the results from these studies indicate a strong connection between enhanced TGFβ-1 and AF propensity. In agreement with these findings, our results demonstrate significantly higher expression of active TGFβ-1 in both ID and IR atria compared to control, suggesting a significant association between overexpression of TGFβ-1 and AF.

Recently, increased expression of MMP-9 has been identified as an independent risk factor of AF as it contributes to atrial structural remodeling and dilation.[145] MMPs

(Matrix Metalloproteinases), responsible for the degradation of ECM proteins, are expressed at a very low level in the healthy myocardium. However, substantial upregulation of MMPs have been associated with heart failure and AF.[171] In a case-cohort-based study, Huxley *et al.* reported that increased MMP-9 was associated with an increase in risk for AF.[175] Nakano *et al*. also reported significant increase in MMP-9 levels in atrial biopsies of human patients with both paroxysmal and persistent AF.[145] A progressive increase of the MMP-9 level was reported with the severity of the disease (from paroxysmal to persistent AF). In animal models of AF, Chen *et al.* reported that during rapid atrial pacing in a porcine model, there was an increased expression and activity of MMP-9 in its active form.[166] In a similar study Hoit *et al*. reported that in rapid atrial pacing canine model there was increased MMP-9 activity in the atrium.[173] In addition, it has been postulated that MMP-9 modulates the activation of TGFβ-1 and thereby contributes to atrial fibrosis. Therefore, the results of these studies indicated that MMP-9 significantly contributes towards atrial remodeling and render the atria susceptible to AF. In the present study, our results demonstrated a significant up-regulation of active MMP-9 in the ID and IR atria in confirmation with the suggested link between up-regulation of MMP-9 during AF. Overall, the overexpression of TGFβ-1 and MMP-9 suggested that the ID and IR animals have increased vulnerability towards atrial fibrosis, which could be one of the reasons that these animals are highly susceptible to AF. However, when we investigated for atrial fibrosis itself, no fibrotic deposits were to be found in either of the diseased atria, suggesting that there are alternate mechanisms that a play role in the development of AF in the animals with insulin-dysregulation.

# CONCLUSIONS

Using transesophageal atrial pacing, we demonstrated that both the insulin-deficient and insulin-resistant mice have greater vulnerability and propensity to atrial fibrillation induction. Although we previously reported alterations in atrial glucose transport, we did not report any atrial fibrosis, glycogen or AGE accumulation in the atria of the subjects with insulin dysregulation in the current study. Therefore, the findings from this study indicate that insulin dysregulation, resulting in impaired glucose uptake, is sufficient to provide a metabolic arrhythmogenic substrate and could be an early pathogenic factor of AF.

# CHAPTER VI

# CONCLUSION

The goal of this study was to investigate the mechanisms underlying diabetes-induced atrial fibrillation. To this end, the regulation of insulin sensitive glucose transporter 4 and 8 in the healthy and diabetic atria (type 1 and type 2) were investigated. In addition, the vulnerability of the diabetic subject to atrial fibrillation was tested. The findings from this study indicate that, 1) The major cardiac isoform GLUT4 and novel isoform GLUT8 are insulin sensitive in the healthy atria and are regulated by the downstream insulin signaling pathway, 2) The expression and regulation of the insulin sensitive GLUTs in the atria are downregulated during insulin-deficient type-1 diabetes and insulin resistance, 3) Both insulin deficiency and insulin resistance significantly increase the vulnerability and propensity to atrial fibrillation induction, and 4) The animals with insulin dysregulation do not demonstrate a fibrotic atria along with unaltered glycogen and AGE accumulation compared to the healthy animals. Collectively, our data suggest that in the absence of structural remodeling and atrial fibrosis, disturbances in the insulin and glycemic states were sufficient to increase the susceptibility towards AF. This finding is in agreement with our central hypothesis that,

*"Dysregulation of glucose metabolism in the diabetic atria is underlying the diabetes-induced development of AF."*

The heart utilizes glucose as a major energy substrate as it generates ~30 % of its total energy (ATP) from glucose oxidation during physiological condition.[21] Therefore, cardiac glucose uptake and utilization is crucial for proper cardiac function. This is crucial to the fact that the atria, which contains the pacemaker of the heart, significantly contributes to the overall cardiac function. During glucose oxidation 38 molecules of ATP are generated from each molecule of glucose. It can be suggested that reduced glucose uptake due to the dysregulation of major cardiac GLUT isoforms (GLUT4 and GLUT8) would lead to an overall reduction in ATP generation during the absorptive state. Decline in ATP generation in turn could potentially cause abnormality in the function of major cardiac ion pumps such as the $Na^+/K^+$ ATPase, membrane bound $Ca^{2+}$ ATPase and sarcoplasmic reticulum $Ca^{2+}$ATPase (SERCA) pumps.[295;351-357] Impaired function of these major ion pumps could cause an imbalance between intracellular and extracellular $Na^+$, $K^+$ and $Ca^{2+}$ concentrations leading to ectopic focus/impulse formation which initiates the perpetuation of atrial fibrillation.[130;131;133;135;177;358;359] Membrane bound $Ca^{2+}$ ATPase and the SERCA pumps are responsible for the clearance and recycling of intracellular $Ca^{2+}$ during diastole. However, impaired SERCA function (due to the lack of available ATP) could lead to alteration in $Ca^{2+}$ handling and eventually cytosolic $Ca^{2+}$ overload. Intracellular $Ca^{2+}$ overload is responsible for 1) the prolongation of the action potential duration (APD);[130;135] 2) the formation of delayed and early afterdepolarization (DAD and EAD respectively);[358;360;361] 3) sarcoplasmic reticulum spontaneous $Ca^{2+}$ release during diastole;[358;362] and 4) mitochondrial $Ca^{2+}$ overload, all of

which are arrhythmogenic (directly or indirectly). Prolongation of the APD allows the formation of delayed afterdepolarizations (DADs) during the relative refractory period and subsequently allows the formation of early afterdepolarizations (EADs). EADs and DADs that are large enough to reach the threshold potential of the atrial myocyte can initiate ectopic firing.[358;359;363] It has been reported that repetitive DAD formation can cause atrial tachycardia.[363] In addition, increased EAD formation has been observed in AF patients and during atrial pacing in large animal models.[360;364] During intracellular $Ca^{2+}$ overload the mitochondria itself accumulate a significant amount of $Ca^{2+}$ via the $Ca^{2+}$ uniporter ion channel.[365;366] Mitochondrial $Ca^{2+}$ overload initiates increase in the production of reactive oxygen species (ROS) and free radicals, which are detrimental to the mitochondria itself and cause damage to the mitochondrial DNA.[367] This results in increased intracellular ROS and oxidative stress. Mitochondrial damage and enhanced oxidative stress have been significantly associated with AF.[367-369] Figure 1 depicts the proposed mechanisms by which alteration in GLUT trafficking/glucose uptake could lead to the development and maintenance of AF.

Overall, the results obtained from this study indicate that insulin dysregulation, resulting in impaired glucose uptake, is sufficient to provide a metabolic arrhythmogenic substrate and could be an early pathogenic factor of AF. Therefore, better understanding of the regulation of glucose transport may lead to the discovery of novel therapeutic targets for the treatment of cardiovascular complications associated with diabetes, including atrial fibrillation.

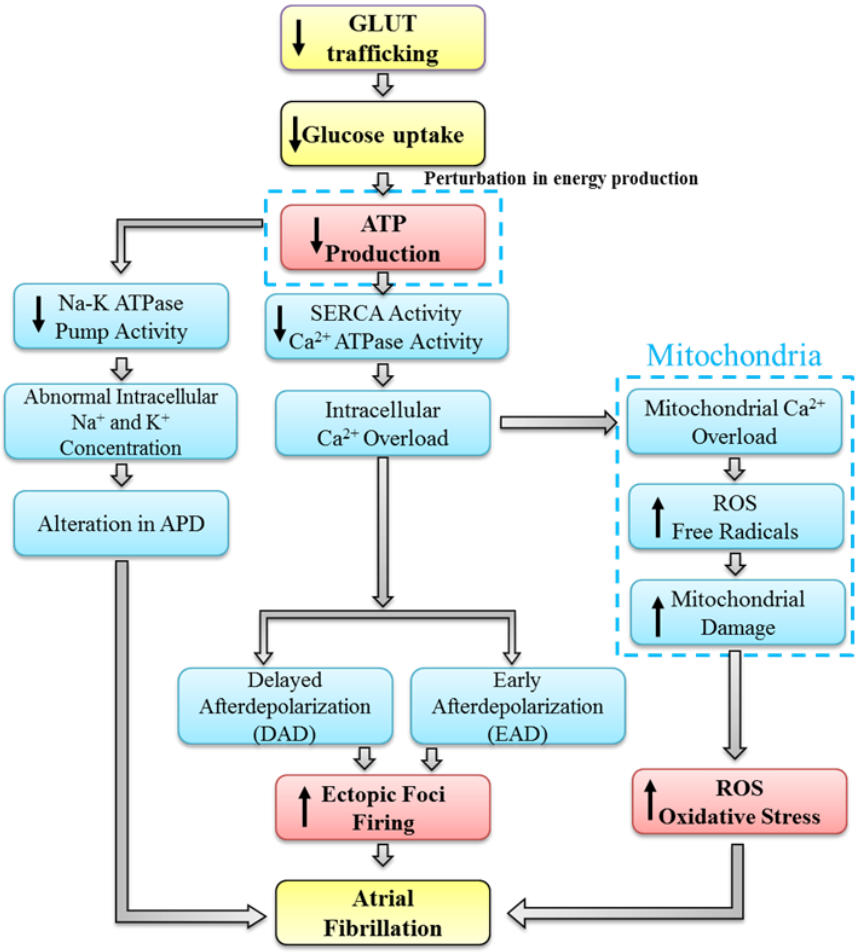


**Figure 1: Schematic diagram depicting the proposed role of impaired glucose uptake in the initiation of atrial fibrillation.** ATP: Adenosine tri phosphate; APD: Action potential duration; ROS: Reactive oxygen species; SERCA: Sarcoplasmic calcium ATPase pump; $Ca^{2+}$: Calcium ion; $Na^{+}$: Sodium ion; $K^{+}$: Potassium ion.

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# APPENDIX A

## ABBREVIATIONS

| | |
|---|---|
| AF | Atrial Fibrillation |
| AGE | Advanced Glycation End Products |
| AKT | Protein Kinase B; Serine/Threonine Specific Protein Kinase |
| AMPK | AMP (5’ Adenosine Mono Phosphate) Activated Protein Kinase |
| APD | Action Potential Duration |
| AS160 | Akt Substrate of 160kDa |
| ATP | Adenosine Tri-Phosphate |
| CAMK | Calcium/Calmodulin dependent Protein Kinase |
| CVD | Cardio Vascular Disease |
| CML | Nε-Carboxymethyllysine |
| DADs | Delayed After Depolarization |
| ECM | Extra Cellular Matrix |
| ER | Endoplasmic Reticulum |

| | |
|---|---|
| GADAs | Glutamic Acid Decarboxylase |
| GAP | GTPase Activating Protein |
| GLUT | Glucose Transporter |
| HLA | Human Leukocyte Antigen |
| IA2A | Tyrosine Phosphatase |
| ID | Insulin-deficient |
| IL-1β | Interlukin-1 beta |
| IR | Insulin-resistant |
| IRS-1 | Insulin Receptor Substrate-1 |
| LAA | Left Atrial Appendage |
| MHC | Major Histocompatibility Complex |
| MMP | Matrix Metalloproteinase |
| mRNA | Messenger RNA |
| mTORC2 | Mammalian Target of Rapamycin Complex 2 |
| NADH | Reduced Nicotinamide Adenine Dinucleotide |
| NCX | Sodium/Calcium Ion Exchanger |
| NFκB | Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells |

| | |
|---|---|
| PAS | Phospho Akt Substrate |
| PFK-1 | Phospho Fructo Kinase-1 |
| PI3-K | Phosphoinositide 3-Kinase |
| PDK-1 | Phosphoinositide Dependent Kinase-1 |
| PIP2 | Phosphatidylinositol 4,5-bisphosphate |
| PIP3 | Phosphatidylinositol (3,4,5)-trisphosphate |
| RAA | Right Atrial Appendage |
| RAGE | Receptor for Advanced Glycation End products |
| RNA | Ribonucleic Acid |
| RTK | Tyrosine Kinase Receptor Family |
| sRAGE | Soluble Receptor for Advanced Glycation End products |
| SA Node | Sinoatrial Node |
| SMAD | Mothers Against Decapentaplegic Homolog |
| SNAP-23 | Synaptosomal-Associated Protein 23 |
| SR | Sinus Rhythm |
| STZ | Streptozotocin |
| T1Dx | Type-1 diabetes/ type-1 diabetic animal |

| | |
|---|---|
| T2Dx | Type-2 diabetes/ type-2 diabetic animal |
| TCA | Tricarboxylic Acid |
| TGFβ-1 | Transforming Growth Factor beta 1 |
| TGN | Trans Golgi Network |
| TIMP | Tissue Inhibitor of Matrix Metalloproteinase |
| TNF-α | Tumor Necrosis Factor Alpha, TNFα |
| SERCA | Sarcoplasmic Reticulum Calcium ATPase Pump |
| SSS | Sick Sinus Syndrome |

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