Molecular and Cellular Signaling Mechanisms Elucidating Aldose Reductase Mediated Ischemia-Reperfusion Injury in the Myocardium

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ABSTRACT

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This dissertation serves to analyze the molecular and cellular signaling mechanisms by which aldose reductase (AR) contributes to ischemia/reperfusion (I/R) injury, specifically in the myocardium. It provides a comprehensive overview of the pathophysiology associated with acute coronary syndromes, the mechanisms of normal myocardial substrate metabolism, and how metabolism is altered under ischemic conditions. Specific chapters in this dissertation aim to elucidate whether AR pathway mediated I/R injury is linked to changes in glycogen synthase kinase 3β (GSK3β) phosphorylation, and whether an increased flux via AR and resultant I/R injury is mediated by changes in autophagy.

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Acute Coronary Syndromes

Cardiovascular diseases which are defined as diseases of the heart and vasculature are the number one cause of death in men and women in the United States [1]. It has been estimated that CVD and stroke related costs for 2010 are approximately $503.2 billion and are expected to triple by the year 2030 [1]. Through several studies, it has become increasingly evident that poor nutrition as well as lack of physical activity play large roles in cardiovascular disease induction and progression [2]. Diabetes is a major risk factor for developing CVD and heart failure; however there is an increased prevalence of people who suffer from diabetic cardiomyopathy, defined as heart failure independent of coronary artery disease [3]. The increased prevalence of diabetes and CVD in the United States can be attributed to the sedentary lifestyle and poor eating habits, however other factors can play a role including age, smoking, and a previous family history of CVD.

The most common cause of ischemic disease is atherosclerosis [4-5]. With disease progression, atherosclerotic plaque buildup in the vasculature can erode and rupture causing a thrombus to develop in arteries which may ultimately reduce or stop blood flow necessary for tissue survival. Acute coronary syndromes (ACS) are conditions which abruptly stop blood flow to the heart due to development of thrombosis on atherosclerotic plaques. Plaque disruption and thrombosis development are the main cause of ACS, which include unstable angina, acute myocardial infarction and sudden ischemic death [4-7]. A reduction in blood flow ultimately leads to myocardial infarction and/or tissue death (necrosis). Patients with acute coronary syndromes are at high risk for myocardial infarctions and/or death and the risks are largely associated with the composition of the plaque.

Angina describes chest pain, or discomfort associated with myocardial infarction and is a typical symptom of patients who present with ACS. Patients who report short episodes of chest pain as a result of activity such as exercise that increases myocardial oxygen demand are described clinically as presenting with stable angina. Unstable angina, on the other hand, is characterized as the unpredictable occurrence of chest pain either at rest or during exercise and without elevation of ST (period of systolic depolarization) segment on the electrocardiogram (ECG) or without detectable biomarkers of necrosis. If biomarkers of necrosis such as Troponin I or Troponin T are detected, patients are described clinically as presenting with non ST elevation myocardial infarction (NSTEMI) [8-11]. Those patients categorized with ST segment elevation (STEMI) typically also have detectable levels of necrotic biomarkers in their blood. Use of clinical myocardial damage biomarkers are no longer limited to those of myocardial necrosis such as the troponins, which are the gold standard markers for cardiac injury, but have expanded to include markers of hemodynamic stress, inflammation and vascular damage [12]. However a search for markers of myocardial ischemia as well as other stages that precede myocardial necrosis including disruption of the plaque, oxidative stress, and tissue hypoxia is warranted [13]. Identification of early stage biomarkers may aid in the clinical outcome for patients. Currently, the ECG is a widely available strategy utilized to detect changes in ischemia due to its low cost, high feasibility, and widely available characteristics [14]. Rapid diagnostic testing as well as early detection and treatment are critical in identifying those patients who could benefit most from therapeutic interventions and to prevent irreversible damage [15]. Therefore, understanding the pathophysiological mechanisms which result in the progression of atherosclerosis, including factors that contribute to plaque formation and plaque disruption are

critical in understanding the underlying risks associated with ACS and in identifying potential biomarkers to identify patients most at risk for developing ACS.

Pathophysiology of ACS:

One of the most common causes of ACS is plaque formation as a result of atherosclerosis and subsequent rupture of the plaque [16]. Development of atherosclerosis or atherogenesis, occurs due to a number of factors which together are the root cause for plaque formation. Increased lipids such as low density lipoprotein (LDL) cholesterol in the blood is transported and deposited in the lumen of blood vessels resulting in the thickening of artery walls and abnormal features of the blood vessel due to endothelial cell injury [17-18]. A pathological inflammatory response ensues due to the initial damage that occurs in the blood vessel wall with increased infiltration and adhesion of monocytes to the surface of the vessel. Monocytes eventually differentiate into a macrophage cell in an attempt to ingest the oxidized LDL particles [19]. Ingestion of oxidized LDL particles by macrophages, now known as 'foam cells,' further escalates the inflammatory response which leads to vascular smooth muscle cell (VSMC) activation [20]. As the disease progresses, activated VSMCs begin to migrate to the neointima portion of the vessel where they begin to proliferate and activate the extracellular matrix generating a fibrous cap [21]. The inflammatory events that occur in the setting of atherosclerosis are correlated with increased episodes of ischemia in patients who present with ACS [20]. Atherosclerotic plaques are divided into two categories, unstable and stable and the likelihood of a plaque becoming unstable and rupturing depends on the type and composition of the plaque [22-23]. Typically, unstable atherosclerotic plaques include a thin fibrous cap and a lipid-rich core that usually occupies greater than 40% of the plaque volume [24]. Stable plaques, on the other hand, develop slowly,

are characterized by having a supportive fibrous cap that is rich in extracellular matrix, and lipid content containing less than 40% of the plaque volume. Unstable plaques which are more prone to rupture are an impending cause of ACS [25]. Although plaque rupture is the most common cause of ACS, other less common causes include drug use, spontaneous coronary artery dissection and coronary embolism [9].

In the context of diabetes, several studies have demonstrated how the metabolic changes, triggered by high blood glucose, accompany the structural and functional modifications in the diabetic myocardium [26-27]. Elevated blood glucose levels are prevalent in patients admitted to hospitals with ACS and are often an indicator of poor prognosis in these patients [28-30]. Because of several contributing factors occurring together including hyperglycemia, increased platelet aggregation and inflammation, diabetic patients with ACS are at greater risk for developing adverse cardiovascular complications [31]. Pathophysiological changes and adverse effects mediated by hyperglycemia including increased inflammation, apoptosis and cellular injury and oxidative stress alter the efficient functioning of the cardiomyocytes and contribute to the increased risk of the diabetic population developing cardiovascular complications. [29, 32]. Build-up of atherosclerotic lesions leading to infarction as well as development of diabetic cardiomyopathy are processes that progress slowly over time and may be asymptomatic impacting the severity of the disease. Patients often do not seek medical help until severe damage has already occurred.

Genetics

It has long been appreciated that coronary artery disease is linked to family history. Gaining insight into possible genetic factors which act to influence ACS has been the focus of several

studies. Understanding pathophysiological features of atherosclerosis and the critical steps which lead to progression of this disease has been a guide in determining possible genetic factors linked to ACS. Therefore, genes which encode some of the main molecules that significantly impact atherosclerosis including platelet function and aggregation (thrombus development), lipid metabolism, characteristics of the blood vessel and vascular function are main targets for genetic characterization. In the search for candidate genes, most studies have looked at polymorphisms or variants in a particular gene, in order to identify whether they occur more or less in patients affected with a myocardial infarction [33-34]. Increased risk of MI has been linked to several polymorphisms. High blood levels of coagulation factor VII and variants in the cholesterol ester transfer protein are associated with several conditions including diabetes mellitus, glucose metabolism, platelet and leukocyte function, and hyperlipidemia and have been associated with increased risk of MI [35]. Despite the fact that several polymorphisms have been identified, several inconsistencies between studies exist and not all polymorphisms have been shown to be associated with the risk of MI. Hence, clinical application of these polymorphism results has been limited. Regardless, the identification of possible targets has provided a platform for understanding the genetic basis of disease onset and progression and with more work could be utilized to improve patient prognosis.

Treatment:

The availability of treatments for acute coronary syndromes has grown over the past few years due to increased awareness of their pathophysiology. Currently available therapies aim to restore blood flow to infarcted tissue such as the use of thrombolytic therapy, percutaneous coronary intervention (PCI) and coronary bypass [36-37]. Treatment of myocardial infarction is aimed

toward restoring blood flow to infarcted areas (reperfusion) as soon as possible in order to salvage myocardial tissue and to reduce infarct size. PCI has emerged as the gold standard option for reperfusion of infarcted tissue. Several clinical trials have demonstrated the importance of early treatment since prompt treatment was associated with improved clinical outcomes in [15, 38] While widely available, one of the main disadvantages of fibrinolytic therapy is that platelet aggregation is increased due to increased thrombin production [39]. Therefore, combining pharmacological therapies which target both platelet aggregation (anti-platelet) and the thrombus (thrombolytic agent) has been shown to be effective in improving clinical efficacy[40]. While PCI alone is effective in achieving complete perfusion, this option is hindered largely due to the lengthy amount of time taken to implement this treatment. Since patient outcome is largely determined by time taken to treat patients, combining pharmacological mechanical therapy can reduce delays in treatment and can also aid in reperfusion before any PCI is performed [39, 41].

Favorable outcomes are determined mainly by prompt reperfusion of infarcted tissue. However, the injury which occurs during reperfusion is well established, and a limitation of the current therapy is impacted by the amount of irreversible damage that occurs during reperfusion [42-43]. Calcium overload, accumulation of oxygen-derived free radicals, and apoptosis all accompany reperfusion and are associated with increased tissue damage and poor outcomes in patients[39, 44]. To further add to the conundrum, it has been estimated that only one-third of those undergoing PCI actually receive adequate myocardial reperfusion and in some patients, adequate coronary artery recanalization is achieved but a condition known as no-reflow may occur where adequate myocardial reperfusion is not achieved [45]. The resulting infarct size and scar in patients with AMI is one of the major determining factors of future cardiac events or the

development of left ventricular remodeling [14]. Understanding myocardial substrate metabolism both with and without pathology will be critical to improve treatment plans for individuals and to minimize damage after an ischemic insult or MI.

Stem and progenitor cell therapy in patients with AMI have been proposed to be used in conjunction with, or after PCI [46]. After recanalization, heart failure development is a major problem due to the significant loss of cardiomyocytes and lack of cardiac tissue regeneration. Extensive basic and clinical research focuses on the regenerative properties of resident cardiac stem cells and bone marrow (BM) derived cells, with the aim to repair damaged cardiac tissue in hopes of ameliorating ventricular remodeling that leads to heart failure [47].

Several cell types including fetal cells, human umbilical cord derived stem cells, embryonic stem cells and skeletal myoblasts have been studied as potential candidates for cardiac tissue regeneration [48]. While studies have demonstrated improved contractile function and effective tissue regeneration after myocardial infarction via embryonic and fetal stem cell transplantation, the availability and ethical issues which surround the use of these cell types has greatly hindered research efforts and therefore urged the need to utilize other cell types [49]. The decrease in cell death, increase in the regeneration of myocardial tissue, and enhanced neovascularization are some of the proposed mechanisms shown to contribute to the beneficial effects of cell therapy.

Several methods are currently available for the delivery of cells to the heart. These include, cell mobilization, peripheral and intracoronary infusions, and direct intramyocardial injections [48]. The use of pharmacological agents, such as granulocyte colony stimulating factor (G-CSF), is an attractive target due to its non invasive nature and has also been studied extensively in clinical trials. However, studies demonstrating the lack of beneficial effects with G-CSF have

questioned this method. Intracoronary infusions are the most popular method for cell delivery currently used and the safety of intracoronary infusions have been established by several clinical trials, although no significant differences in morbidity and mortality are observed in patients who received BM derived stem cells [50]. While several methods are available, including cell mobilization and intramyocardial injections, not much is known about the safety and efficacy of these delivery methods [51]. Furthermore, identifying the patients who would benefit most from cell therapy, the dose of cells to be delivered, the optimal cell type to be delivered and the mechanisms by which cell therapy exhibit beneficial effects all need further evaluation. More clinical trials are needed in order to establish safety and efficacy of stem cell therapy in improving cardiac outcomes in patients with AMI.

Ischemic Preconditioning:

The term ischemic preconditioning (IPC) is a procedure where brief episodes of ischemia and reperfusion in alternating cycles are performed on a heart and have been shown to reduce infarct size. Originally described by Murry, Jennings and Reimer, IPC performed on dogs was found to reduce infarct size and preserve ATP protecting the heart against lethal ischemia [52]. Since this observation, several studies have focused on this phenomenon and have tried to elucidate the mechanisms by which IPC protects the myocardium against lethal ischemia. There are two windows of protection with IPC termed ‘early’ and ‘late’ windows. The early window occurs rapidly and protection may last approximately 1-2 hours after IPC whereas the late window occurs about 24 hours after IPC and the protection can last for up to 3 days [53]. IPC has been shown to improve cardiac function through reductions in infarct size, apoptosis, endothelial dysfunction, and reactive oxygen species[54]. Improvement in metabolic status of the heart

including reductions in the rate of ATP utilization, depleted glycogen stores, and decreased lactate and proton accumulation have been proposed and shown to protect against lethal ischemia, however, these mechanisms are unlikely to explain the intense protection conferred by IPC [55]. Neuroendocrine triggers including bradykinin and opiods have been identified and shown to be released during ischemia initiating IPC protection through adenosine receptor mediated pathways [55-57].

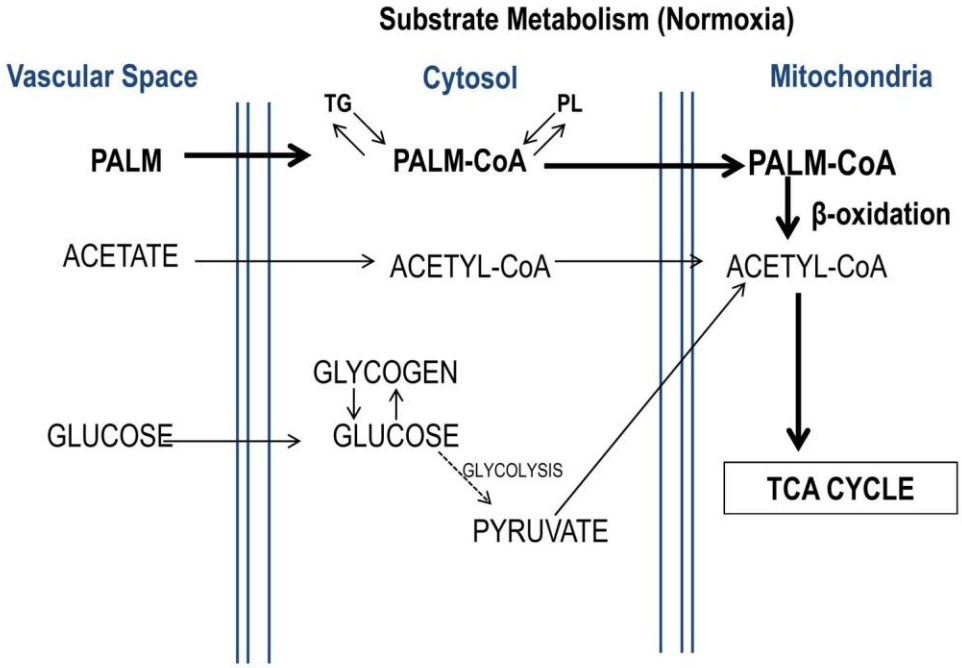
Adenosine receptors signal via phospholipases and diacylglycerol (DAG) to activate PKC and IPC protection. Studies utilizing Protein Kinase C (PKC) inhibitors such as chelerythrine and staurosporine completely abolished protection via preconditioning, demonstrating the essential role of PKC pathway in preconditioning [58-59]. It has been proposed that the K_{ATP} channel is one of the targets of PKC and a proposed hypothesis is that PKC mediates cardioprotection by activating the K_{ATP} channel and targeting the mitochondrial permeability transition pore (mPTP), both of which are important factors when considering cardiomyocyte viability after stress induction [60-61]. Several other kinases including mitogen-activated protein kinase (MAPK), PI3 kinase, AKT and ERK have been shown to be involved in preconditioning by inhibiting formation and opening of mPTP [55]. Various PKC inhibitors and activator peptides of PKC translocation have been developed and studied in mediating cardioprotective effects. These peptides can be effectively delivered into cardiomyoctes via tat-protein derived peptides and the cardioprotective effects mediated by these tat protein-derived peptides including δ-PKC inhibitor peptide δV1-1 and ε-PKC activator peptide ψεRACK, have been shown to reduce damage after ischemia reperfusion (I/R) [62-63]. Notably, ε-PKC activator peptide was demonstrated to exhibit ischemic preconditioning like effects. The effect of PKC regulating tat peptides have also been examined for their effect on ischemia reperfusion.

The application of preconditioning in a clinical setting has garnered a great deal of attention, however since most patients with ACS already present with acute MI, identifying patients who would benefit most from this therapy has been difficult. The beneficial effects of preconditioning require application of the therapy before the onset of lethal ischemia. Because mimicking the exact animal experimental methodology in clinical patients is problematic for ethical reasons, surrogate end points have to be utilized [64]. Clinical coronary angioplasty studies where balloons are inflated sequentially causing brief episodes of ischemia have demonstrated attenuations in creatine kinase (CK) release, lactate production and elevations in ST segment [65-67]. Reductions in myocardial infarct and improvement in left ventricular ejection fraction (LVEF) was also observed in a separate study where coronary angioplasty balloons were inflated and deflated for up to six cycles following stent placement (ischemic post conditioning) [68]. Because of the extensive research investigating the mechanisms of IPC, numerous agents which mimic IPC have emerged and are available for use in humans including adenosine agonist AMP579, sildenafil, erythropoietin, and atrial natriuretic peptide (ANP) [56, 69-71].

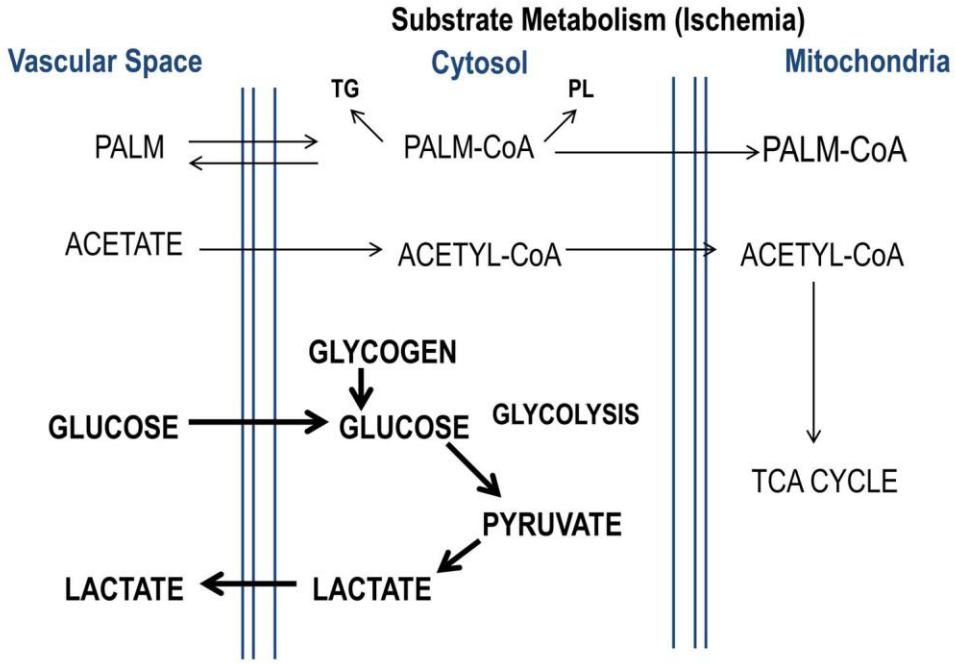
Metabolism of heart (Normoxia)

The heart is a dynamic organ which can utilize several different substrates including fatty acids (FAs), glucose amino acids, lactate, and ketone bodies [72]. To meet the energy demands of contractile function, the heart utilizes enormous amounts of substrate. The normal heart under aerobic conditions relies primarily on the oxidation of FAs rather than any of the other substrates for energy production due to the significant amounts of ATP produced per molecule of fatty acid (Figure 1 adapted from [73]).

Figure 1:



Under normal conditions, fatty acids are the preferred substrate for energy production yielding significant amounts of ATP per mole of fatty acid. Glycolysis yields <2% of ATP under normal physiological conditions [76]



Under ischemic conditions, the heart switches from primarily utilizing fatty acids to glucose oxidation and glycolysis. Oxidative phosphorylation is significantly impaired.

Under normal fasting conditions, FAs are readily available in the blood and become rapidly oxidized by the heart for ATP generation [74]. After a meal, blood glucose is increased greatly resulting in a subsequent increase in insulin production. The presence of insulin in the blood suppresses FA lipolysis which allows the heart to use glucose instead of FAs. Oxidation of FA yields the majority of acetyl-CoA, approximately 60-90%, while pyruvate formed via glycolysis yields 10-40% [72, 75]. Acetyl-CoA formed after FAs and glucose oxidation is fed into the Kreb's Cycle producing NADH and carbon dioxide. ATP is ultimately generated through oxidative phosphorylation which occurs within the mitochondria. As electrons pass through the respiratory chain in the mitochondria, a proton gradient created across the inner mitochondrial membrane serves as the driving force by which ATP is produced. Protons are shuttled through the ATP Synthase allowing phosphorylation of ADP [76]. In a normal healthy heart, substrate metabolism remains robust to meet the energy demands for contractile function.

Metabolism of heart (Ischemia)

Appreciating substrate metabolism in the normal healthy heart is critical in order to comprehend substrate metabolism in stressed states (Figure 1 adapted from [73]). In times of stress, such as during myocardial infarction, there is an imbalance between the rate of oxygen consumption and metabolite delivery necessary to meet the energy demands of the working heart [77]. During ischemia, the heart rapidly shifts from primarily oxidizing FAs for energy, to oxidizing pyruvate generated via glycolysis as an adaptive or maladaptive mechanism in efforts to protect the myocardium during ischemic stress. Studies using magnetic spectroscopy have been instrumental in establishing this metabolic shift and also to illustrate the imbalances in substrate metabolism utilization of the failing heart [78]. Earlier, it is mentioned that approximately 60-

90% of acetyl-CoA is formed via FA oxidation generating 95% of ATP [75]. The production of ATP via FAs oxidation is however more energetically costly than glucose oxidation. Beta oxidation of FA consumes about 12% more oxygen than glucose per ATP molecule generated. During the time of an ischemic insult, the heart switches to glucose utilization which dramatically reduces oxygen consumption and thus allows for continued ATP production, albeit at a much lower yield.

GLUT 1 (insulin insensitive) and GLUT4 (insulin sensitive) are the two isoforms present in the myocardium and have been shown to be responsible for glucose uptake in the heart [79-80]. After a meal when glucose and insulin are increased or in the event of an ischemic insult, GLUT4 translocates from intracellular compartments to the plasma membrane and facilitates glucose uptake by cardiomyocytes [81]. Once inside the cell, glucose becomes phosphorylated by the enzyme hexokinase forming glucose-6-phosphate (G-6-P). Once formed, G-6-P can either enter the glycolytic pathway or be converted into glycogen for storage. The fate of G-6-P to either enter glycolysis or to be converted into glycogen depends on several factors. Firstly, the cytosolic ratio of NADH/NAD+ greatly influences glycolytic flux since the enzyme glyceraldehyde-3 phosphate dehydrogenase (GAPDH) utilizes NAD+ in order to convert glyceraldehyde-3-phosphate into 1,3 diphosphoglycerate. The formation of 1,3 diphosphoglycerate further proceeds along in the glycolytic pathway creating pyruvate. Secondly, the rate of glycolysis or glycogen synthesis depends on the activity of phosphofructokinase which converts fructose-6-phosphate into fructose 1,6 bisphosphate. This is the key rate-limiting regulatory step in glycolysis and one which commits glucose to the glycolytic pathway. The lack of oxygen during ischemia forces the myocardium to rapidly

uptake glucose and shunt towards the glycolytic pathway. Earlier steps begin as previously described with GLUT4 transporters translocating to the plasma membrane to rapidly uptake glucose into the cardiomyocytes starved for energy. The duration and severity of ischemia will determine the extent of abnormalities observed in metabolism and the contribution glucose provides to energy production. During periods of severe ischemia where flow is eliminated, ATP becomes significantly depleted which leads to a rapid increase in glucose uptake and glycolytic flux. If severe ischemia is prolonged over time, inhibition of glycolysis will eventually occur due to an accumulation of glycolytic metabolites/cofactors and tissue necrosis will develop. A moderate ischemic insult, on the other hand, will result in a shift towards anaerobic metabolism with an increase in lactate production. It was mentioned earlier that the yield of ATP via glucose oxidation is far lower than that of FA oxidation; however, maintenance of glycolytic flux during ischemia is key in preserving the myocardium and salvaging tissue. Even though lower than FA oxidation, the ATP generated during glycolysis is sufficient to preserve myocardial tissue after an ischemic insult since it supplies enough energy to support functioning of enzymes including the Na+/K+ ATPase as well as the Ca2+-ATPase. Several studies in the literature have shown the benefits of sustaining glycolytic flux during ischemia and preservation of myocardial tissue [82-83]. Elucidating myocardial substrate metabolism can aid in the development of therapeutic agents which could target these substrates in order to increase glucose oxidation and improve myocardial function during and after I/R.

Because increased fatty acid beta oxidation during I/R is associated with negative outcomes, a beneficial strategy would be to develop therapeutic agents which will increase glucose and pyruvate oxidation and decrease fatty acid oxidation. There are several ways this can be

accomplished and will now be discussed in more detail. A measure that can be applied to increase myocardial glycolysis is by increasing glucose uptake. Animal studies have illustrated the beneficial effects glucose-insulin-potassium (GIK) in improving cardiac function, while clinical studies have varied greatly [84-85]. Because insulin is known to suppress lipolysis in adipocytes, insulin emerges as a potential therapeutic agent serving to increase glucose oxidation. This has been demonstrated clinically in patients with ischemic heart disease that were administered infusions of insulin which improved certain indices used to evaluate heart function including improved left ventricular ejection fraction [86]. GIK infusions administered to patients, improved patient outcome as a result of decreased FFA lipolysis and a reduced availability of FFA, therefore shifting β-oxidation to glycolysis and glucose oxidation [87-92]. Furthermore, decreased infarct size, a reduction in ventricular dysrhythmias and an overall increase in survival were all associated with use of GIK [93-94]. However, numerous clinical studies utilizing GIK have varied greatly in its ability to attenuate injury after acute myocardial infarction. In most of the clinical trials, GIK treatment was a fixed dose cocktail which failed to adjust to intravenous glucose administration [95]. Immediate increases in glucose concentrations, depending on the GIK cocktail used, immediate increases in glucose levels could result (when compared to pretreatment levels). Hyperglycemia is known to be associated with a host of negative outcomes including thrombus formation and inflammation [96-97]. Additionally, most of the clinical trials utilizing GIK did not take the metabolic status of patients into account, which could influence the effect of GIK [95]. Glucose levels in patients either before or after GIK administration varied between studies and the time and delivery of GIK administration greatly varied between clinical studies [95]. Because of these variations and inconclusive data, GIK is not currently utilized as an adjunctive therapy to for AMI. Adjunct to

reperfusion and thrombolytic therapies, studies demonstrated that infusion of GIK in diabetic patients decreased mortality [98]. In addition to increasing glucose uptake, therapies aimed at inhibiting fatty acid oxidation or directly activating PDH have also been studied as methods to increase glycolytic flux. Clinical studies in patients with heart failure that have utilized dichloroacetate (DCA), a PDH kinase inhibitor, have demonstrated improved cardiac contractility and improved glucose oxidation [99-100]. Carbohydrate metabolism is increased via direct activation of PDH and provides sufficient ATP needed for sustaining heart function through pyruvate oxidation. Acipimox, a niacin derivative and antilipolytic agent, is another therapy utilized, in the aim to inhibit FA oxidation [101-102]. Studies utilizing Acipimox have shown inhibition of FA oxidation and increases glucose oxidation [103].

It is established that decreasing FA β-oxidation while increasing glucose uptake and oxidation is key in maintaining optimal contractile function after I/R *in vitro*, however, the role of FA β-oxidation in protecting ischemic myocardium is evolving. Studies utilizing heart specific lipoprotein lipase knockout (hLpL0) mice models demonstrated that hearts were able to increase glucose uptake and glucose oxidation via increased PDH activity, while FA oxidation rates were decreased [104]. In these studies, it was observed that following abdominal aortic constriction, hLpL0 mice died suggesting that increased glucose oxidation alone was insufficient to protect the myocardium after chronic injury [104]. The concept of fatty acid β- oxidation inhibition arose due to studies illustrating a correlation between increased serum fatty acids and increased mortality. It was observed by Oliver et al, that the death rate was higher in patients with high levels of fatty acids in their serum compared to those with lower serum fatty acids [105]. The level of fatty acids in the serum was shown to be a primary determinant of glucose oxidation.

Increased serum fatty acids effectively inhibit glucose oxidation [106-107]. A major step in fatty acid oxidation is the delivery of FAs to the mitochondria and the key enzyme which mediates this process is Carnitine Palmitoyltransferase-I (CPT-1) [108]. Additionally, L-carnitine is important in the delivery of FA to the mitochondria via L-carnitine mediated transport and influences PDH activity and glucose oxidation [108-109]. Pharmacological agents aimed at inhibiting CPT-1 effectively reduce FA oxidation and also activate PDH. The beneficial effects of L-Carnitine administration has been shown clinically to increase glucose oxidation, stimulate PDH and improve contractile function upon reperfusion [110]. Ranolazine is a novel agent tested extensively in various animal models and has demonstrated efficacy by increasing glucose oxidation and in some instances inhibiting FA oxidation. In isolated mitochondria studies, trimetazidine, another anti-ischemic drug, has demonstrated a similar effect of increased glucose oxidation [111]. In hearts treated with trimetazidine, oxidative phosphorylation is inhibited when palmitoyl carnitine is the substrate suggesting that the drug inhibits FA oxidation [112].

Mitochondria pore and Ischemia-Reperfusion Injury:

As is mentioned earlier, recanalization therapy is widely accepted as the gold standard of treatment of myocardial infarction, however it is also widely acknowledged that the dysfunction observed during reperfusion is in part due to irreversible damage that occurs during ischemia. Therefore several studies have focused on improving metabolism during ischemia in order to prevent ischemic injury and to promote improved functional recovery of the myocardium during reperfusion. Despite these efforts, injury that occurs during reperfusion is exacerbated in part due to increased reactive oxygen species (ROS). Current therapy aims to restore blood flow to infarcted tissue; however, an inflammatory response ensues upon reperfusion in tissue deprived

of blood and oxygen. White blood cells including neutrophils flood infarcted areas inducing inflammatory factors and free radicals in response to tissue damage. Leukocyte accumulation due to increased adhesion to the endothelial surface contributes to further damage as blood flow in capillaries may become blocked or reduced. Free radical species released by neutrophils cause damage to proteins, lipids as well as DNA resulting in further damage. When considering substrate metabolism upon reperfusion, oxidative phosphorylation and oxygen consumption levels return to normal and a reduction in myocardial lactate levels are observed [74]. Several studies have shown that stimulation of glucose oxidation during reperfusion improves functional recovery due to a decrease in Ca^{2+} accumulation [113].

The mitochondria are the main energy centers of the cardiomyocytes and generate the energy through oxidative phosphorylation that is necessary for efficient contractile functioning. Maintenance of mitochondria is vital for the survival of the myocardium after a stressful event [114-116]. Consisting of an outer and inner membrane, the mitochondria must maintain a pH gradient and a certain membrane potential to drive ATP production needed for maintenance of ion homeostasis. During ischemia, oxidative phosphorylation reactions that occur in the mitochondria are disrupted, resulting in decreased ATP production, increase in phosphate, decrease in the intracellular pH, and an increase in intracellular Ca^{2+} concentrations. The acidosis that transpires during ischemia activates the Na^{+}/H^{+} which works to restore pH levels. Because ATP production during ischemia is severely compromised, the activity of this antiporter is disrupted causing Na^{+} overload. As mentioned earlier, maintenance of glycolysis during I/R is required to supply ATP necessary for Ca^{2+} and Na^{+} ion pumps to function efficiently. Intracellular Na+ increases during ischemia have been shown to occur due to increased uptake of

Na^+ via the Na^+/H^+, and a decrease in intracellular pH [117]. Following imbalances of the Na+/H+ antiport is the Na+ dependent rise in intracellular Ca^{2+} via the Na^+/Ca^{2+} exchanger. Studies have demonstrated decreases in Ca^{2+} accumulation when the Na^+/H^+ exchanger is inhibited [117-119]. During reperfusion, the Na^+/Ca^{2+}antiporter tries to restore intracellular Na^+ levels, however, Ca^{2+} overload proceeds are the conditions which greatly favor the opening of the mPTP [120-121].

The extent of injury to the mitochondria is highly dependent on the duration and severity of injury during I/R that the mitochondria are exposed to, and ultimately decides the fate of the cell. The extent to which the pore remains open eventually will decide which mechanism of cell death (i.e. apoptosis or necrosis [121-123]. Under normal physiological processes, generation of ROS from the mitochondria does occur. During reperfusion, the sudden influx of oxygen leads to a rapid induction of oxidative stress due to a much higher generation of ROS as well as a decrease in antioxidant defense systems such as superoxide dismutase and reduced glutathione [124]. The increased oxidative stress is accompanied by Ca^{2+} overload due to disruption in the activities of Ca^{2+}ATPase and Na^+/K^+ ATPase, ion pumps which work to increase calcium efflux and decrease calcium influx respectively. Calcium overload which ensues effectively inhibits oxidative phosphorylation reactions and causes the opening of mPTP. Once the mPTP opens, mitochondria lose the pH gradient and the membrane potential dissipates, both of which are required to generate ATP [125]. Eventually mitochondria become uncoupled with ATP depletion leading to cell death by necrosis.

Autophagy:

While most studies have focused on improving tissue viability and regeneration, recent studies on autophagy have shown that processes which eliminate dead/damaged cells are also important in improving viability. Autophagy (Auto=self) and (phagy=eat) describes a process triggered by a cell in order to rid itself of misfolded or damaged proteins, protein aggregates, and damaged organelles. The process of autophagy occurs under normal physiological conditions and is necessary to maintain cellular homeostasis [126]. Upregulation of autophagy under pathological conditions such as myocardial ischemia has been the focus of recent studies to determine whether the process under pathophysiological conditions is adaptive or maladaptive [127]. While some have shown conferred cardioprotection with autophagy upregulation, other studies have demonstrated the opposite[128] . Understanding the induction of autophagy, as well as critical components of the autophagic process is warranted before discussion of autophagy mediated cardio protection or lack thereof can be addressed.

The importance of constitutive activation of autophagy under basal conditions in the myocardium is noted in animal models, and in patients who present with Danon's disease. Development of cardiomyopathy, as a result of left ventricular dilation and contractile dysfunction due to defects in lysosomal associated membrane protein 2 (LAMP2) is ultimately what patients with Danon's disease face [129]. Deficiency in LAMP2 hinders the lysosome's ability to digest and eliminate engulfed proteins resulting in the accumulation of damaged macromolecules including mitochondria [130]. Cardiac specific knockdown of Atg 5 gene in mouse models have further strengthened the significance of autophagy in maintaining cardiac homeostasis, as these animals develop heart failure [131].

Several proteins/kinases are involved in the initiation of autophagy induction and comprise complex signaling pathways involved in activating the process of autophagy. Mammalian target of rapamycin (mTOR), phosphtidylinositol 3 kinase (PI3K), and AMP activated protein kinase (AMPK) are major players in the signaling cascade of autophagy induction and regulate its upregulation by sensing cellular status [132]. mTOR and AMPK are key sensors of nutrient status in a cell and are activated under periods of nutrient deprivation or starvation [132-133] . AMPK is located upstream of mTOR and when activated, AMPK phosphorylates and inhibits mTOR.

The process of autophagy involves formation of an autophagosome which functions to engulf damaged proteins and organelles for elimination [134]. The discovery of Atg (autophagic) genes allowed for a greater understanding of the critical components involved in autophagosome formation. More than 15 various Atg genes are required in the creation of the autophagosome [135]. Beclin-1 is the mammalian homolog of yeast Atg 6 and is a critical component in the early steps of the process [136]. Together with the class III PI3K, beclin-1 functions to recruit various proteins assist in the elongation phase of autophagosome development [137-139]. Once formed, fusion of the autophagosome containing damaged proteins or organelles with lysosomes, occurs. The high pH level of lysosomes aids in the digestion and degradation of all materials inside the autophagosome. For experimental investigation, several methods exist in order to observe or detect autophagy in tissue or cells. Microtubule associated protein 1A/1B light chain 3 (LC3) is used in several studies to identify autophagosome formation [140]). Digestion of macromolecules through the autophagic process provides the necessary substrates including fatty

acids and amino acids to generate fuel in the form of ATP for the production of new cellular components [128].

Aldose Reductase:

So far, the glucose metabolism pathways during I/R that have been well established prior to 1997 have been discussed here. We were the first group to demonstrate impaired glycolysis after an increased flux via the aldose reductase (AR)/polyol pathway in the diabetic and non-diabetic hearts. AR, a member of the aldo-keto reductase family is a monomeric NADPH dependent enzyme and the first, rate- limiting step in the polyol pathway [141-144]. It effectively utilizes NADPH to convert glucose into sorbitol (Figure 2).

Figure 2:

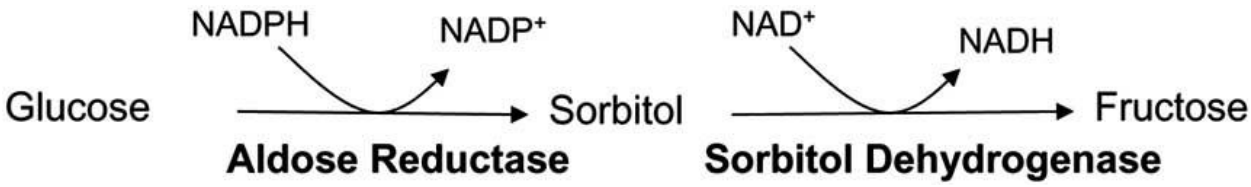


Figure 2 as shown in [141]: ***Glucose flux via Aldose Reductase. Glucose is metabolized by AR to Sorbitol. Sorbitol dehydrogenase metabolizes Sorbitol to Fructose utilizing NAD$^+$.***

The second part of the pathway involves the enzyme sorbitol dehydrogenase (SDH) which utilizes NAD^+ to convert sorbitol to fructose (Figure 2). The polyol pathway was originally discovered in seminal vesicles where it was hypothesized that fructose was produced in order to sustain the developing spermatozoa [145]. Aldose reductase is widely expressed in several tissue types including lens, heart, and especially in the inner medulla of the kidney [146]. The production of sorbitol in the inner medulla of the kidney has been proposed to regulate and protect against the high osmotic forces [147]. Aside from glucose, AR metabolizes other

substrates efficiently including retinoids, 4-hydroxynonenal (4-HNE), 2-methylpentanal, methylglyoxal and glyceraldehydes [148-150]. The enzyme, however, has a greater preference for glucose than any of the other substrates and binds to glucose with a much higher affinity. Most of the substrates mentioned above bind to AR with K_m values in the 8-50 micromolar range while AR has an anomer specific preference for D-glucose and binds to this specific glucose anomer with K_m values in the micromolar range (0.66 μmol/L) [142, 151-152]. The activity of AR increases under elevated blood glucose levels as well as ischemic conditions [141, 153]. Under ischemic conditions, an increase in nitric oxide (NO) has been shown to contribute to increased activation of AR. Nitric oxide was further implicated in aldose reductase activation as use of NO inhibitors attenuated the increase in aldose reductase activity [154]. Furthermore, early studies confirmed, through the use of the aldose D-xylose as a substrate that myocardial NADPH reducing activity is due to increased activity of AR, as opposed to aldehyde reductase [154]. An increased glucose flux via AR inhibits glycolytic flux. SDH utilizes NAD^+ for sorbitol to fructose conversion resulting in an increased cytosolic ratio of $NADH/NAD^+$ [141]. In glycolysis, GAPDH also requires NAD^+ for converting glyceraldehyde-3-phosphate to 1,3 diphosphoglycerate conversion. A competition between the two pathways develops due to limited NAD^+ availability. Glucose flux via AR during ischemia negatively impacts the myocardium by increasing oxidative stress, impairing ATP production as a result of altered glucose metabolism, and impairing calcium homeostasis [155]. All of the aforementioned are conditions which favor the opening of the mitochondrial permeability transition pore (mPTP) [156-160]. Preserving mitochondrial function is essential for normal recovery after I/R [121, 157, 159, 161]. Once opened, the mPTP has been implicated in upregulation of apoptotic and necrotic cell death mechanisms due to loss of membrane potential and ATP depletion [156-158].

Animal models have become useful in demonstrating the negative effects of AR flux and expression in various pathologies. Because the activity of AR in mice is much lower than that in humans or rats [146, 162], a transgenic mouse model was created in which human levels of AR (hAR) are expressed. This transgenic mouse has AR expression and activity comparable to that found in humans. Several studies have provided convincing evidence showing the negative association of increased flux via AR and certain pathologies. Expression of human AR in macrophages of atherosclerotic plaques and has also been implicated in vascular injury due to increased growth of VSMCs and increased generation of ROS [163]. Transgenic hAR mice subjected to I/R displayed greater injury as demonstrated by several indices [141, 157, 164-165]. These studies demonstrated using both the isolated Langendorff perfusion system *ex vivo* and *in vivo* heart preparations, that hearts from hAR mice had decreased contractile function, increased generation of ROS, decreased production of ATP and increased damage to the mitochondria after I/R injury compare to wild-type counterparts [157, 162]. Studies utilizing rat models have shown increases in AR activity in hearts subjected to I/R.

While extensive evidence demonstrating inhibition of AR to be protective, there are conflicting data sets which argue the opposite. The role of AR as an essential mediator of the protective effects of late phase preconditioning (PC) has been addressed in earlier studies which show increased AR expression as a result of PC [166]. These studies allude to the antioxidant properties of AR which functions to neutralize against the accumulation of the toxic aldehyde 4-HNE under I/R conditions, as well as protect against oxidative stress mediated arterial wall injury[167]. Despite these findings, several studies have established that inhibition of AR does not cause accumulation of toxic products such as malonyldialdehyde [168-172]. Further insight into the role of ALDH2 as well as more complete measurements of 4-HNE will help to elucidate

the role of AR in ischemic hearts as a protective enzyme. It is clear from our studies and others that increased injury after I /R due to elevated levels of reactive oxygen species and poor functional recovery are associated with increased AR levels in mice overexpressing AR [162, 173]. AR null mice, in contrast, were protected from injury associated with I/R [174]. Studies which demonstrate the ability of AR to attenuate accumulation of toxic lipid aldehydes utilized canine models of pacing induced heart failure, however AR expression in these models has been shown to be decreased [175]. In chronic diabetic rats, increases in both flux and expression of AR and SDH were increased, and in patients with ischemic and diabetic cardiomyopathies AR expression was also increased [3, 176]. These observances taken together, stress the importance of examining the role of AR in rodent models of heart failure.

AR inhibition has been shown to reduce oxidative stress, and increase ATP levels promoting glucose oxidation and glycolysis [154, 162]. Additionally, reduction of cardiovascular complications including decreased restenosis and atherosclerosis has been shown in rodent models as a result of AR inhibition. Aldose reductase knockout (ARKO) models, however, do not exhibit increased protection from atherosclerosis. Compensation from other genes as a result from global AR elimination could have an effect on the phenotype and explain the lack of atheroprotection observed in ARKO mice [177]. Increases in cytosolic NAD^+/NADH ratio due to inhibition of AR increases the availability of NAD^+ and improves flux via glycolysis. Furthermore, experimental studies have shown decreased mPTP opening and a reduction in apoptotic cell death mechanisms which led to a reduction in injury after I/R after treatment with ARIs. In the clinical setting, studies have shown that inhibition of AR improves cardiac function in patients with diabetes. A study involving diabetic patients with neuropathy demonstrated that the left ventricular abnormalities seen in these patients were partially reversed [80]. These

studies taken together highlight AR as an attractive therapeutic target in the quest to salvage ischemic myocardium. The beneficial effects of AR inhibition are discussed in greater detail below.

Aldose Reductase and Diabetes:

Cardiovascular complications including hypertension, acute myocardial infarction and angina which ultimately result in congestive heart failure are the leading cause of death in diabetic populations [178-179]. Secondary microvascular complications including diabetic neuropathy, nephropathy, and retinopathy arise due to chronic hyperglycemia and its effects on endothelial dysfunction in the microvasculature. Endothelial dysfunction as a result of high blood glucose is a characteristic early event of atherosclerosis which eventually leads to cardiomyopathy [180-181]. The use of diabetic animal models has elucidated some of the pathophysiological mechanisms such as altered glucose metabolism and increased polyol flux that mediate development of complications through aberrant signaling. Diabetic animal models have revealed abnormal substrate metabolism and molecular signaling which contributes to impaired myocardial function. This is in particular due to decreased glucose uptake due to a decrease in the GLUT 1 and GLUT4 transporters which mediate glucose uptake. Additionally, isolated diabetic rat heart models have shown that glycolysis is impaired, in part due to the increase in FFAs. Under normal insulin conditions, lipolysis is inhibited thus increasing free circulating FFAs. Under diabetic conditions and low insulin levels however, this inhibition is relieved. The increases in FFAs are then able to further inhibit glucose uptake, further impairing glucose metabolism. Consistent with the observation of decreased myocardial uptake in diabetic animal models, is decreased PDH activity. As explained earlier, increased acetyl CoA in mitochondria

via FA oxidation triggers inhibition of PDH activity. PDH kinase is largely responsible for the activity of PDH. Dichloroacetate, a PDH kinase inhibitor has been utilized in diabetic rat models and has been shown to improve contractile function and increase glucose oxidation [108, 182]. Production of advanced glycation end products (AGEs) as a result of increased polyol flux, and the phosphorylation of fructose further contribute to the conundrum of AR in mediating adverse effects especially in the diabetic setting. The interaction of AGEs with the receptor for advanced glycation end products (RAGE) activates a mechanism implicated in increased injury after I/R [141, 183]. Figure 3 demonstrates a comprehensive visual of the interactions between the AR pathway, glycolysis as well as AGE generation (Figure 3 as shown in [141]).

Figure 3:

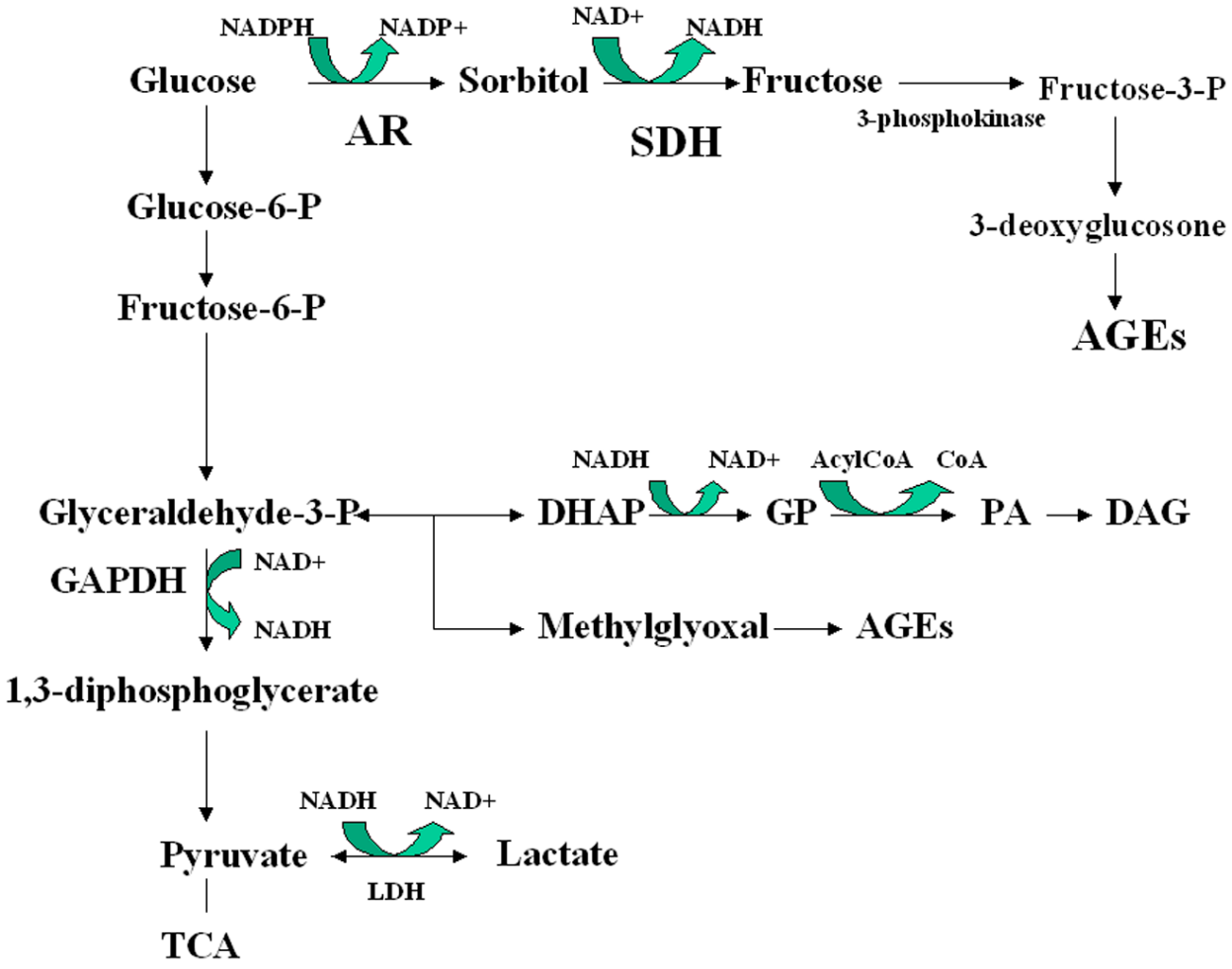


Figure 3: Comprehensive overview of flux via Aldose Reductase, glycolysis and advanced glycation end product (AGEs) generation.

In experimental studies, transgenic animals over-expressing hAR and made diabetic utilizing streptozotocin displayed greater losses in nerve conduction velocity and greater degeneration of nerve fibers when compared to wild-type diabetic mice [171]. To confirm the role of AR in contributing to this negative phenotype, transgenic, diabetic animals were treated with an ARI and significant improvement in nerve conduction velocity and reductions in nerve fiber degeneration was observed [171]. Additionally, further studies using animal models where AR

was genetically ablated displayed significant reductions in nerve fiber atrophy and increased nerve conduction velocity. For the most part, use of ARIs in diabetic animal models have either delayed onset of complications or prevented them altogether. For clinical application and usefulness, reversing secondary complications which arise in diabetic populations is ideal. This is because the secondary complications develop slowly over time and clinical symptoms from these complications which present in diabetic patients usually occurs after prolonged exposure to hyperglycemia and significant damage has already occurred. Treatments which delay progression of disease would be beneficial to prevent severe damage but would greatly depend on early detection of complications. Genetic variations in the human AR gene (ALD2 or AKR1B1) have been studied and linked to several diabetic complications including nephropathy, neuropathy and retinopathy [142, 184-186]. The onset of cardiorenal complications has been associated with genetic polymorphisms of AR in a type 2 diabetic Chinese population [187]. Similarily, diabetic macroangiopathy was also associated with genetic AR polymorphisms in a Japanese type 2 diabetic population [188].

Clinical studies of ARIs/complications:

AR is an attractive target in diabetics due to its impact on glucose metabolism. The presence of AR polymorphisms has been linked to several diabetic complications including cardiovascular disease and renal complications. Human studies utilizing ARIs have demonstrated that in patients with neuropathy, administration of the ARI Zopolrestat for one year increased cardiac output and left ventricular ejection fraction (LVEF) when compared to patients treated with placebo [80]. In a different study, Tolrestat, another type of ARI, was found to hinder the progression of diabetic autonomic neuropathy [189]. The beneficial effects of ARI

administration in renal function have been established. Both Tolrestat and Zopolrestat administration to type 1 diabetic patients was effective in reducing urinary albumin secretion [190-191]. Positive effects of AR inhibition are further supported in studies utilizing diabetic animal isolated heart models that have been perfused with ARIs and have been protected from adverse effects of high glucose [171]. Neuropathy consists of defects in nerve conduction velocity as a result of elevated blood glucose levels. Clinical utility of ARIs in treatment of diabetes has been complicated due in part to the distinctive nature of complications in each patient. To date, the majority of clinical trials have tested effects of ARIs on neuropathy. Data from these trials are largely inconclusive due to a number of inconsistencies that are likely a result of differences in experimental design and in methodology. Use of Alrestatin, Tolrestat, Ponalrestat, Fiderestat and Sorbinil have all been extensively studied in clinical trials have produced a range of results from no effect to some improvement, especially in trials investigating effects on neuropathy [192]. Additionally, toxicity problems in patients associated with the use of ARIs, especially Sorbinil, have proven to remain a tremendous problem [193]. In the diabetic setting, the potential of inhibiting AR in the heart has been emphasized in these key studies of diabetic complications in human subjects. However, recapitulating human AR biology in animal models is necessary to further understand the mechanisms of human disease.

CHAPTER 2: STATEMENT OF THE PROBLEM

It has been established that an increased flux via aldose reductase (AR)/polyol pathway leads to impairment in metabolic ion and energy homeostasis and increases reactive oxygen species production which in part, leads to the opening of the mitochondrial permeability transition pore. The involvement of AR in mediating cardiovascular disease complications including restenosis, atherosclerosis, myocardial ischemia as a result of oxidative stress, endothelial dysfunction, and mitochondrial damage has become increasingly apparent. The mechanisms by which AR modulates cardiac injury is evolving. In my thesis, I investigated two important aspects which are likely to regulate tissue viability. Hence, my focus has been on elucidating mechanisms of I/R injury, and clearance of dead/damaged proteins and organelles by autophagy (Figure 1).

The objective of this thesis is to determine the signal transduction mechanisms by which the AR pathway contributes to the vulnerability of the myocardium to ischemic challenge. The aim in Chapter 3 is to elucidate whether AR pathway mediated I/R injury is linked to changes in GSK3β phosphorylation. Phosphorylation GSK3β has been demonstrated to be cardioprotective by reducing apoptosis and oxidative stress, as well as inhibiting mitochondrial pore opening [194]. We utilized transgenic mice broadly overexpressing hAR, as well as AR knockout mice (ARKO) to analyze changes in GSKβ phosphorylation. We demonstrate that an increased flux via aldose reductase decreased phosphorylation of GSK3β and is linked to decreased cardiac recovery upon reperfusion. The focus in chapter 4 is to determine whether an increased flux via AR and resultant I/R injury is mediated by changes in autophagy. Our findings indicate that increased flux through the polyol pathway decreases autophagy and is associated with the worsening of the myocardium during I/R injury. Finally in chapter 5, we discuss possible mechanisms by which AR mediates I/R injury via GSK3β and possible future

directions/experiments that can be performed to gain a better understanding of how cardioprotection is conferred via inhibition of GSK3β.

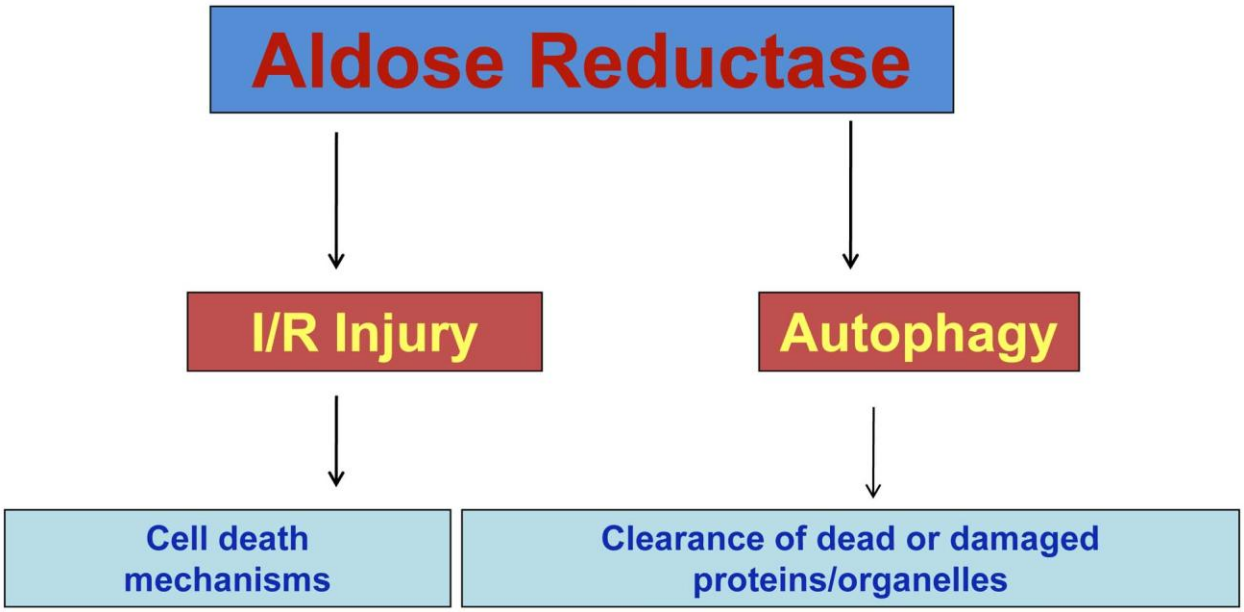


Figure 1: The purpose of this thesis is to determine the signaling mechanisms by which an increased flux via Aldose Reductase mediates ischemia-reperfusion injury. This thesis will focus on two distinct endpoints: Cell death mechanisms and mechanisms which mediate clearance of dead or damaged proteins/organelles.

Chapter 3: Aldose Reductase modulates cardiac glycogen synthase kinase 3 beta phosphorylation during ischemia-reperfusion.

The following work has been submitted as a manuscript. The data has been adapted from the original manuscript version for this thesis.

ABSTRACT:

Our objective was to investigate if AR mediated ischemia reperfusion (I/R) injury is linked to changes in phosphorylation of glycogen synthase kinase 3β (p-GSK3β), a key signaling protein that mediates cardioprotection and regulates numerous substrates including transcription factors and metabolic proteins.. We hypothesized that AR mediates I/R injury and impairs myocardial recovery through decreased phosphorylation of GSK3β. Hearts from wildtype (WT), AR transgenic (ARTg) and AR knockout (ARKO) mice were isolated and perfused with and without GSK3β inhibitors SB216763 and lithium chloride (LiCl) under normoxic (30 minutes), ischemic (30 minutes) and reperfusion (60 minutes) conditions. Lactate dehydrogenase (LDH) release, a marker of injury, was measured in the perfusate after reperfusion in the presence and absence of GSK3β inhibitors. Left ventricular developed pressure (LVDP) measured to determine myocardial function was decreased in hearts from ARTg mice compared to WT and ARKO after I/R, while LDH release was increased in ARTg mice ($p<0.05$). GSK3β phosphorylation was decreased in ARTg hearts as compared to WT and ARKO ($p<0.05$). Increased Cytochrome C and decreased Bcl-2 expression was observed in ARTg hearts after I/R injury ($p<0.05$). GSK3β phosphorylation and Bcl-2 expression was increased and Cytochrome C expression was decreased in ARKO as compared to WT ($p<0.05$). ARTg hearts perfused with SB216763 and LiCl had significant improvement in LVDP and exhibited decreased LDH release and decreased mitochondrial pore opening ($p<0.05$). Treatment with SB216763 and LiCl increased p-GSK3β and Bcl-2 expression and decreased Cytochrome C in ARTg hearts ($p<0.05$). Ad-hAR overexpressing cells, exposed to hypoxia (0.5% O_2) for 30 minutes and reoxygenation conditions (20.9% O_2) for 60 minutes had greater LDH release compared to control HL-1 cardiomyocytes ($p<0.05$). Analogous to I/R experiments, GSK3β phosphorylation was decreased and correlated

with increased Cytochrome C and decreased Bcl-2 expression in ad-hAR overexpressing cells ($p<0.05$). Treatment with PI3K/AKT inhibitor LY294002 increased cardiac injury demonstrated by increased LDH release in ARTg, WT, and ARKO hearts and in ad-hAR overexpressing cells. In summary, our data demonstrate that AR mediates I/R injury, in part, via modulation of GSK3β phosphorylation .

INTRODUCTION:

While recanalization therapy using thrombolytic or surgical approaches have helped salvage ischemic myocardium, damage observed during reperfusion phase attenuates the benefits of these interventions [195-196]. Therapeutic strategies that target both the ischemic and reperfusion components of myocardial injury are likely to afford protection against acute myocardial ischemia-reperfusion (I/R). In the quest for novel therapeutic strategies for acute I/R injury, we have focused on interventions that protect against myocardial I/R injury by modulating substrate metabolism. In this context, we and others have demonstrated that the aldose reductase (AR) pathway contributes to myocardial I/R injury and that the inhibition of AR protects hearts from I/R damage [141, 157, 162, 173, 195-199].

AR, a member of the aldo-keto reductase family is a monomeric NADPH dependent enzyme and the first, rate- limiting step in the polyol pathway [141-144]. Glucose flux via AR increases under ischemic conditions, [141, 153] even in the absence of diabetes, and negatively impacts the myocardium by increasing oxidative stress, impairing ATP production as a result of altered glucose metabolism, and impairing calcium homeostasis, conditions which favor the opening of the mitochondrial permeability transition pore (mPTP) [156-160]. Preserving mitochondrial function is essential for normal recovery after I/R [121, 157, 159, 161]. Opening of the mPTP has been implicated in upregulation of apoptotic and necrotic cell death mechanisms due to loss of membrane potential and ATP depletion [156-158].

Elucidation of the mechanisms that contribute to cell death, have allowed for new insights and increased awareness for therapeutic strategies that can be used in combination with

recanalization therapies. GSK3β beta has emerged as a potential therapeutic target in the quest to treat myocardial ischemia-reperfusion injury and ischemic disease. Under normal conditions, GSK is constitutively active and phosphorylates downstream targets. There are two isoforms of GSK 3 which exist, GSK3α and GSK3β. GSK3α and GSK3β are both regulated by phosphorylation and desphosphorylation; GSK3α on the Serine 21 and GSK3β on Serine 9 sites and Tyrosine 216 sites. Of the two isoforms the phosphorylation and inhibition of GSK3β has been shown to contribute to enhanced cardioprotection, and has been demonstrated to be critical for survival as ablation of this isoform is embryonically lethal. The role of GSK3β in mediating cardioprotection has been demonstrated in studies that have utilized various cardioprotective agents which increased the phosphorylation and inhibition of GSK3β resulting in a delayed opening of the mPTP and decreased apoptosis [194]. Various models of GSK3β inhibition including siRNA protein knockdown and small molecule pharmacological inhibitors have demonstrated that the threshold for ROS and Ca2+ induced mPTP opening is increased resulting in cardioprotection [194]. Furthermore, in studies utilizing transgenic mice with constitutive activation of GSK3β where the Serine 9 site is converted to Alanine, abolished these protective effects. *In vivo* studies of I/R where inhibitors of GSK3β were added either before ischemia or before reperfusion demonstrated protective effects through a reduction in necrosis [200]. GSK3β is most notably known for its role in metabolism and as was explained in the introductory portion of this thesis, dysregulation of metabolism is an important factor and crucial contributor to decreased cardioprotection. In addition to mediating glycogen storage by activating glycogen synthase (GS), GSK3β when active is known to phosphorylate and inhibit an important enzyme that regulates glucose oxidation, pyruvate dehydrogenase (PDH). Maintaining glycolysis and glucose oxidation during I/R is key in preserving ischemic myocardium and a decreased activity

of PDH is linked to decreased glucose oxidation [201]. Protection and preservation of cardiac tissue after administration of dicholoracetate, an activator of PDH, further supports the importance of maintaining glucose oxidation after I/R [202]. Interestingly, inhibition of GSK3β has been shown confer protection by decreasing inhibition of PDH, therefore rendering the enzyme active and increasing glucose oxidation [203]. The involvement of GSKs in various pathologies of the heart including hypertrophy, metabolism, cardiac cell death, and ischemic disease has been established in numerous studies utilizing various model systems. In addition to its role in cardiac pathology, GSK3β has been implicated in other diseases including cancer, Alzheimer's disease and bipolar disorders[204]. GSK3β, a serine/threonine kinase, has been identified by several studies as a key signaling protein which mediates cardioprotection and reduces cell death [203, 205]. GSK3β phosphorylates numerous substrates including transcription factors and metabolic proteins, and is involved in several cellular processes such as gene transcription, apoptosis and cell division [194, 206]. A wide spectrum of signaling pathways have been associated with the phosphorylation and inhibition of GSK3β on the Serine 9 site [207-208]. Upstream pathways known to phosphorylate and therefore inhibit GSK3β include the PI3K/AKT, P70S6K/mTOR, protein kinase A and C (PKA, PKC) pathways. Regulation of GSK3β also occurs as a result of autophosphorylation on the Tyrosine 216 site which instead of inhibiting GSK3β, constitutively activates the kinase [203]. The canonical Wnt pathway is also involved in the regulation of GSK3β through protein interactions. In this process, a protein complex formation of β-catenin, Axin, GSK and adenomatous polyposis coli (APC) proteins inhibit phosphorylation of downstream targets by GSK3β. Once this protein formation is disrupted, inhibition of GSK3β is relieved.

We have previously demonstrated that increased metabolic flux through AR mediates I/R injury, in part, via changes in PKCα/β dependent signaling [209] and mPTP opening [157]. A number of studies through the use of various experimental animal models have suggested that activation of specific PKC isoforms are involved in mediating cardiovascular injury through increases in cell proliferation and apoptosis [210]. Various isoforms of PKC exist, however in cardiac tissue, activation of α and β isoforms specifically have been implicated in mediating cardiac pathologies. The expression and activation of the β isoform has been shown to be increased under pathological conditions such as I/R and diabetes [211-212]. Similarly PKCα has been implicated as a negative regulator of cardiac hypertrophy. The use of PKCα null mice showed decreased susceptibility to heart failure and improved contractile function [213-214].

Since phosphorylation of GSK3β is a key determinant of mPTP opening, we investigated if AR mediates I/R injury, in part, by adversely influencing GSK3β phosphorylation in murine hearts. It is unclear if phopshorylation of GSK3β is impacted by AR during I/R. We utilized adult HL-1 cardiomyocytes in an *in vitro* I/R injury model as well as *ex vivo* intact hearts subjected to I/R. Since mice have an AR protein expression and activity that is several fold lower than that in rats and humans [142, 162], we employed transgenic mice overexpressing human aldose reductase (ARTg) as well as an aldose reductase knockout mouse model (ARKO) to determine whether altered flux via AR influenced GSK3β phosphorylation.

METHODS:

2.1 Animals

All animal experiments were approved by the Institutional Animal Care and Use Committees of Columbia University, New York University School of Medicine, Baylor college of medicine and conformed to the guidelines outlined in the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Pub. No. 85-23, 1996). Male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were used as control wild-type (WT) mice. Male mice weighing 25–30 g at age 12-14 weeks were used in all experiments and maintained in a temperature-controlled room with alternating 12:12-h light-dark cycles. Mice transgenic for human aldose reductase (ARTg) were obtained from Dr. Mitsuo Itakura (University of Tokushima, Japan) and a colony was established at our facility at NYU Medical Center. Briefly, these transgenic mice were developed by injecting full-length hAR cDNA with a mouse major histocompatibility antigen class I promoter [215]. Aldose reductase knock-out (ARKO) mice was generated as described recently [216]. The hAR transgenic and ARKO mice have been backcrossed >10 generations to develop a C57BL/6J background.

2.2 Reagents

The primary antibodies used were anti-phospho-GSK3β/totalGSK3β, anti-phospho-Akt (Thr308 and Ser473)/total-Akt IgG, (Cell Signaling, USA); anti-cytochrome C IgG (BD Pharmingen, USA); anti-beta-actin IgG (BD Biosciences Pharmingen, USA). The secondary antibodies used were goat-anti-rabbit IgG-peroxidase antibody and rabbit-anti-mouse IgG-peroxidase antibody (Sigma, USA). All primary antibodies were diluted 1:1000 prior to use in western blot studies. GSK3 inhibitors Lithium Chloride and SB216763, and PKCα/β inhibitor Gö6976 were

purchased from Sigma, USA. PI3K/Akt inhibitor LY294002 was purchased from Calbiochem, USA.

Isolated perfused heart preparation:

Experiments were performed using an isovolumic isolated heart preparation as published and modified for the use in mice hearts [157, 162]. Mice were anesthetized using a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). After deep anesthesia was achieved, hearts were rapidly excised, placed into iced saline, and retrogradely perfused at 37ºC in a nonrecirculating mode through the aorta at a rate of 2.5 mL/min. Hearts were perfused with modified Krebs-Henseleit buffer containing (in mM) NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, glucose 5, palmitate 0.4, BSA 0.4, and 70 mU/L insulin. The perfusate was equilibrated with a mixture of 95% O_2-5% CO_2, which maintained perfusate PO_2 > 600 mmHg. Left ventricular developed pressure (LVDP) was measured using a latex balloon in the left ventricle. LVDP and coronary perfusion pressure were monitored continuously on a 4-channel Gould recorder.

Ischemia/reperfusion protocol:

Hearts from WT, ARTg and ARKO hearts were perfused with Krebs-Henseleit buffer throughout the I/R protocol. After an equilibration period of 30 min, global ischemia was performed for 30 min followed by 60 min of reperfusion. Perfusate temperature was maintained at 37ºC at all times during the protocol (i.e., during baseline, ischemia, and reperfusion).

To determine the link between AR and GSK3β after I/R injury, experiments were performed in the presence of GSK3β inhibitors; SB216763 (3μM) and lithium Chloride (LiCl; 3mM) as well as PI3K/Akt inhibitor (LY294002, 10μM). Hearts from WT and ARTg mice were perfused with

modified Krebs-Henseleit buffer containing 3 μM SB216763 or 3mM LiCl. The doses of the inhibitors used in this study were based on the literature [217-219]. Inhibitors were present at the start of the equilibration period and continued throughout ischemia and reperfusion.

2.5 Mitochondrial swelling as a measure of MPTP opening.

We employed mitochondrial swelling as a measure of MPTP opening. For measurement of MPTP opening, mitochondria were suspended in freshly prepared swelling buffer (0.2 M sucrose, 10 mM Tris-MOPS, pH 7.4, 5 mM succinate, 1 mM phosphate, 2 μM rotenone, and 1.0 μM EGTA-Tris, pH 7.4) at 0.5 mg/ml, and swelling of mitochondria was monitored by decrease in absorbance at 540 nm in the presence of $CaCl_2$ (5–100 μM). Extent of pore opening was expressed in terms of changes in absorbance at 540 nm per minute in the presence and absence of Ca^{2+}.

2.6 Culture and Transfection of HL-1 Cardiomyocytes

Immortalized HL-1 cardiomyocytes were a gift from Dr William Claycomb (Louisiana State University, New Orleans, LA, USA) [220]. HL-1 cell line was chosen because they were derived from cardiac muscle cells obtained from mice [220]. Cells were plated on gelatin/fibronectin-coated six-well plates at a density of 4×10^5 cells/well. Human AR was overexpressed in HL-1 cardiomyocytes through viral mediated transfection. Appropriate empty vector was transfected as a control. For siRNA transfection, HL-1 cells were cultured in six-well plates and transfected with control or 40nM GSK3β siRNA using Lipofectamine RNAiMax reagent (Invitrogen, USA) according to the manufacturer's instructions. HL-1 cardiomyocytes were subjected to hypoxic stress for 30 minutes (0.5% O_2) using an In Vivo 400 hypoxic

workstation maintained at 37°C, followed by 60 minutes of reoxygenation (20.9%O_2). In specific experiments, cardiomyocytes were treated with either GSK3β siRNA or PI3K/Akt (10μM) and PKCα/β inhibitors (1μM).

2.7 Western Blot Analysis

The tissue and cell protein concentration was determined using a DC Protein Assay kit (Bio-Rad). Equal amounts of protein were separated by SDS-PAGE (4–12% gradient gels), and proteins were transferred to a nitrocellulose membrane (Invitrogen). After blocking in 5% dry milk in 20 mM Tris-HCI, pH 7.5, 250 mM NaCl, 0.1% Tween 20 (TBST), membranes were incubated overnight with target primary antibodies (1:1000 dilution) according to the manufacturer's instructions. Membranes were incubated sequentially with secondary antibody for 1 hr. Blots were visualized with an ECL Horseradish Peroxidase Western Blot Detection System (Cell Signaling, USA), and quantitative analysis was performed using Image Quant TL software (Amersham).

2.8 LDH Measurements

Cardic injury due to I/R stress was assessed by measuring LDH release in the perfusates that were collected at the end of reperfusion. In HL-1 cardiomyocytes, injury due to hypoxia/reoxygenation (H/R) stress was measured in supernantants that were collected after H/R. LDH was measured using the commercially available kits (Pointe Scientific, Inc.) as published earlier.

2.9 Statistical Analysis

All data are presented as mean value ±standard error of the mean (SEM). Statistical significance of differences between various groups of heart was determined by ANOVA. Post hoc comparisons were performed with Tukey or Dunnett procedures using GraphPad software as indicated. Statistical significance was ascribed to the data when $P<0.05$.

RESULTS:

GSK3β phosphorylation expression in WT, ARTg and ARKO mice

We utilized the isolated Langerdorff perfusion system in order to establish the influence, if any, of AR mediated ischemic injury on the phosphorylation and inhibition of GSK3β. Previous studies have shown that the phosphorylation and inhibition of GSK3β is cardioprotective [203, 205]. We first examined GSK3β phosphorylation at both the Ser9 and Tyr216 sites on GSK-3β after a 30 minute baseline period only. Results show that there are no differences in GSK3β phosphorylation in WT, ARTg and ARKO hearts after baseline (Figure 1A and 1B).

We then determined whether GSK3β phosphorylation was influenced after I/R. Induction of I/R resulted in decreased inhibition of GSK3β demonstrated by decreased levels of p-GSK3β phosphorylated at Ser9 and increased levels of p-GSK3β phosphorylated at Tyr216 in ARTg mice hearts as compared to WT mice (Figure 1C, 1D; $p<0.05$). Phosphorylation of GSK3β on the Ser9 site was also significantly increased in ARKO hearts as compared to WT (Figure 1C). Furthermore, ARTg hearts displayed a more than threefold increase in apoptosis compared to WT and ARKO hearts as measured by cytosolic Cytochrome C expression (Figure 1E, $p<0.05$). Similarly, anti-apoptotic protein, Bcl-2 expression was lower in ARTg hearts compared to WT and ARKO hearts (Figure 1F; $p<0.05$).

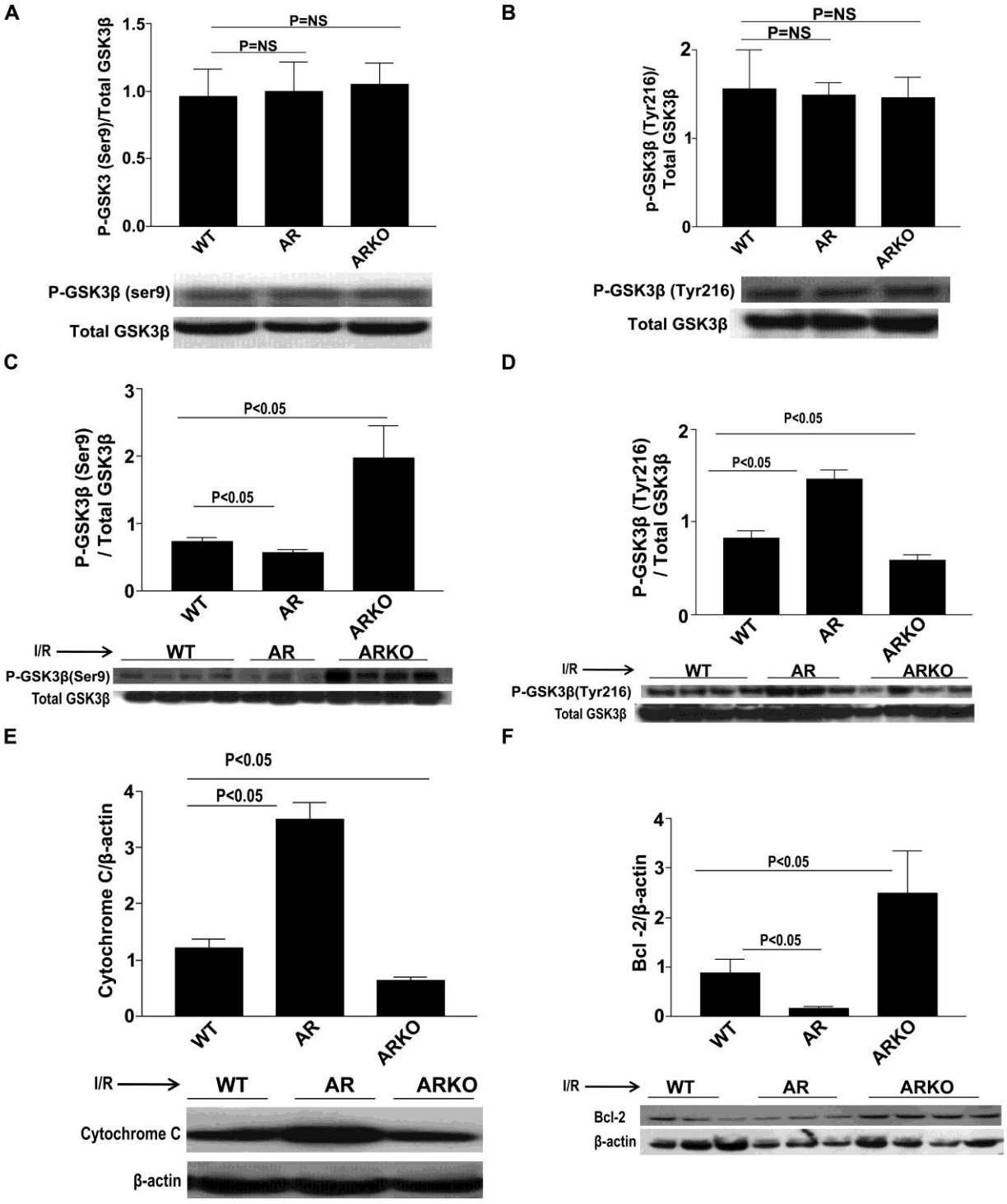


Figure1: GSK3β phosphorylation expression in WT, ARTg and ARKO mice*. Western blot analysis for* ***a.)*** *p-GSK3β-Ser9 and* ***b.)*** *p-GSK3β-Tyr216 under baseline conditions.* ***c.)*** *p-GSK3β-Ser9 and* ***d.)*** *p-GSK3β-Tyr216 under I/R conditions.* ***e.)*** *cytochrome C and* ***f.)*** *Bcl-2 in untreated WT, ARTg and ARKO mice hearts subjected to I/R. No differences were observed in p-GSK3β expression after baseline conditions. ARTg hearts showed decreased p-GSK3β inhibition compared to WT hearts after I/R. ARKO hearts had significant increases in p-GSK3β (Ser9) ($p<0.05$). ARTg hearts displayed a more than threefold increase in apoptosis compared to WT and ARKO hearts as was measured by Cytochrome C expression ($p<0.05$). Bcl-2 expression was significantly downregulated compared to WT and ARKO hearts. ARKO hearts displayed significant decreases in apoptotic levels compared to WT ($p<0.05$). WT-denotes non transgenic mice, AR-denotes AR transgenic mice (ARTg), and ARKO-denotes AR null mice. (N=4-16/group).*

Pharmacological inhibition of GSK3β protects mice hearts upon I/R

Because ARTg hearts displayed decreased inhibition of GSK3β, we next sought to further test the role of GSK3β in mediating I/R injury. WT and ARTg hearts were treated with GSK3β inhibitors SB216763 and lithium chloride (LiCl), and were perfused under normoxic conditions for 30 minutes, followed by 30 minutes of ischemic conditions and 60 minutes of reperfusion conditions. LiCl targets the inhibitory phosphorylation site of GSK3β-Ser9 and works to increase phosphorylation, while SB216763 targets the stimulatory phosphorylation site GSK3β-Tyr216 and works to decrease phosphorylation. Treatment with SB216763 and LiCl protected WT and ARTg mice hearts significantly as shown in figures 2a and 2b. Improvement of LVDP (Figure 2A, $p<0.05$) was observed in WT and ARTg hearts treated with both SB216763 and LiCl. Similarly, figure 2B shows decreased release of LDH (Figure 2B, $p<0.05$) in WT and ARTg hearts upon treatment with both inhibitors. Treatment with LiCl and SB216763 significantly inhibited GSK3β in both WT and ARTg hearts (Figures 2C and 2D; $p<0.05$). WT and ARTg hearts perfused with both GSK3β inhibitors displayed markedly reduced apoptotic levels as was demonstrated by reduced cytochrome C (Figure 2E; $p<0.05$) expression and increased Bcl-2 expression (Figure 2F; $p<0.05$).

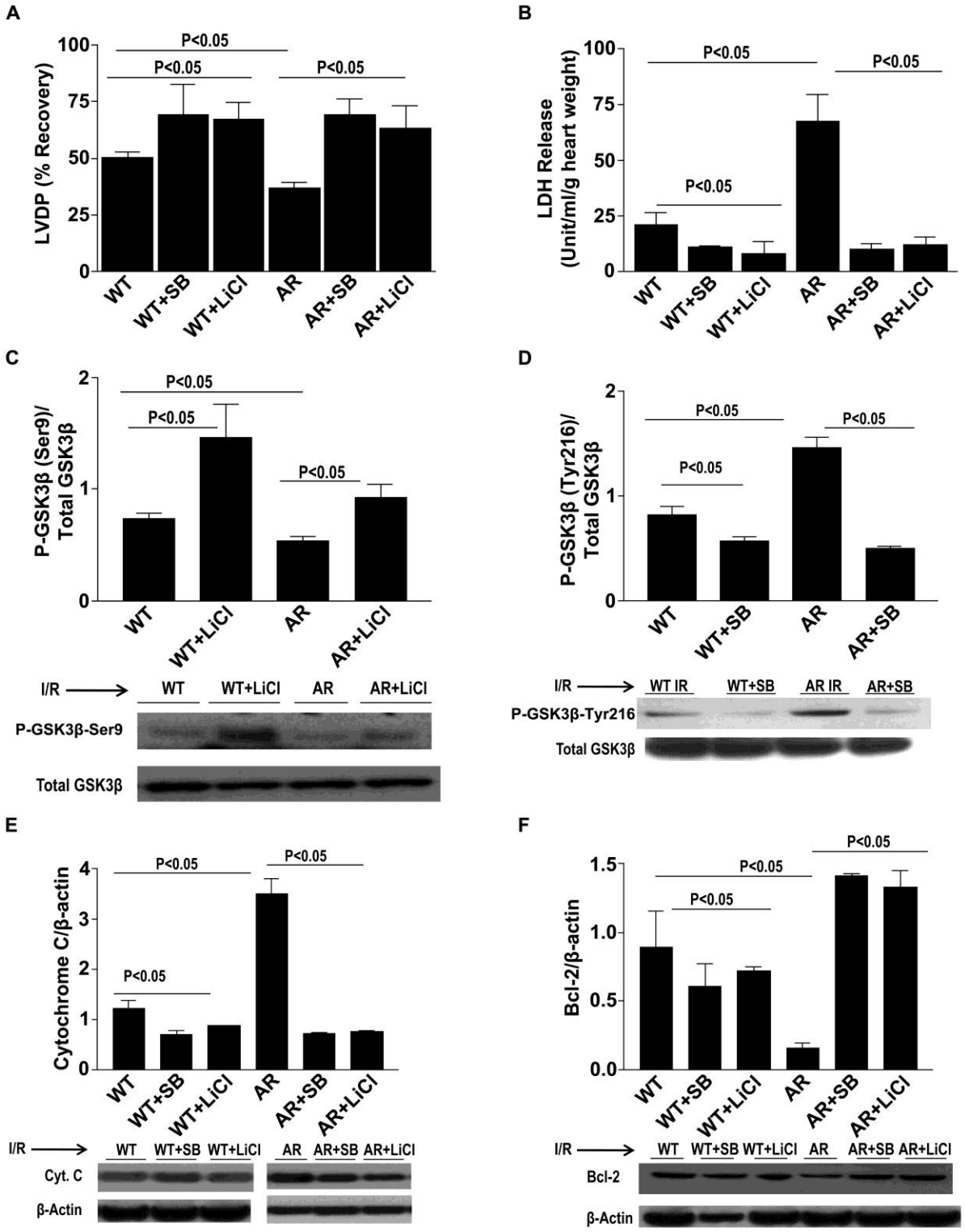


Figure 2: ***<u>Pharmacological inhibition of GSK3β protects mice hearts upon I/R</u>***. *Determination of myocardial ischemic injury and function with and without GSK3β inhibitor treatment as shown by a.) left ventricular developed pressure (LVDP) recovery and b.) lactate dehydrogenase (LDH) release. Western blot analysis for c.) p-GSK3β-Ser9 and d.) p-GSK3β-Tyr216 e.) Cytochrome C, and f.) Bcl-2 in WT and ARTg hearts perfused with and without GSK3β inhibitors SB216367 and LiCl. Treatment with SB216367 and LiCl protected WT and ARTg mice hearts, significantly increased p-GSK3β inhibition ($p<0.05$) and decreased apoptosis. WT-denotes non transgenic mice, AR-denotes AR transgenic mice, and ARKO-denotes AR null mice. (N=4-16/group).*

Pharmacological inhibition of GSK3β abolished mPTP pore opening in mitochondria from WT and ARTg hearts, as shown by the mitochondrial swelling changes in response to increasing amounts of added calcium (Figure 3).

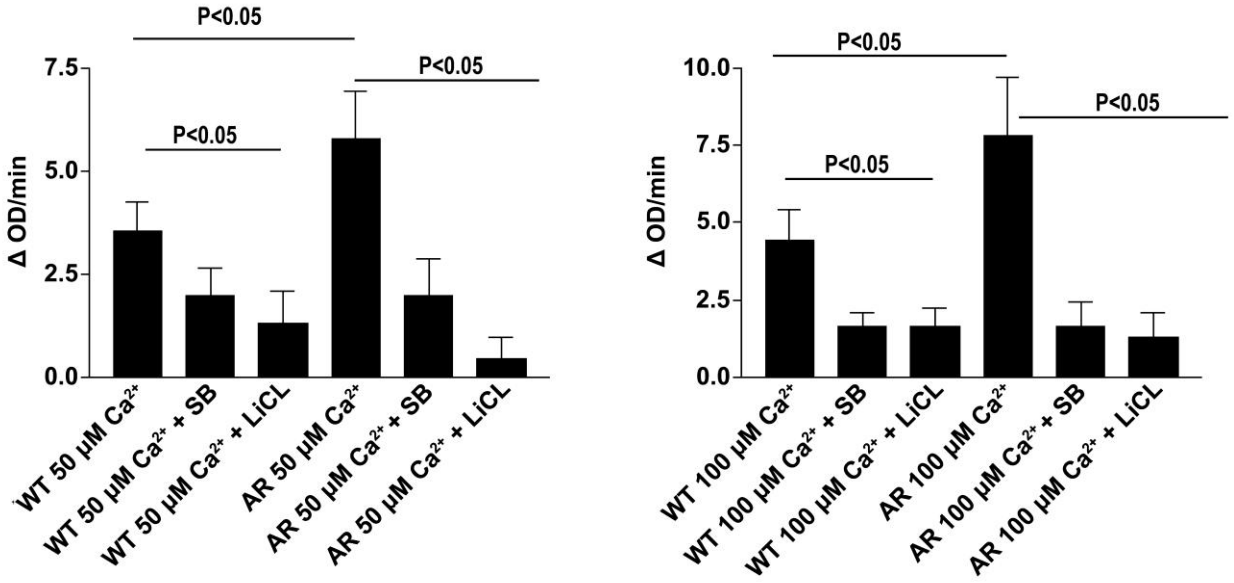


Figure 3: ***<u>Mitochondrial permeability in WT and ARTg hearts treated with and without GSK3β inhibitors SB216763 and LiCl and subjected to I/R.</u>*** *Mitochondrial permeability transition pore opening (mPTP) was determined by monitoring swelling of mitochondria by measuring light scattering at 540 nm in the presence and absence of varying amounts of added calcium. Pore opening was reduced in WT and ARTg hearts treated with SB216367 and LiCl in response to increasing calcium concentrations (p<0.05) WT-denotes non transgenic mice, AR-denotes AR transgenic mice (ARTg). (N=4-16/group).*

PI3K/Akt is a well-established upstream kinase that has been shown to phosphorylate and inhibit GSK3β at the serine residue. We investigated Akt phosphorylation expression patterns in WT, ARTg and ARKO hearts after I/R. To determine whether Akt is a key player by which AR mediates I/R injury, we utilized the PI3K/Akt inhibitor LY294002 during I/R. Functional recovery was impaired in WT, ARTg and ARKO hearts in the presence of LY294002 as was demonstrated by significant decreases in LVDP (figure 4A; $p<0.05$). As shown in figure 4B, LDH release was significantly increased in WT, ARTg, and ARKO hearts subjected to I/R stress in the presence of LY294002. Marked reductions in p-Akt levels were observed in WT, ARTg, and ARKO hearts treated with LY294002 (Figure 4C; $p<0.05$). Consequentially, p-GSK3β levels were decreased in all three groups after I/R when in the presence of the PI3K/Akt inhibitor

(Figure 4D; $p<0.05$). Inhibition of the PI3K/Akt pathway resulted in significant increases in apoptotic levels as was measured by Cytochrome C expression (Figure 4E; $p<0.05$). These data indicate that reduction in GSK3β phosphorylation is in part due to reduced PI3K/Akt phosphorylation.

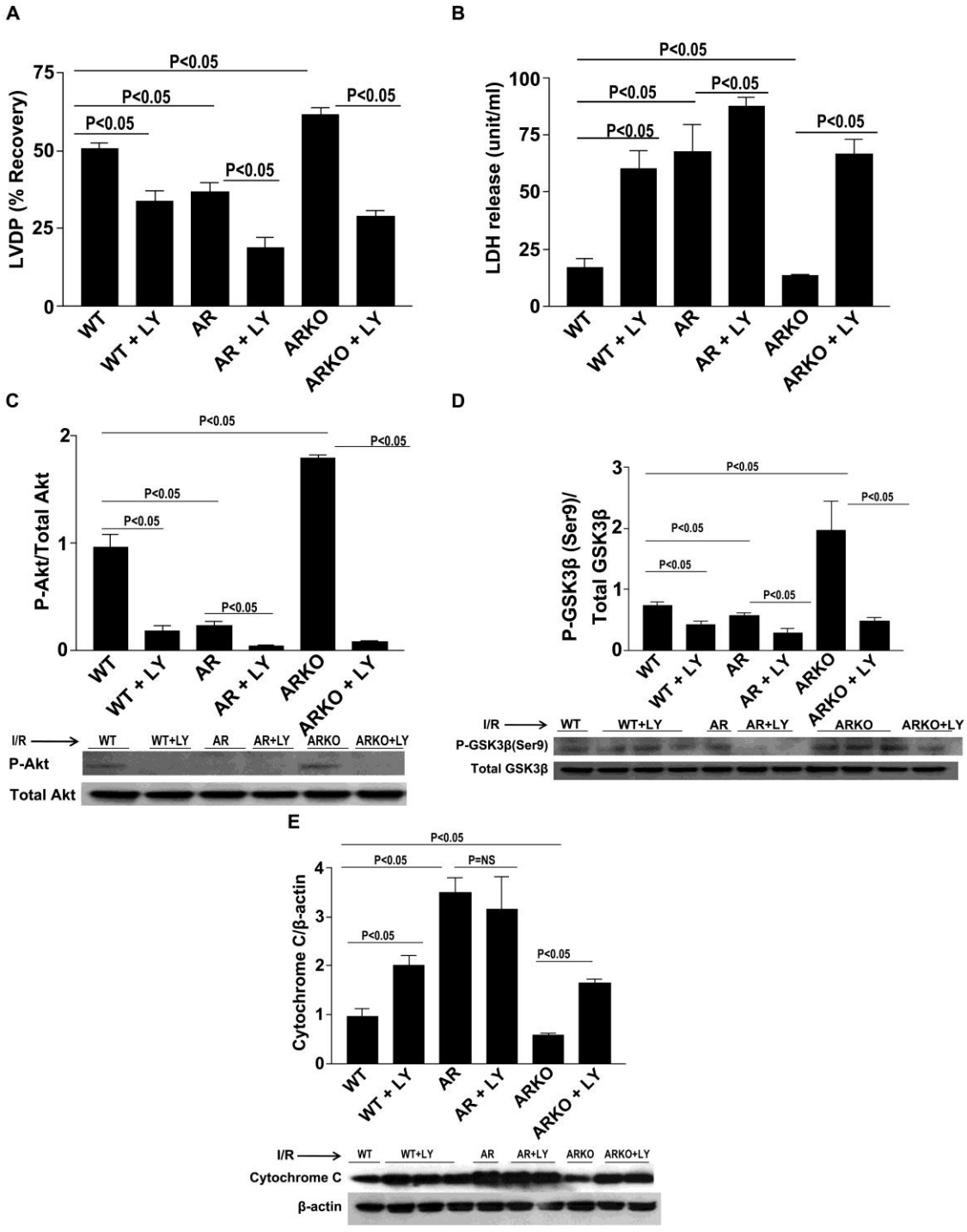


Figure 4: <u>Inhibition of PI3K/Akt</u>. *Determination of myocardial ischemic injury and function, as shown by* ***a.)*** *left ventricular developed pressure (LVDP) and* ***b.)*** *Lactate Dehydrogenase (LDH) release in WT, ARTg and ARKO perfused with and without PI3K/AKT inhibitor LY294002. Western blot analysis for* ***c.)*** *p-Akt,* ***d.)*** *P-GSK3β-Ser9 and* ***e.)*** *Cytochrome C in WT, ARTg and ARKO hearts perfused with and without PI3K/Akt inhibitor LY294002. Treatment with LY294002 decreased p-Akt and p-GSK3β levels in all 3 groups ($p<0.05$). Cytochrome C levels were increased in WT and ARKO hearts ($p<0.05$), but not ARTg upon perfusion with LY294002. WT-denotes non transgenic mice, AR-denotes AR transgenic mice (ARTg), and ARKO-denotes AR null mice. (N=4-16/group).*

Akt and GSK3β phosphorylation expression in hAR overexpressing HL-1 Cells

We employed the murine cardiomyocyte HL-1 cell line to identify where the signaling mechanisms are occurring in the myocardium. We utilized siRNA to inhibit GSK3β and to determine whether GSK3β knockdown would decrease cellular injury and apoptosis. Dose dependent concentrations of siRNA were utilized in order to determine the least amount of siRNA required to efficiently knock down GSK3β. Approximately 60% GSK3β knockdown was achieved in hAR overexpressing HL-1 cardiomyocytes with a 40nM siRNA concentration (Figure 5A). LDH measured in the supernatants after GSK3β siRNA transfection and H/R stress was markedly decreased in both control and hAR expressing cells (Figure 5B). As was expected, GSK3β siRNA knockdown in control and hAR expressing cells significantly reduced p-GSK3β levels (Figure 5C) and consequentially reduced cytochrome C levels (Figure 5D ; $p<0.05$).

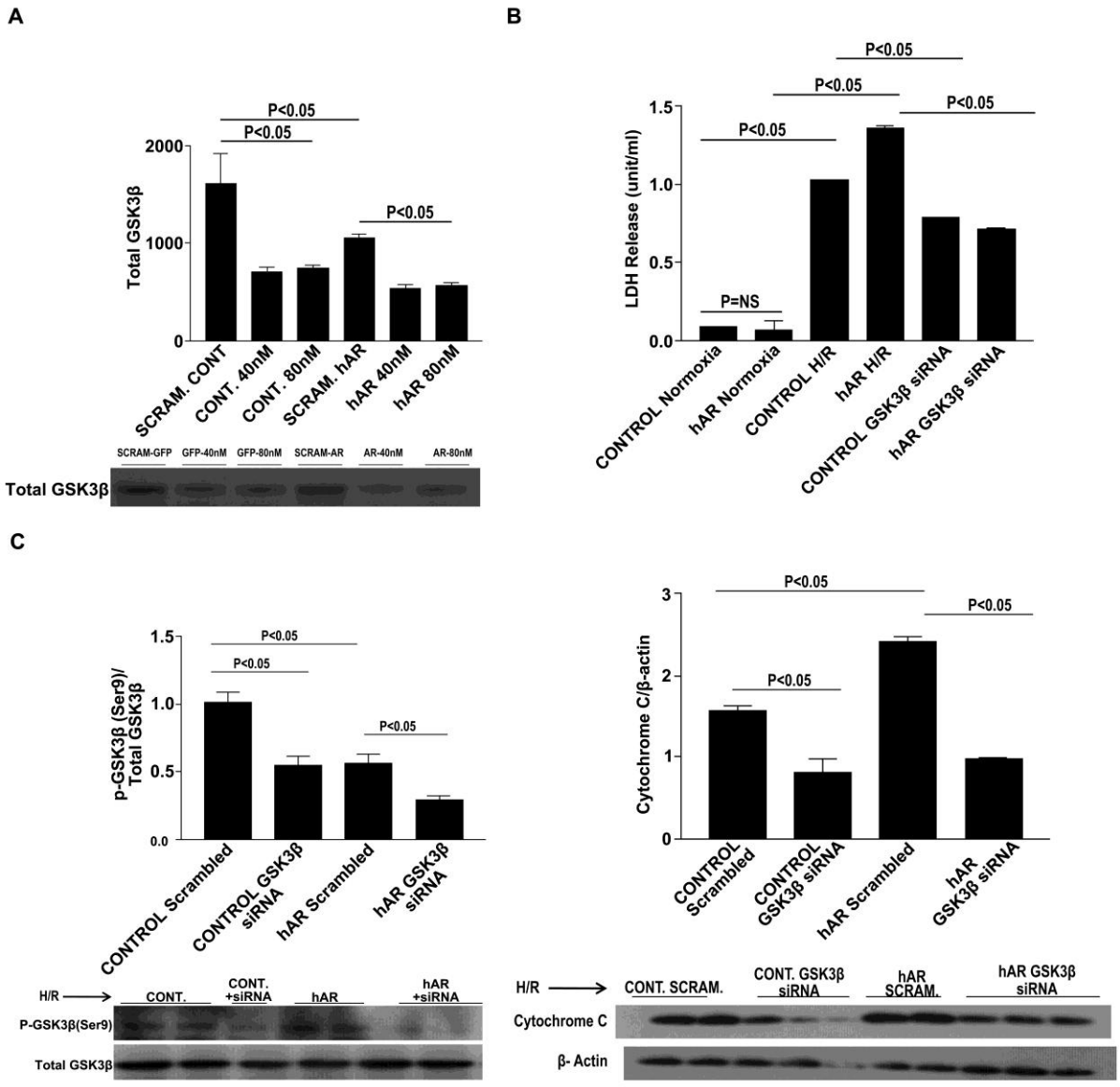


Figure 5: *<u>GSK3β siRNA knockdown in HL-1 cells.</u> Expression of GSK3β with concentration dependent siRNA knockdown. Determination of cardiac injury as determined by* ***b.)*** *LDH release in control and hAR siRNA knockdown of GSK3β *. Western blot analysis of* ***c.)*** *p-GSK3β and* ***d.)*** *Cytochrome C expression in control and hAR overexpressing cells transfected with GSK3β siRNA . No change in LDH release was observed in control and hAR overexpressing cells exposed to 30 minutes of Normoxia alone. Thirty minutes of hypoxia followed by 60 minutes of reoxygenation increased LDH release ($p<0.05$) while inhibiting GSK3β with siRNA significantly reduced LDH release and injury ($p<0.05$). GSK3β siRNA knockdown significantly reduced p-GSK3β expression in both control and hAR overexpressing cells. Inhibition of GSK3β significantly reduced apoptotic levels in both control and hAR overexpressing cells. *Error bars not visible. Control Normoxia: SEM±0.003, Control hAR SEM±0.006, Control GSK3β siRNA: ± 0.005****)***

HL-1 cells expressing hAR and empty control vectors were exposed to H/R stress to determine p-Akt and p-GSK3β patterns. Similar to what was found in ARTg hearts during I/R, the

expression of p-Akt was significantly reduced in ad-hAR expressing cells during H/R stress (Figure 6A; $p<0.05$). Decreased expression of p-Akt corresponded with decreased phosphorylation and inhibition of GSK3β. We analyzed the expression of p-GSK3β at both the Ser9 and Tyr216 residues in hAR overexpressing cells and found that phosphorylation of GSK3β was significantly decreased on the Ser9 site and increased on the Tyr216 site (Figures 6B and 6C; $p<0.05$), after H/R. As was observed in *ex vivo* heart I/R injury, decreased phosphorylation and inhibition of GSK3β correlated with increased cell death after H/R as was measured by the increased expression of Cytochrome C and reduced Bcl-2 levels in hAR overexpressing cells (Figure 6D and 6E). Additionally, cardiac injury as measured by LDH release in supernatants from hAR overexpressing cells after H/R was significantly increased (Figure 6F). HL-1 cells overexpressing hAR as well as control cells were treated with the PI3K/Akt signaling inhibitor LY294002 during H/R to establish if Akt phosphorylation is a key event which mediates injury. As shown in figure 6G, both control and hAR overexpressing cells treated with the signaling inhibitor displayed marked increases in LDH release after H/R which was parallel to experiments in the *ex vivo* I/R heart preparations. In addition to the increased LDH release, LY294002 treatment significantly reduced p-GSK3β expression (Figure 6H; $p<0.05$) and resulted in increased apoptotic levels as shown by decreased Bcl-2 levels (Figure 6I; $p<0.05$).

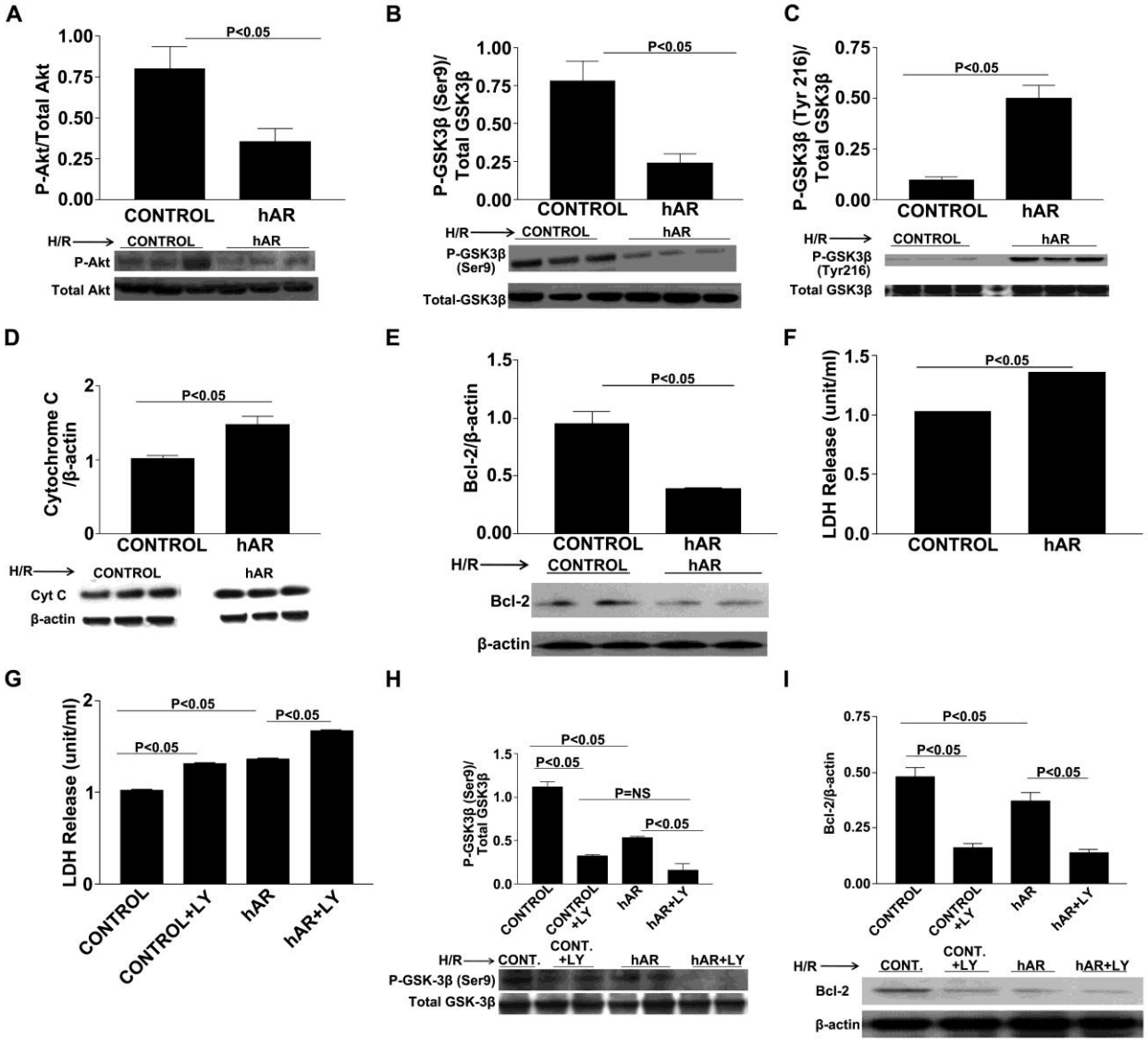


Figure 6: *<u>HL-1 Cardiomyocyte studies</u>. Western blot analysis for* ***a.)*** *p-Akt,* ***b.)*** *p-GSK3β-Ser9 and* ***c.)*** *p-GSK3β-Tyr216)* ***d.)*** *cytochrome C and* ***e)*** *Bcl-2 in control and hAR overexpressing HL-1 cells subjected to H/R alone. Determination of cardiomyocyte H/R injury as shown by* ***f.)*** *Lactate Dehydrogenase (LDH) release in control and hAR overexpressing HL-1 cell supernatants* **g.)** LDH release in control and hAR overexpressing HL-1 cells treated with and without PI3K/Akt inhibitor LY294002. Western blot analysis for* ***h.)*** *p-GSK3β-Ser9 and* ***i.)*** *Bcl-2 in control and hAR overexpressing HL-1 cells treated with and without PI3K/Akt inhibitor LY294002. Cells were incubated with LY294002 (10 μM) or its vehicle control DMSO for 1 hr, followed by 30 min of hypoxia and 1 hr reoxygenation (H/R). Treatment with LY294002 decreased p- Akt levels, decreased inhibition of GSK3β and increased apoptosis in both control and hAR overexpressing cells. LDH release in supernatants in both control and hAR overexpressing cells was increased with PI3K/Akt inhibitor LY294002 treatment. *Error bars not visible. Control SEM±0.006 and hAR SEM±0.011.*

We have shown earlier that flux via AR leads to activation PKCα/β and that inhibition of PKCα/β with Gö6976 attenuates I/R injury [209]. In addition, PI3K/Akt pathway has been shown to be a downstream target of PKCα/β [221]. To investigate if AR driven changes in

PKCα/β influence GSK3β phosphorylation, we treated HL-1 cells with PKC α/β inhibitor Gö6976 and subjected them to H/R stress. PKC inhibition resulted in increased p- Akt (Figure 7A; $p<0.05$) and p-GSK3β (Figure 7B; $p<0.05$) in ad-hAR expressing HL-1 cells. Furthermore, treatment of HL-1 cells with Gö6976 resulted in a marked reduction of injury as shown by decreased LDH release (Figure 7C, $p<0.05$). These data establish that AR modulates p-GSK3β, in part, via PKCα/β in cardiac cells.

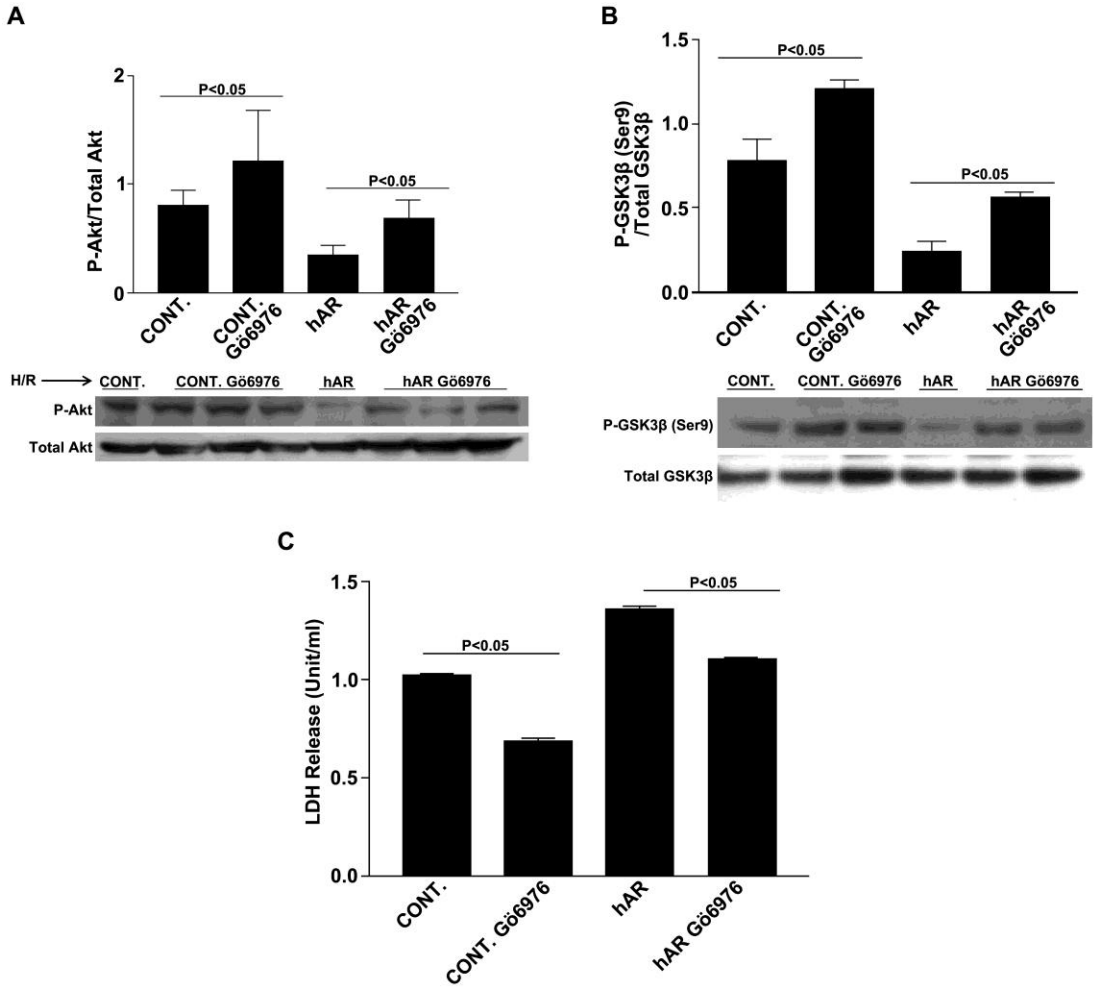


Figure 7: *<u>PKCα/β inhibition.</u> Western blot analysis for **a**.) p-Akt and **b**.)p-GSK3β. Determination of cardiomyocyte injury as shown by **c.**) LDH release. Treatment with Gö6976 resulted in decreased cellular injury and increase in p-GSK3β. Cells were incubated with Gö6976 (1μM) or it's vehicle control DMSO for 1hr, followed by 30 min of hypoxia and 1 hr reoxygenation (H/R).*

DISCUSSION

Previous studies demonstrated the important role of AR in mediating myocardial I/R injury [141-142, 157, 162, 164, 173, 197-199, 209, 222]. Our goal in this study was to investigate if AR mediated I/R injury is linked to changes in phosphorylation of GSK3β and whether these changes would correlate with the impaired ability of the myocardium to recover after an ischemic insult. Consistent with earlier results, we show that transgenic mice over-expressing human AR have increased I/R injury [157, 164, 209]. Hearts from ARKO mice demonstrated a significant increase in LVDP recovery as compared to WT mice. Our earlier studies showed that inhibition of AR in WT mice reduced injury and also improved function. We have previously shown that after treatment with Zopolrestat, an aldose reductase inhibitor (ARI), WT mice had significant decreases in the opening of the mPTP. This can be explained in part because the expression of AR and flux via the enzyme increases after ischemia. Here, we show that inhibition of AR (ARKO) significantly protects the myocardium as a result of increased phosphorylation of GSK3β and a reduction in apoptosis. We demonstrate that AR mediates I/R injury, in part, via decreased phosphorylation of GSK3β (Ser9) thereby impairing mitochondrial function, as well as functional recovery of the heart. Phosphorylation of GSK3β was significantly decreased in ARTg mouse hearts as well as in hAR overexpressing HL-1 cells. Further experiments indicated that upon pharmacological inhibition of GSK3β, significantly higher levels of p-GSK3β was observed in ARTg hearts subjected to I/R and hAR overexpressing cells subjected to H/R. The increases in levels of p-GSK3β correlated with decreased apoptosis in GSK3β inhibited ARTg hearts. Our efforts to further elucidate the signaling mechanisms that contribute to the decreased phosphorylation of GSK3β highlight AKT which functions upstream of GSK3β as a key player in mediating I/R injury. Inhibition of

PI3K/AKT pathway resulted in significant cardiac injury and impairment cardiac recovery function. Treatment with PI3K/AKT inhibitor decreased p-AKT and p-GSK3β levels.

Two mammalian isoforms of GSK3 have been identified; GSK3α and GSK3β [203]. During zebrafish cardiogenesis, GSK3α has been shown to be important for cardiomyocyte survival [223]. Further studies aided in clarifying the role of GSK3α by demonstrating improved glucose tolerance and insulin sensitivity as a result of global GSK3α loss in a murine model [224]. The GSK3β isoform regulates numerous cell processes including apoptosis and proliferation and has been shown to enhance cardioprotection in several studies [225-227]. The activity of GSK3β is regulated by phophorylation [194, 203, 206]. The phosphorylation and inhibition of GSK3β is a major component of its cardioprotective role. Several studies have shown that the inhibition of GSK3β reduces apoptosis and confers cardioprotection depending on the degree and duration of inhibition [203, 208]. As is mentioned above, drastic inhibition through complete ablation of GSK3β is embryonically lethal due to TNF induced apoptosis. Furthermore, short-term inhibition of GSK3β has been demonstrated to be protective, and as the duration of inhibition increases, the protective effects are lost due to heart failure as a result of NFκB inhibition, TNF-induced apoptosis and myocyte loss [203]. The contribution of sustained GSK3β inhibition to hypertrophy and growth of cardiac cells is evident since studies have demonstrated that constitutive activation of GSK3β alleviated these effects. The question of whether GSK3β inhibition is protective after I/R has been disputed and the answer varies depending on several factors. One important factor in considering the protective effects of GSK3β inhibition is the model used in the study. Cardioprotection from preconditioning was inhibited in cardiomyocytes isolated from transgenic mice which overexpressed constitutively active GSK3β

due to replacement of Serine 9 with Alanine. This has been supported by several other studies which have demonstrated protection of the myocardium via GSK3β inhibition through other means including post conditioning, opiod-induced cardioprotection and via inhibition from the upstream PI3K/AKT pathway. One study however, that utilized a GSK3α/β double knock-in model found that GSK3β inhibition is not necessary for protection in mice [205]. In this double knock-in model, both phosphorylation sites, Serine 21 on GSK3α and Serine 9 on GSKβ, were switched to alanine thereby preventing any phosphorylation on those sites. Mice hearts from this model were isolated and subjected to I/R studies and it was found that infarct size after pre and post conditioning was greatly reduced compared to wild-type littermates suggesting that inhibition of GSK3β is not critical or necessary for protection. It has been argued that cardiac specific overexpression of constitutively active GSK3β is linked to upregulations in atrial natriuretic peptide (ANP) which is known to alter cardioprotective signaling. Similarly, the double knock-in model has been questioned since upregulation of other compensatory mechanisms could be contributing to the reduction of infarct size observed [205]. Furthermore, Nishino et al showed that pharmacological inhibition of GSK3β did not confer cardioprotection after preconditioning in mice, however when inhibitors were administered to rats, a reduction in infarct size was observed. These differing results suggest a species specific response to pharmacological inhibition of GSK3β. It is clear from these varying results that additional studies are needed to clarify the role of GSK3β in mediating caridoprotection are warranted. Therefore, when considering GSK3β as a therapeutic target, it is imperative to consider the end point in clinical outcomes since both the sustained activation and complete or sustained inhibition are both detrimental. The use of cardioprotective agents in a cardiomyocyte model has been shown to phosphorylate and inhibit GSK3β and further protects by inhibiting mitochondrial

permeability transition pore (mPTP) opening [206]. Cardiomyocytes from mice with a constitutively active GSK3β were not protected upon treatment with cardioprotective agents. Other studies have shown a reduction in infarct size and an improvement in post ischemic cardiac function with the use of GSK3β inhibitors [203, 206] [228-229]. Consistent with these data of cardioprotection achieved by inhibiting the activity of GSK3β, our data show significantly enhanced phosphorylated GSK3β upon treatment with GSK3β inhibitors after I/R injury which correlated with enhanced cardioprotection and a reduction in apoptosis in ARTg mice hearts. Analogous to heart *ex vivo* I/R experiments, siRNA knockdown of GSK3β in hAR overexpressing cells were protected against H/R stress.

Several kinases upstream converge and inhibit GSK3β. GSK3β has emerged as the integration point of many of these pathways and plays a central role in transferring protective signals downstream to target(s) [206, 230]. One of these upstream regulators includes AKT/PKB, a Ser/Thr kinase that has been shown to phosphorylate and inhibit GSK3β on the serine residue [207]. Fujio et al. have shown that AKT functions to promote cardiomyocyte survival and protects against I/R in the mouse hearts [231]. Our results show that the metabolic flux through AR mediates I/R injury, in part, via decreased AKT activation. Furthermore, in hearts from WT and ARKO mice perfused with PI3K/AKT signaling inhibitors, we observed marked increases in LDH and impaired functional recovery during reperfusion. The PI3K/AKT inhibitor resulted in marked decrease in the levels of phosphorylated GSK3β that consequentially led to increased cell death and apoptosis. These data lead us to believe that AKT and GSK3β are key kinases which participate in the signaling mechanisms that mediate functional and cardiac recovery after I/R injury as a result of increased flux via AR. Decreased phosphorylation of these kinases

resulted in the impairment of cardiac recovery which contributed to the vulnerability of the myocardium to recover after an ischemic insult.

Studies by us [209] and others [232-233] have demonstrated that increased flux via AR leads to activation of PKC (α/β) in euglycemic and hyperglycemic conditions. Furthermore, previous reports in the literature have alluded to the selective inhibition of PI3K/Akt as a result of PKC activation in endothelial cells [234]. The mechanism of PI3K/Akt inhibition has been attributed to activation of the PKCβ isoform in particular, due to hyperglycemia. Hence, we investigated the role of PKC activation and GSK3β phosphorylation. Naruse et. al., have demonstrated that inhibition of PKCβ resulted in improved glomerular endothelial cell function and improved insulin action [234]. Furthermore, translocation of PKCβII from the cytosol to membrane has been shown to contribute to I/R injury and either genetic or pharmacological inhibition of PKCβII protected ischemic cardiac tissue [212]. Additionally, activation of PKCα has been shown to contribute to cardiac hypertrophy and inhibition of this PKC isoform led to attenuation of inflammation, cardiomyocyte growth and cardiac hypertrophy [235]. Here, we demonstrate that inhibition of PKC, specifically α and β isoforms reduced cellular injury as demonstrated by reductions in LDH release upon treatment with PKC α/β inhibitor Gö6976. Furthermore, we demonstrate that Akt and GSK3β phophorylation was increased in both control and ad-hAR HL-1 cells treated with Gö6976. Taken together, our data demonstrate that increases in AR flux leads to PKC activation followed by, decreases in Akt and GSK3β phosphorylation, and is linked to increases in I/R injury (Figure 8).

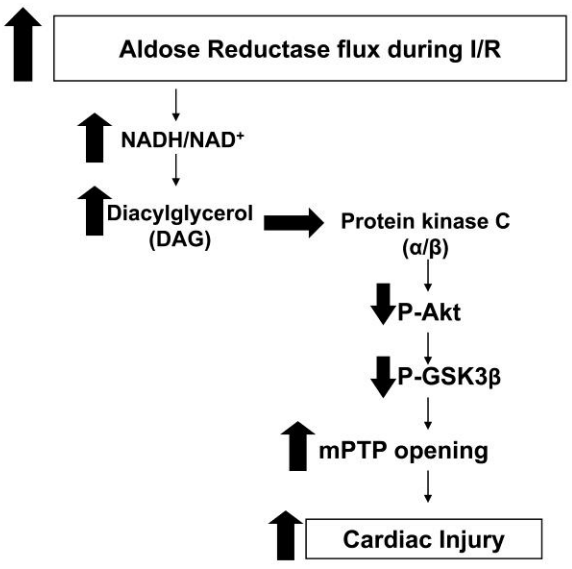


Figure 8: *Schematic diagram. Increased flux via AR after I/R resulted in generation of diacylglycerol (DAG) and activation of PKCα and β isoforms leading to decreased phosphorylation of Akt and GSK3β kinases. Decreased GSK3β ultimately decreased cardioprotection via increased apoptotic mechanisms and mitochondrial permeability opening.*

Limitations:

While this study does not show the beneficial effects of AR inhibition with the use of an ARI, our group shows that p-GSK3β Serine 9 is significantly increased in the left anterior descending coronary artery (LAD) ligation *in vivo* model, after treatment with Zopolrestat, an ARI, compared to untreated groups (data not shown). Secondly, the 30-minute ischemia and 60 minute reperfusion end points have been argued to be the time at which most of the myocardium is in heart failure with near complete loss of ATP. This may be correlated to the decreased p-GSK3β observed in ARTg hearts compared to WT and ARKO. Post translational modifications of proteins such as phosphorylation of GSK3β do not occur consistently, but rather have peak moments where phosphorylation is increased. It may have been appropriate to conduct time course studies in order to evaluate p-GSK3β levels in response to I/R.

In summary, our data demonstrates decreased levels of p-GSK3β in ARTg mice hearts as compared to WT hearts. Inhibition of GSK3β increased levels of p-GSK3β and was associated with decreased injury, improved functional recovery, and decreased apopotosis after I/R in ARTg hearts. ARKO mice had increased levels of p-GSK3β, improved functional recovery, and decreased apopotosis after I/R as compared to WT and ARTg hearts. Furthermore, we show that AR modulates changes in levels of p-GSK3β via AKT pathway. Taken together, our data demonstrate that AR mediates I/R injury, in part, via modulation of GSK3β phosphorylation.

Our next phase of studies will determine mechanisms by which AR modulates GSK3β phosphorylation during I/R.

Chapter 4: The Role of Autophagy in Aldose Reductase Mediated Ischemia-Reperfusion Injury: Good or Bad Guy?

Abstract:

Increased flux of glucose via the aldose reductase (AR) pathway is a key player in mediating ischemia/reperfusion (I/R) injury. The role of autophagy in AR mediated I/R injury is unknown, therefore we sought to investigate whether an increased flux via AR impacts autophagy. cDNA plate array specific for autophagic genes demonstrated significant reductions in beclin-1 transcript levels in ARTg hearts compared to WT. Utilizing an isolated Langendorff perfusion system, hearts from adult wildtype (WT) mice, AR transgenic (ARTg) and AR knockout (ARKO) mice were isolated and and perfused under under normoxic conditions for 30 minutes, followed by 30 minutes of ischemia and 60 minutes of reperfusion. Western blot analysis revealed significant reductions in beclin-1 protein expression in ARTg hearts and in hAR overexpression HL-1 cardiomyocytes. Use of GSK3β inhibitors (SB216763 and LiCl) demonstrated no further reductions in beclin-1 protein levels in WT, ARTg and ARKO hearts. Autophagy inhibitor 3-methyladenine (3-MA) was utilized to determine whether autophagy is a key player in AR mediated I/R injury. Lactate dehydrogenase (LDH), a marker of cardiac injury, and the functional recovery marker, LVDP, were measured in the perfusate after reperfusion in the presence and absence of autophagy inhibitor 3-MA. Hearts from WT, ARTg and ARKO mice displayed significant increases LDH release. LVDP was significantly impaired after reperfusion in WT, ARTg and ARKO hearts after perfusion with 3-MA and correlated with increased Bcl-2 expression. Mammalian target of rapamycin (mTOR) is the key sensor of nutrient status in a cell. Expression of mTOR was significantly increased in ARTg hearts compared to WT and ARKO. Inhibition of mTOR with rapamycin significantly increased beclin-1 levels in WT, ARTg and ARKO hearts. Inhibition of mTOR improved functional recovery and decreased injury marker LDH. Furthermore, treatment with rapamycin increased

expression of p-Akt and p-GSK3β. Taken together, our results indicate induction of autophagy in ARTg hearts is impaired and is correlated with the decreased ability of the myocardium to recover after an ischemic insult.

INTRODUCTION:

Conditions that trigger autophagy has been shown to occur under the I/R setting [236]. Several studies have demonstrated increased activation of autophagy under I/R conditions, however, the exact role of autophagy in the I/R setting has been under great debate; with existing evidence both for, and against the protective effects of autophagy induction [236]. It is clear that the role of autophagy under pathological conditions whether protective or maladaptive, warrants further investigation as there are inconclusive data on the subject. Most of the studies on the topic have varied in the experimental models and designs, and so it is possible that the protective effects of autophagy are dependent on the duration, as well as the severity of ischemia and reperfusion. Additionally, the levels of autophagy, as well as upstream signaling pathways are critical in determining protective or detrimental effects of autophagy [237].

Previous studies have shown that increased flux via AR is linked to increases in cardiac I/R injury [154-155]. Mechanisms by which AR mediates injury include decreased energy metabolism, increased reactive oxygen species (ROS), mPTP opening, impaired ion homeostasis, and involvement of the Jak/STAT pathway [157, 162, 209]. While several studies have provided insight into both the protective and detrimental effects associated with upregulation of autophagy during I/R, it is unknown whether an increased flux via aldose reductase (AR) influences any changes in autophagy. Therefore, the first goal of chapter 4 is to determine whether AR influences autophagic induction, and second, to elucidate the role, if any, of autophagy in aldose reductase (AR) mediated I/R injury. In order to determine whether there are any I/R related autophagy effects, we analyzed the expression of autophagy related gene, beclin-1, in WT, ARTg and ARKO hearts.

MATERIALS/METHODS:

All animal experiments were approved by the Institutional Animal Care and Use Committees of Columbia University, New York University School of Medicine, Baylor college of medicine and conformed to the guidelines outlined in the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Pub. No. 85-23, 1996). Male C57BL/6J mice were used as control wild-type (WT) mice. Male mice weighing 25–30 g at age 12-14 weeks were used in all experiments. Mice transgenic for human aldose reductase (ARTg) were obtained from Dr. Mitsuo Itakura (University of Tokushima, Japan) and a colony was established at our facility at NYU Medical Center. Aldose reductase knock-out (ARKO) mice was generated as described recently [216].

Reagents

The primary antibodies used were anti-Beclin-1 IgG, anti-Bcl-2 IgG, anti mTOR IgG/anti-Total mTOR IgG (Cell Signaling, USA); anti-cytochrome C IgG and anti-beta-actin IgG (BD Biosciences Pharmingen, USA). The secondary antibodies used were goat-anti-rabbit IgG-peroxidase antibody and rabbit-anti-mouse IgG-peroxidase antibody (Sigma, USA). Autophagy inhibitor 3-methyladenine (3-MA) was purchased from Sigma-Aldrich, USA.

Isolated perfused heart preparation:

Experiments were performed using an isovolumic isolated heart preparation as published and modified for the use in mice hearts [157, 162]. Mice were anesthetized using a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). After deep anesthesia was achieved, hearts were rapidly excised, placed into iced saline, and retrogradely perfused at 37ºC in a nonrecirculating

mode through the aorta at a rate of 2.5 mL/min. Left ventricular developed pressure (LVDP) was measured using a latex balloon in the left ventricle.

Ischemia/reperfusion protocol:

To determine the link between AR and autophagy after I/R injury, hearts from WT, ARTg and ARKO mice were perfused in the presence of autophagy inhibitor; 3-methyladenine (3-MA-10mM). Rapamycin (1 mg/kg, i.p.) was administered to WT, ARTg and ARKO mice 30 minutes prior to rapid excision of the heart.. The doses of the inhibitors used in this study were based on publications in the literature [238]. Inhibitors were present at the start of the equilibration period and continued throughout ischemia and reperfusion.

Western Blot Analysis

The tissue and cell protein concentration was determined using a DC Protein Assay kit (Bio-Rad). Equal amounts of protein were separated by SDS-PAGE (4–12% gradient gels), and proteins were transferred to a nitrocellulose membrane (Invitrogen). After blocking in 5% dry milk in TBST, membranes were incubated overnight with target primary antibodies (1:1000 dilution) according to the manufacturer's instructions. Membranes were incubated sequentially with secondary antibody for 1 hr. Phosphorylated and total mTOR expressions were transferred overnight in 4˚C and blocking was in 5% BSA in TBST. Blots were visualized with an ECL Horseradish Peroxidase Western Blot Detection System (Cell Signaling, USA), and quantitative analysis was performed using Image Quant TL software (Amersham).

2.8 Lactate Dehydrogenase (LDH) Measurements

Cardiac injury due to I/R stress was assessed by measuring LDH release in the perfusates that were collected at the end of reperfusion. In HL-1 cardiomyocytes, injury due to hypoxia/reoxygenation (H/R) stress was measured in supernantants that were collected after H/R. LDH was measured using the commercially available kits (Pointe scientific, Inc., USA) as published earlier.

2.9 Statistical Analysis

All data are presented as mean value ±standard error of the mean (SEM). Statistical significance of differences between various groups of heart was determined by ANOVA. Post hoc comparisons were performed with Tukey or Dunnett procedures using GraphPad software as indicated. Statistical significance was ascribed to the data when $P<0.05$.

RESULTS:

Our aim was to determine whether autophagy was impacted by an increase in flux via AR. To do this, we utilized the Langendorff perfusion system of isolated *ex vivo* heart preparations. We examined beclin-1 levels as an indicator of autophagy upregulation. We performed a cDNA plate array for specific autophagic genes including beclin-1, Atgs 3, 7 and 10, and BNIP3. Our results demonstrate that in ARTg hearts, beclin-1 is significantly downregulated at the transcript level compared to WT hearts (Figure 1A). Western blot analysis further confirmed this finding by illustrating a significant reduction in beclin-1 levels at the protein level in hearts from ARTg animals compared to WT and ARKO (Figure 1B). HL-1 cardiomyocytes overexpressing human aldose reductase (hAR) and subjected to H/R also demonstrated a decrease in beclin-1 levels compared to controls (Figure 1C). The reduced induction of autophagy in ARTg hearts correlated with a decrease in phosphorylation of Akt and GSK3β, as well as an increase in apoptosis as was demonstrated in the previous chapter by an increase in cytochrome C and decrease in Bcl-2 expression.

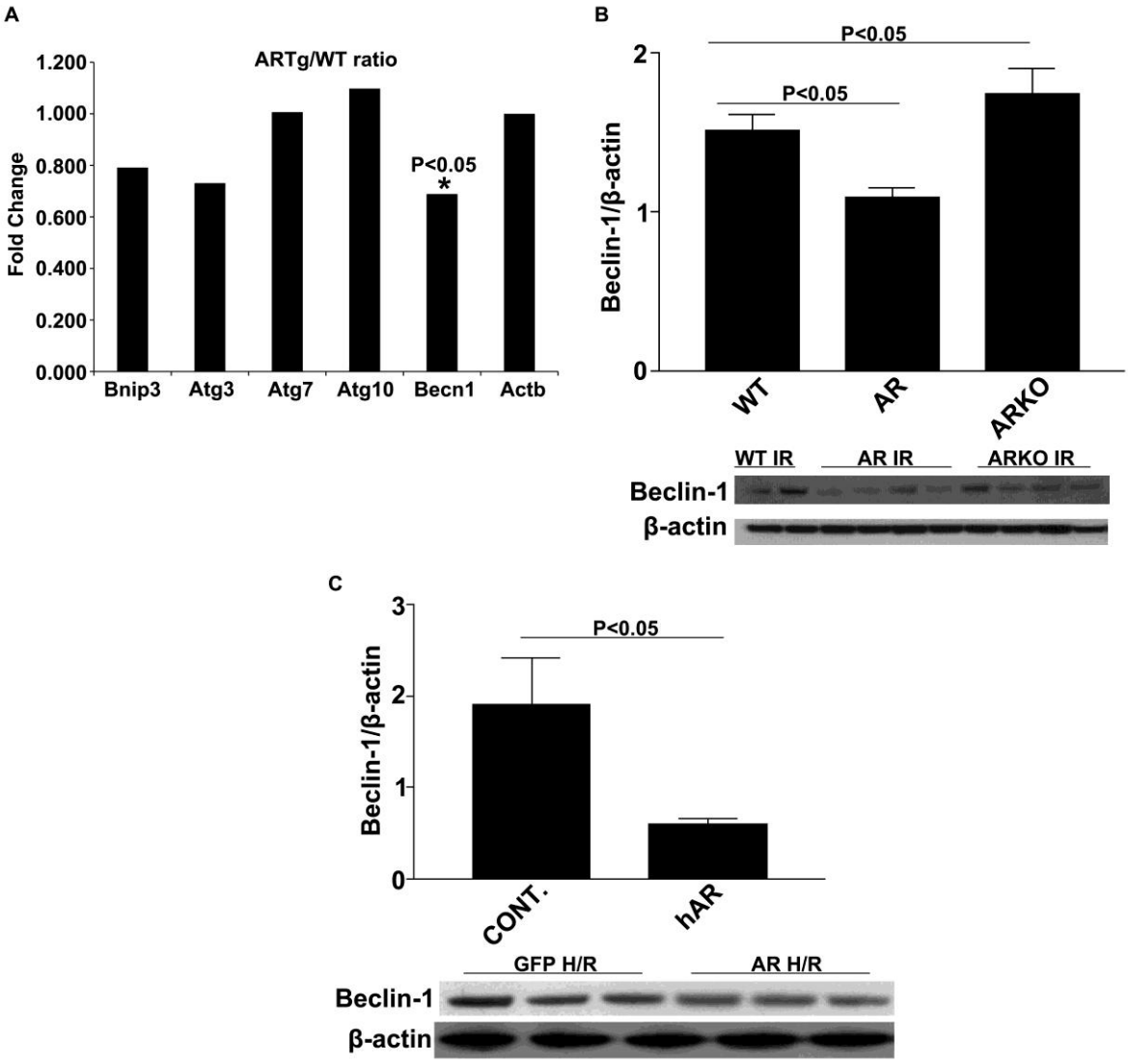


Figure 1: Expression of Autophagy. ***a.)*** *cDNA plate array of specific autophagic genes.* ***b.)*** *western blot protein expression of Beclin-1 in WT, ARTg and ARKO hearts and* ***c.)*** *protein expression of beclin-1 in control and hAR expressing HL-1 cardiomyocytes. Beclin-1 was significantly downregulated at both the transcript and protein expression in ARTg hearts compared to WT and ARKO in cardiac tissue ($p<0.05$). Similarly, hAR overexpressing cardiomyocytes displayed significant decreases in beclin-1 expression.*

Because ARTg hearts displayed decreased upregulation of autophagy which was associated with a decrease in phosphorylation of GSK3β, we next sought to determine whether autophagy was GSK3β dependent or independent. We determined whether inhibition of GSK3β influenced changes in beclin-1 levels. WT and ARTg hearts were treated with GSK3β inhibitors SB216763 and lithium chloride (LiCl), and were perfused under normoxic conditions for 30 minutes, followed by 30 minutes of ischemic conditions and 60 minutes of reperfusion

conditions. Western blot analysis indicated no further changes in beclin-1 levels (Figure 2) in hearts of GSK3β inhibition, suggesting that autophagy is unlikely to be downstream of GSK3β in our model.

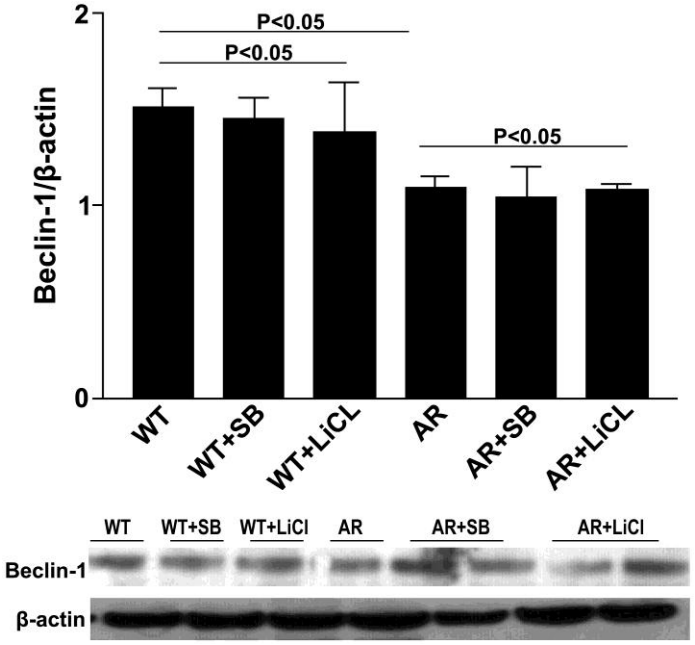


Figure 2: *Expression of beclin-1 with GSK3β inhibition. Protein expression of* ***a.)*** *beclin-1 with and without GSK3β inhibitors SB216763 and LiCl. No further changes in beclin-1 were observed via GSK3β inhibition*

To determine whether autophagy is a key player in AR mediated I/R injury, WT, ARTg and ARKO hearts were perfused with autophagy inhibitor 3-MA under normoxic (30 min), ischemic (30 min) and reperfusion conditions (60 min). Our data demonstrate that inhibition of autophagy significantly decreased autophagy as demonstrated by decreased beclin-1 levels (Figure 3A). Perfusion with 3-MA decreased cardiac function and increased injury after I/R. Left ventricular developed pressure (LVDP) was found to be significantly decreased in hearts from WT, ARTg and ARKO hearts upon perfusion with autophagy inhibitor 3-MA (Figure 3B). Furthermore, LDH measured after I/R as a marker for cardiac injury was found to be significantly increased in all three groups upon perfusion with 3-MA (Figure 3C). Apoptosis was increased in hearts from

all three groups as indicated by a decrease in Bcl-2 protein levels in WT, ARTg and ARKO hearts (Figure 3D).

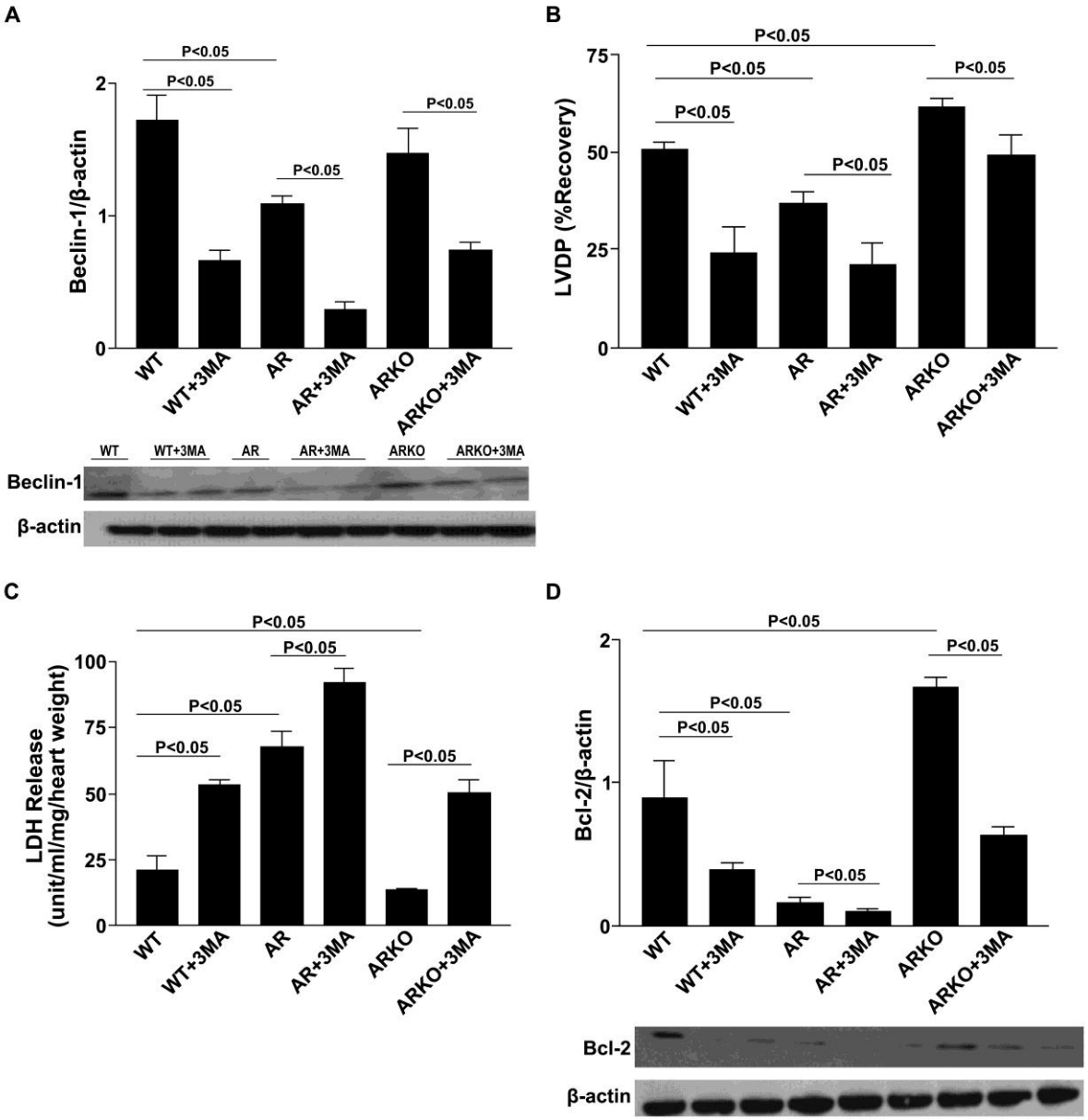


Figure 3:Inhibition of Autophagy. Western blot analysis of a.)beclin-1 in WT, ARTg and ARKO hearts treated with and without 3-methyladenine. Determination of myocardial ischemic function and injury with and without autophagy inhibitor 3-methyladenine treatment as shown by b.)LVDP and c.)LDH release. Western blot protein analysis of d.) Bcl-2 in WT, ARTg and ARKO hearts treated with and without 3-methyladenine. Use of 3-methyladenine resulted in significant decreases in beclin-1 expression in WT, ARTg and ARKO hearts. furthermore, inhibition of autophagy decreased functional recovery and increased cardiac injury and apoptosis.

Mammalian target of rapamycin (mTOR) is the key sensor of nutrient status in a cell. Upon phosphorylation, it initiates the induction of autophagy. We analyzed expression of

phosphorylated mTOR (p-mTOR) in WT, ARTg and ARKO mouse hearts after I/R injury. Western blot analysis revealed that protein expression of p-mTOR was significantly increased in ARTg hearts compared to WT and ARKO hearts (Figure 4A). The observed increase in p-mTOR expression in ARTg hearts correlated with a decrease in beclin-1 levels, a finding consistent with data in the literature showing inhibition of autophagy when mTOR is phosphorylated [239]. Therefore, to determine whether mTOR is a key regulator of autophagy after I/R, hearts from WT, ARTg and ARKO were perfused with inhibitor of mTOR, rapamycin. Treatment of mice with rapamycin significantly improved LVDP and decreased LDH release in WT, ARTg and ARKO hearts (Figure 4B and 4C). As expected rapamycin significantly decreased phosphorylation of mTOR in WT, ARTg and ARKO hearts (Figure 4D). Use of rapamycin increased beclin-1 expression as demonstrated by western blot analysis in ARTg hearts when compared to WT and ARKO (Figure 4E). Furthermore, treatment with rapamycin increased p-Akt and p-GSK3β expression (Figure 4F and 4G) and reduced apoptosis levels as demonstrated by decrease in cytosolic Cytochrome C levels in ARTg hearts (Figure 4H). These data indicate that mTOR is a key factor in regulating the induction of autophagy after I/R. In these studies, reduced induction of autophagy is associated with the impairment of the myocardium to recover after an ischemic insult. Increased phosphorylation of mTOR correlated with a decreased induction of autophagy and contributed to I/R injury.

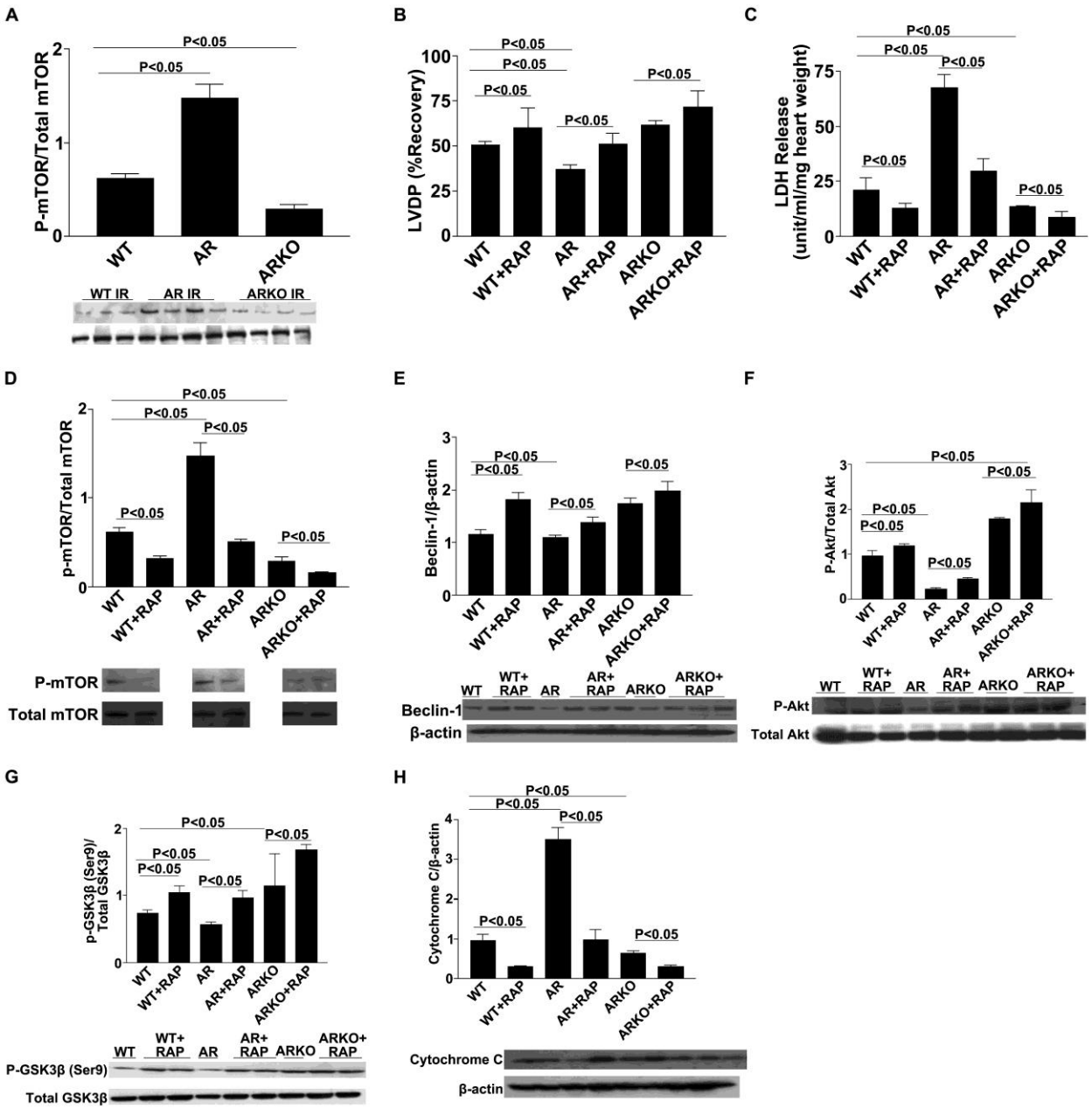


Figure 4:Role of mTOR in autophagy induction. *Western blot analysis of a.)p-mTOR in WT, ARTg and ARKO hearts. Determination of myocardial ischemic function and injury with and without mTOR inhibitor rapamycin treatment as shown by b.)LVDP and c.)LDH release. Western blot protein analysis of d.) p-mTOR; e.) beclin-1; f.)p-Akt and g.)p-GSK3β; and h.)cytochrome C in WT, ARTg and ARKO hearts treated with and without rapamycin. Use of rapamycin resulted in significant increases in beclin-1 expression in WT, ARTg and ARKO hearts. Furthermore, rapamycin increased functional recovery and decreased cardiac injury and apoptosis.*

DISCUSSION:

In this study we investigated the role of autophagy induction in AR mediated I/R injury. As we have shown before, increased flux via the AR pathway negatively impacts the myocardium by increasing oxidative stress, impairing ATP production as a result of altered glucose metabolism, and impairing calcium homeostasis, conditions which favor the opening of the mitochondrial permeability transition pore [240-243]. Studies that have utilized the isolated Langendorff perfused heart model have shown increased beclin-1 expression with preconditioning or resveratrol treatment, strategies that have been established to achieve cardioprotection [244-246]. We show that autophagy related gene, beclin-1 is downregulated in ARTg hearts compared to WT and ARKO indicating decreased autophagosome formation and decreased induction of autophagy. A decreased beclin-1 expression in ARTg hearts correlated with a decrease in p-GSK3β expression, an increased cytosolic Cytochrome C, and decreased Bcl-2 protein expression. Furthermore, when WT, ARTg and ARKO hearts were treated with autophagy inhibitor 3-MA, our study revealed that functional recovery in was markedly impaired. Consistent with this observation is the increased LDH released in all three groups after perfusion with 3-MA. These results indicate that induction of autophagy in ARTg hearts is impaired and is correlated with the decreased ability of the myocardium to recover after an ischemic insult. Additionally, inhibition of autophagy impaired functional recovery and increased cardiac injury in WT and ARKO hearts.

Induction of autophagy usually occurs under periods of stress such as nutrient deprivation or starvation. In these times, induction of autophagy will generate the necessary substrates needed for generation of nutrients to support the cell and prevent further damage. There are several triggers for autophagy induction, one of which is starvation and has been discussed earlier as a

reason why autophagy is an evolutionary conserved process that occurs amongst eukaryotes. It is required to maintain cellular and tissue homeostasis especially in certain periods including embryonic development. Disregulation of autophagy has been shown to contribute to a myriad of diseases including cancer, diabetes, neurodegenerative diseases such as Alzheimer's and cardiac diseases [247].

There are several studies which have shown that autophagy is upregulated during the I/R setting [248-249]. Key events that occur during I/R which contribute to the impairment of the myocardium such as nutrient and ATP depletion, calcium accumulation, ROS accumulation and mPTP opening are all events which trigger the induction of autophagy. Whether upregulation of autophagy is protective or not is a question that remains to be answered. The use of the isolated heart models as well as *in vivo* heart models of ischemia reperfusion has further added to the controversy of the protective mechanisms offered by autophagy, or lack thereof. Cardioprotective agents such as sulfaphenazole as well as ischemic preconditioning, a method known to confer cardioprotection, have both been demonstrated to stimulate autophagy by increasing beclin-1 levels as well as the LC3II/LC3I ratio and consequentially reduce cell death. Adding to the confusion however, are studies where heterozygous knockdown of beclin-1 (beclin-1 +/-) in mice resulted in a reduction of infarct size. In cellular studies under hypoxia reoxygenation conditions, treatment with autophagy inhibitor 3-MA reduced cellular injury and decreased apoptosis [248, 250]. In contrast to this, other studies have shown that inhibition of autophagy with use of 3-MA or knockdown of beclin-1 was protective in cardiomyocytes after simulated ischemia reperfusion [251]. Cellular studies utilizing HL-1 cardiomyocytes have indicated protective effects associated with upregulation of autophagy [236].

Mammalian target of rapamycin known for its role in mitochondrial metabolism, growth and proliferation, also plays a key role in nutrient regulation [252]. In the present study, we examined the role of mTOR in regulating autophagy in WT, ARTg and ARKO hearts. This study demonstrates that intraperitoneal administration of rapamycin, inhibitor of mTOR, induced cardioprotection as is demonstrated by a reduction in functional recovery, upregulation in beclin-1 protein expression and a reduction in apoptotic markers.

This study establishes that an increased flux via AR reduced induction of autophagy and is associated with the vulnerability of the myocardium to recover after an ischemic insult. Earlier chapters discussed the importance of maintaining mitochondrial integrity after I/R in order to preserve functional recovery. One of the mechanisms by which autophagy in this setting may be protective is via mitophagy. Mitochondria that have been damaged during I/R conditions can potentially cause nearby mitochondria to become damaged. During I/R, increased calcium accumulation in the mitochondrial matrix causes swelling and ultimately forces the mPTP to open dissipating membrane potential and depleting ATP. Opening of the mitochondria is accompanied by cytochrome C release into the cytosol which activates apoptotic pathways. Similarly, increased mPTP opening causes a rapid rise in ROS and calcium release which ultimately will cause further damage to neighboring mitochondria. Elimination of damaged mitochondria through autophagy, termed mitophagy, has been shown to be critical in maintaining cellular integrity and preventing cytotoxicity [253-256].

Limitations:

3-methyladenine is a widely used inhibitor of autophagy [257-258]. In these studies we utilized 3-MA to investigate the effects of autophagy inhibition in AR mediated I/R injury. Some studies have shown that use of 3-MA may potentially confound results because it is a non specific inhibitor and targets other proteins which may implicate the finding in results. Specifically, 3-MA has been shown to promote glycogen breakdown and inhibit flux through phosphofructokinase [259].

Chapter 5: Summary, Concluding Remarks, and Future Directions.

Summary:

My research has provided new insights into the signaling mechanisms mediating cardiac ischemic injury and autophagy. Importantly, findings from my thesis research have established the hierarchy of AR driven signaling events which mediate cardiac I/R injury. I have opted to present a brief review of my findings as bullets below.

Research findings in chapter 3 demonstrate:

1. Increased flux via AR decreased phosphorylation of GSK3β
2. The decrease in GSK3β phosphorylation is mediated in part via increased PKCα/β activation and decreased Akt phosphorylation.
3. Decreased phosphorylation of GSK3β was linked to increased mPTP opening and reduced cardioprotection

The focus of chapter 4 is to investigate whether an increased flux via AR influences any change in autophagy induction and whether autophagy is linked to AR mediated I/R injury.

Research findings in Chapter 4 demonstrate:

1. Increased flux via AR decreased autophagy related gene Beclin-1
2. Decreased Beclin-1 expression correlated with increased phosphorylation of mTOR and decreased phosphorylation of Akt and GSK3β (shown in chapter 3)
3. Inhibition of autophagy with 3-Methyladenine correlated with decreased functional recovery, increased injury and increased apoptosis
4. Inhibition of mTOR with rapamycin increased Beclin-1 expression, improved cardiac function, reduced injury, and decreased apoptosis.

In the next part of this chapter, I provide insights into the important questions raised by my research that will require further investigation.

Concluding Remarks and Future Directions:

We have demonstrated that an increased flux via AR decreased the phosphorylation of GSK3β leading to increased injury, and decreased cardiac function. We further showed that inhibition of GSK3β utilizing specific and nonspecific inhibitors (SB216763 and LiCl respectively) conferred cardioprotection in AR transgenic mouse heart that has undergone ischemia-reperfusion (I/R). Currently, small molecule inhibitors of GSK3 that exist are not isoform specific but target both α and β isoforms. Therefore, utilizing GSK3 β transgenic mice will be key in further confirming the cardioprotective role of GSK3β inhibition. Two models that have been used to study the role of GSK 3 in cardiac tissue are the overexpression of cardiac specific constitutively active GSK3β, and the GSK3β knock-in model where the Serine 9 phosphorylation site is converted into Alanine therefore inhibiting phosphorylation.

We and other have previously demonstrated that increased flux via AR leads to activation of PKC (α/β) in euglycemic and hyperglycemic conditions. After glucose flux via the polyol pathway increases after I/R, PKC becomes activated through increased diacylglycerol (DAG) levels influenced by increases in the NADH/NAD^+ ratio. PKC activation, specifically the β isoform has been implicated in impairment of the myocardium to recover after an ischemic insult [212]. In our studies, we hypothesized that the increased PKC activation observed after I/R contributes to impairment of the myocardium through a decreased inhibition of AKT and GSK3β. Furthermore, we expected that the inhibition of PKC (α and β isoforms) would result in an increase in AKT and GSK3β phosphorylation. We utilized the PKC α and β inhibitor, Gö6976, in HL-1 cardiomyocytes and assessed phosophorylation patterns of AKT and GSK3β kinases. Results revealed that inhibition of α and β isoforms of PKC increased phosphorylation of AKT and GSK3β and led to marked reductions in cardiac injury marker, LDH. To further

strengthen our studies and to further illustrate the role of PKC α/β in AR mediated I/R injury, future studies should be conducted utilizing either PKCα -/- and PKCβ-/- mice bred on an ARTg background. These efforts will help establish if ablation of these PKC isoforms will alleviate some of the detrimental effects of AR mediated I/R injury. It might be argued that whole body knockout of PKC would activate other compensatory mechanisms which could confound interpretation of results. Therefore, utilizing cardiomyocyte specific PKC α/β knockout animals might be more informative in efforts to elucidate and further characterize the role of PKC in the heart.

Silent information regulators (sir2) are class III NAD+ dependent histone deacetylases and have been shown to increase lifespan in addition to protecting cells from apoptosis. The mammalian ortholog of sir2 is Sirt1. Studies utilizing cardiac specific overexpression of Sirt 1 demonstrate that a 2.5-7.5 fold increase in cardiac Sirt1 expression conferred cardioprotection and displayed resistance when exposed to oxidative stress [260]. As is explained in the introductory portion of this thesis, an increased flux via the polyol pathway decreases the availability of cytosolic NAD+. Studies from our lab have shown that Sirt1 activity is reduced in ARTg hearts due to a reduced availability of NAD+ necessary for Sirt1's deacetylase activity. Furthermore, it has been shown that autophagy induction is upregulated through NAD+ dependent deacetylation of FOXO1 by Sirt1 [261]. In these studies, it would be interesting to investigate if the decrease in autophagy upregulation which contributes to I/R injury is mediated, in part, via decreased deacetylation of FOXO1. Specific questions include: will increased flux via AR result in increased acetylation of FOXO1 in ARTg relative to WT and ARKO? How does the acetylation of FOXO1 influence autophagy in WT, ARTg and ARKO mice? Protein expression of acetylated

FOXO1 can be assessed in WT, ARTg and ARKO hearts to determine if influenced by the decreased Sirt1 activity. It is hypothesized that increased acetylation of FOXO1 will be observed in ARTg hearts relative to WT and ARKO hearts and will be associated with reductions in autophagy induction.

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