

**Cardioprotective Mechanisms of PPAR  $\gamma$  in Diabetic Heart**

by

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## Abstract

Patients with diabetes exhibit significantly altered renin-angiotensin system (RAS). Recently it has been determined that hyperglycemic conditions induce an increase in angiotensin II (AT II) expression; specifically in cardiomyocytes. Altered RAS has been shown to be associated with an increase in oxidative stress and cardiac dysfunction leading to the development of cardiac hypertrophy. The transient outward potassium current ( $I_{to}$ ) in cardiac myocytes is mainly mediated by members of the Kv subfamily of voltage gated potassium channels and has been shown to be altered in cellular localization and expression during the development of cardiac hypertrophy. However it is not clear as to how AT II affects the pore forming complex at the cell membrane and thus directly affects the  $I_{to}$  current. In the current study, we explored the protective effect of PPAR $\gamma$  ligands on cardiomyocyte  $I_{to}$  by preventing NADPH Oxidase activation and the ensuing ROS formation. Furthermore, short term PPAR $\gamma$  activation in diabetic leptin deficient db/db mice displayed improvements in the membrane association of the molecular components of  $I_{to}$  as well as prolonged QT interval. These findings demonstrate that PPAR  $\gamma$  agonist have the potential to attenuate cardiomyocyte dysfunction associated with diabetes.

Even though we and others have demonstrated that the use of PPAR  $\gamma$  agonists exerts a profound protection against diabetes mediated cardiovascular pathologies, the use of these drugs in a clinical setting is restricted because of its detrimental side effects with long term use.

Therefore, development of new therapeutic agents which can modulate the PPAR  $\gamma$  activity without the adverse effects can be beneficial to mitigate diabetes induced cardiovascular diseases.

The objective of the second study reported in this dissertation is to develop novel dual PPAR  $\gamma/\delta$  agonists without the deleterious side effects associated with full PPAR  $\gamma$  agonists. Docking simulations of 23 novel compounds within the ligand binding domain of PPAR  $\gamma/\delta$  were performed using AutoDock Vina which consistently reproduced experimental binding poses from known PPAR agonists. Comparisons were made and described with other docking programs AutoDock and Surflex-Dock (from SYBYL-X). Biological evaluation of compounds was accomplished by transcriptional promoter activity assays, quantitative PCR gene analysis for known PPAR  $\gamma/\delta$  targets as well as *in vitro* assays for lipid accumulation and mitochondrial biogenesis versus known PPAR agonists. We found one (compound 9) out of the 23 compounds evaluated, to be the most potent and selective dual PPAR $\gamma/\delta$  agonist which did not display the deleterious side effects associated with full PPAR $\gamma$  agonists.

Additionally, we have observed that PPAR  $\gamma$  agonist treatment can offer a protection against I/R injury in the diabetic heart/ The major form of fatality due to diabetes is development of congestive heart failure triggered by myocardial infarction (MI). The impaired insulin signaling in the diabetic heart leads to myocardial energy dysregulation that compromises the

cardioprotective mechanism against ischemic injury. We observed that db/db mice (leptin deficient, type 2 diabetic mice) have increased infarction size compared to wild type mice after ischemia/reperfusion (I/R) injury by TTC stain. We also found that activity of Hypoxia inducible factor-1 (HIF-1 $\alpha$ ), a master transcription factor involved in the cardioprotective response to ischemia, is impaired in db/db hearts. HIF-1 $\alpha$  is known to transcriptionally regulate genes involved in myocardial energetics. We recently found that HIF-1 $\alpha$  transcriptionally regulates the mitochondrial protein frataxin in cardiomyocytes as determined by luciferase assays. In vitro studies indicate that hypoxic conditions increase frataxin protein expression in cardiomyocytes as determined by western analysis. Frataxin plays an important role in the Fe-S cluster biogenesis required to maintain the activity of aconitase, succinate dehydrogenase and complexes in the mitochondria. Interestingly, we observed decreased expression of frataxin in the ischemic diabetic heart. Therefore, we postulate that attenuated HIF-1  $\alpha$ -frataxin signaling in ischemic db/db hearts leads to abnormally enlarged infarction size in response to I/R. The decline in HIF-1 $\alpha$  activity in response to hypoxia was further validated in cardiomyocytes cultured in high glucose media. The significance of frataxin against hypoxic injury was confirmed by utilizing over-expressed frataxin cardiomyocytes via MTT, ATP and aconitase activity assays. Currently we are attempting to identify the hypoxia response element (HRE) in frataxin promoter to further validate the transcriptional activity of HIF-1 $\alpha$ . In addition, we are completing the I/R surgeries on HIF-1 $\alpha$  KO mice to address the cardioprotective nature of HIF-1 $\alpha$ -frataxin signaling against MI.

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## List of Abbreviations

ACE	Angiotensin converting enzyme
ADA	American Diabetes Association
AF	Activation function
AGE	Advanced glycation end products
AV	Atrio-ventricular
AT II	Angiotensin II
CAD	Coronary Artery Disease
CPT I	Carnitine palmitoyltransferase I
DBD	DNA binding domain
DFO	Desferrioxamine
DMOG	Dimethyloxallylglycine
ECG	Electrocardiogram
FABP	Fatty acid binding protein
FATP	Fatty acid transport protein
FDA	Food and Drug Administration
FFA	Free fatty acids
FIH	Factor inhibiting HIF
FRDA	Friedreich's ataxia
G6PD	Glucose-6-phosphate dehydrogenase



GAPDH	Glyceraldehyde – 3 – phosphate dehydrogenase
GLO I	Glyoxalase I
GLUT	Glucose Transporter
HIF-1 $\alpha$	Hypoxia inducible factor – 1 $\alpha$
HRE	Hypoxia response element
Hsc	Heat shock cognate protein
Hsp	Heat shock protein
IRS-2	Insulin receptor substrate – 2
LAD	Left anterior descending coronary artery
LBD	Ligand binding domain
LV	Left Ventricle
I/R	Ischemia/ reperfusion
MG	Methylglyoxal
MHC	Myosine Heavy Chain
MI	Myocardia infarction
MPP	Mitochondrial processing peptidase
NAC	N-acetyl-cystein
NO	Nitric oxide
ODD	Oxygen degradation domain
PPAR	Peroxisome Proliferator Activated Receptor
PHD	Prolyl hydroxylase

PPRE	Peroxisome Proliferator Response Element
pVHL	Von Hippel – Lindau tumor suppressor protein
RAS	Renin Angiotensin System
RLU	Relative light units
ROS	Reactive Oxygen Species
RXR	Retinoid X Receptor
SA	Sino-atrial
SERCA	Sarcoplasmic reticulum Ca <sup>2+</sup> pump
SPPARM	Selective peroxisome proliferator activated receptor modulator
TAD	Transactivation domain
TG	Triglyceride
TZD	Thiozolidinedione
VEGF	Vascular endothelial growth factor

## 1. Introduction

Diabetes mellitus (DM) is at epidemic proportions in United States and is identified as the most common endocrine disorder found in modern society. It occurs due to deficits in insulin secretion or function, or both. This insulin deficiency induces many alterations in the overall metabolism in the body leading to dysfunction and failure of various organs.

DM is categorized in to various types. The most prevalent type of DM are Type 1 DM and Type 2 DM. Type 1 DM results from cell mediated autoimmune destruction of pancreatic  $\beta$ -cells. This leads to absolute deficiency of insulin in the body and the patient have to essentially rely on exogenous insulin for survival. Type 2 DM, which is the most prevalent form of diabetes, is due to insulin resistance and circulating hyperinsulinemic condition. The incidences of type 2 DM is rapidly increasing due to the lifestyle modifications, including dietary habits and lack of exercise.

According to the statistics published by American Diabetes Association, heart diseases are the most common complication associated with diabetes and accounts for highest morbidity and mortality rates. Many of the cardiovascular complications in diabetics have been attributed to coronary heart diseases. However, diabetics suffer from additional cardiac insult referred to as diabetic cardiomyopathy. This was identified as a distinct clinical entity and was defined as diabetes mediated structural and functional changes of the heart, that are manifested independent of other risk factors such as hypertension and coronary artery disease. The most common diabetes mediated structural modifications in the heart are interstitial and perivascular fibrosis and left ventricular hypertrophy. In regards to the functional changes, diabetic cardiomyopathy is

known to develop diastolic dysfunction. Often, this would be followed by development of systolic dysfunction as well.

Many cellular mechanisms are identified that predispose the heart to develop diabetic cardiomyopathy. One such mechanism is alterations of the substrate preference to produce ATP in the heart. Generally, the healthy heart produces approximately 70% of its ATP through oxidation of fatty acids. The rest of the 30% of the energy is provided by glucose oxidation. However, in diabetes, the energy produced by fatty acid oxidation is elevated to more than 90%. This results in an increase delivery of free fatty acids to the myocardium at a rate that exceeds the rate of fatty acid oxidation. This leads to accumulation of ectopic lipid storage in the myocardium and leads to development of lipotoxicity. Also increased fatty acid utilization suppresses the glucose oxidation via Randle cycle. Other mechanisms attributed to the development of diabetic cardiomyopathy are increased oxidative stress, mitochondrial dysfunction, impaired  $\text{Ca}^{2+}$  homeostasis, and increased activity of the Renin Angiotensin System (RAS) in the body. It was identified that the heart has its own functional RAS. Many recent publications confirm the increased activity of RAS in diabetic condition. Furthermore, large scale clinical trials have demonstrated that pharmacological inhibition of RAS can attenuate the development of many cardiac complications.

The increased RAS can adversely impact the heart and alter the function of ion channels in the cardiomyocytes. Alterations in the functions of ion channels lead to changes in the cardiac action potential, and subsequently to the development of arrhythmias which can be fatal. The changes in the electrical conductance are evident by the altered ECG traces of diabetics. We and the others have found that in diabetic animal models, the outward potassium current ( $I_{to}$ ) is significantly attenuated.  $I_{to}$  current plays a critical role in regulating the phase 1 of the cardiac

action potential. Further, previously published reports confirm that altered  $I_{to}$  can lead to prolonged QT segments in the ECG traces. Our data indicates that diabetes alter the channel forming components of  $I_{to}$  which leads to attenuation of the outward  $K^+$  current. Also, we report the treatment of the diabetic animals with a PPAR  $\gamma$  agonist can significantly rescue the myocardium from these adverse effects.

PPAR (peroxisome proliferator activated receptors) are a class of nuclear receptor family. PPAR  $\gamma$  is a subtype of PPAR family, and its critical role in adipogenesis in the adipose tissue is well established. Further, it is well known for its insulin sensitizing effects, and agonists of PPAR  $\gamma$  such as rosiglitazone, pioglitazone (Thiazolidinediones – TZD) had been used clinically for years as a treatment to overcome insulin resistance associated with diabetes. The beneficial effects of using PPAR  $\gamma$  agonists against diabetes mediated cardiovascular pathologies are well demonstrated. Unfortunately, the off target side effects associated with the long term use of rosiglitazone and pioglitazone have raised controversy and the use of these drugs in the treatment of diabetes has been restricted by Food and Drug Administration of USA. The most common side effects that arise with TZDs are weight gain due to adipogenesis, and peripheral edema which can be detrimental for patients with pre existing cardiovascular diseases. Therefore, many research endeavors are focused on developing new PPAR  $\gamma$  agonists, which retain the insulin sensitizing properties without the detrimental adverse effects. This novel class of compounds is called selective PPAR  $\gamma$  receptor modulators (SPPARMs).

We have found that use of PPAR  $\gamma$  agonists can reduce the cardiac damage in db/db diabetic transgenic animals. We and the others have demonstrated that the diabetic hearts sustain extensive cardiac damage when subjected to ischemia/ reperfusion (I/R) injury compared to non diabetic controls. During our studies to understand the signaling pathways that may contribute to

the development of a larger infarction in the diabetic heart, we found that the expression and activation of the transcription factor HIF-1 $\alpha$  (Hypoxia Inducible Factor) is significantly attenuated in the diabetic heart. HIF-1 $\alpha$  gets activated during hypoxic/ ischemic insults and activate myriad of genes that confer protection. HIF-1 regulates the transcription of its target genes by binding to Hypoxia Response Elements (HRE) in the promoter regions. Our preliminary data suggests that HIF-1 $\alpha$  activity in the cardiomyocytes exposed to hyperglycemia is significantly suppressed due to increased methylglyoxal (MG) formation. MG is a highly reactive compound that is formed in hyperglycemic conditions and is known to induce advanced glycation end (AGE) product formation leading to dysfunction of proteins. MG can bind covalently with CPB/ p300 which are co activators required for the proper function of HIF-1 $\alpha$  in response to hypoxia.

Further, our preliminary data suggests that HIF-1  $\alpha$  can transcriptionally regulate proteins adiponectin and frataxin in response to hypoxia in the heart. Adiponectin is predominantly secreted by the adipose tissue; however the presence of cardiac derived adiponectin which exerts its effect via autocrine/ paracrine mechanisms has also been identified. We have identified potential HRE regions in the adiponectin promoter, and also we have observed increased expression of gene and protein expression with elevated HIF-1 $\alpha$  levels in isolated cardiomyocyte systems. The data we have accumulated in this study is currently being formulated into a manuscript.

We also observed that HIF-1 $\alpha$  can regulate the expression of frataxin in response to hypoxia. Frataxin is a nuclear encoded mitochondrial protein which plays an essential role in formation of Fe-S clusters in the cells. Fe-S clusters are prosthetic groups which are required for to maintain the proper function of enzymes like aconitase, succinate dehydrogenase and complex

I and III of the electron transport system. Therefore, frataxin is essential for the normal oxidative phosphorylation in the mitochondria. It also maintains the iron homeostasis in the mitochondria. To better understand the role of HIF-1 $\alpha$  – frataxin signaling during hypoxia, we have constructed a cardiac specific HIF-1 $\alpha$  knockout mouse. Further, to identify the protective effects of frataxin exerted during hypoxia, we have constructed frataxin over-expressed H9C2 cardiomyocyte cell line by utilizing a lentiviral system. Our preliminary data also suggests the presence of HRE in the promoter of frataxin gene. The functional capacity of these HRE sites will be further validated by conducting point mutations and also ChIP assays.

In summary, our data suggests that PPAR  $\gamma$  agonism offers protection against diabetes mediated cardio vascular pathologies. Further, we report that the PPAR  $\gamma$  agonists rescue the diabetic heart from attenuated  $I_{to}$  current via its antioxidant properties. Further, we have identified that PPAR  $\gamma$  can increase the expression of the transcription factor HIF-1 $\alpha$  which can exert a profound protection against hypoxic insult in the diabetic heart. We have found that adiponectin and frataxin can be transcriptionally regulated by HIF-1 $\alpha$  in response to hypoxia in cardiomyocytes.

## **2. REVIEW OF LITERATURE**

### **Diabetes Mellitus and the Heart**

#### **2.1 Diabetes Mellitus**

Diabetes mellitus (DM) is a chronic metabolic disease characterized by defects in insulin secretion, insulin function or both. Insulin deficiency alters the metabolism of carbohydrate, fat and proteins and eventually develops hyperglycemia (1). DM has been reported to be the most common endocrine disorder at present, and appears in epidemic proportions in United States (2). This disease has become a major public health problem because with time, it manifests damage, dysfunction and failure of various organs and consequently gives rise to life threatening complications. These severe complications include cardiovascular diseases, peripheral arterial disease, cerebrovascular diseases, neuropathy, nephropathy, retinopathy and ulceration (1).

##### **2.1.1 Prevalence and pathophysiology of Diabetes Mellitus**

According to the American Diabetes Association (ADA), 25.8 million adults and children (8.3% of population) are diagnosed with diabetes in 2011. Also ADA claims that approximately 7 million Americans are undiagnosed with diabetes and unaware of their disease. The total cost of diagnosed diabetes in 2013 was \$245 billion, indicating that DM has become a major public health burden in the country.



DM is categorized into various types, but the two most prevalent types are Type 1 DM and Type 2 DM.

Type 1 DM, which accounts for 5-10% of diagnosed cases of diabetes, results from cell-mediated autoimmune destruction of pancreatic  $\beta$ -cells. This leads to absolute insulin deficiency in the body, requiring the patient to depend essentially on exogenous insulin for survival. Commonly, Type 1 DM appears in young population, however it can manifest in adult population as well (3). The genetic and environmental factors that lead to  $\beta$ -cell destruction by an autoimmune response are not fully understood. However, Type 1 DM has been shown to be polygenetic trait in mice. The major locus that is responsible for the disease manifestation encodes for major histocompatibility complex, and other 9 loci which contribute for the disease development were also identified (4). Further, it was estimated that approximate annual increase in the risk for developing Type 1 DM in developed nations is 6%, which is postulated to be an outcome of environmental triggers. Some reports validate increasingly common presence of insulin resistance with Type 1 DM, which may be attributed to the higher incidences of obesity in developed countries. Also, administration of high doses of exogenous insulin may contribute to the development of insulin resistance in Type 1 DM as well (5).

Type 2 DM is the most prevalent type of diabetes and accounts for 90-95% cases of all those diagnosed with diabetes. Previously, this was referred to as adult onset-diabetes as majority of the population who are diagnosed with the disease were adults. One of the underlying causes for Type 2 DM is peripheral insulin resistance, which may lead to compensatory hypersecretion of insulin from pancreatic islets causing a decline in function of the cells. The tissues most prominently express insulin resistance are skeletal muscles, adipose tissues and liver due to their high dependency of insulin to uptake glucose. The incidences of Type 2 DM is rapidly

increasing, especially in developing countries due to lifestyle modifications, changes in dietary habits and lack of exercise. Most of the Type 2 diabetics are obese, and obesity itself is a causative factor for the development of insulin resistance. How increased dietary calorie intake, reduced physical activity and obesity leads to development of Type 2 DM is not fully elucidated. Further, Type 2 DM is associated with a strong genetic disposition, even though the genetics are not clearly defined (1, 6, 7).

### **2.1.2 Complications associated with diabetes**

Diabetes is a chronic disease and with time, leads to development of myriad of complications resulting in end organ dysfunction and failure. Nevertheless, diabetes can induce acute metabolic complications such as diabetic ketoacidosis as result of extreme hyperglycemia or coma due to hypoglycemia.

The chronic complications of diabetes occur due to the damage to vasculature. These complications are categorized in to microvascular (damage to small blood vessels) and macrovascular complications (damage due to arteries). These complications account for high morbidity and mortality rates in the population (Figure 2.1).

The microvascular complications include:

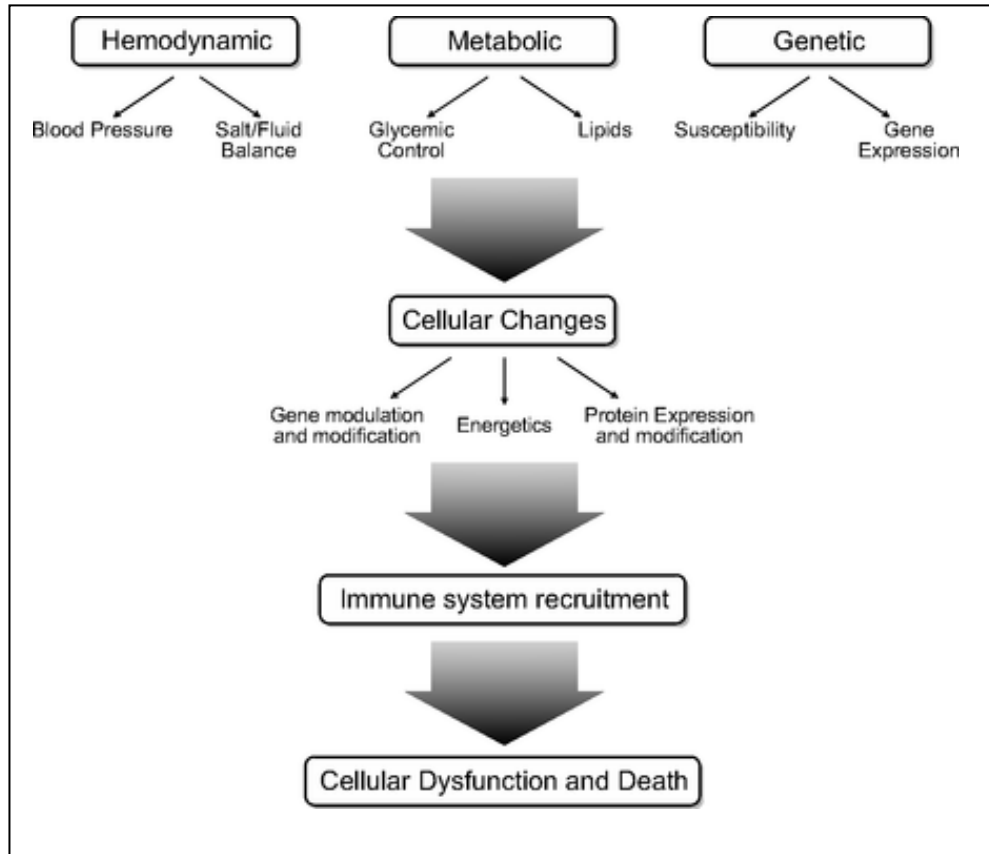
- Retinopathy – damage to the eye. Chronic diabetes leads to development of lesions in retina, leading to blindness.

- Nephropathy – damage to the kidney. This is characterized by proteinuria and decreased glomerular filtration rate which progress over a long period of time. If untreated, leads to end stage renal failure.
- Neuropathy – damage to neurons. Diabetes can damage both peripheral somatic and autonomic neurons. Majority of diabetics eventually develop neuropathy, which may result in amputations in extremities.

The macrovascular complications of diabetes are:

- Coronary artery disease (CAD) – results in myocardial infarctions which may lead to heart failure and death.
- Cerebral artery disease – manifests strokes due to atherosclerosis in the cerebral artery
- Peripheral vascular disease – results from atherosclerosis in the arteries which supply blood to extremities.

Maintaining the optimum glycemic levels during the course of disease is the most effective way to mitigate the development of vascular complications.



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**Figure 2.1 Major areas which contribute to diabetic complications**

The chronic hyperglycemia induces hemodynamic, metabolic and genetic alterations in the body leading to disruption of homeostasis and triggers cellular damage. This leads to inflammation and subsequent cellular dysfunction and death.

### 2.1.3 Diabetic cardiomyopathy

According to the statistics published by ADA, heart diseases were noted on 68% of diabetes related death certificates among population aged 65 years or older. Many of these deaths due to cardiovascular complications have been attributed to CAD. However, it was found that diabetics suffer from additional cardiac insults termed as “diabetic cardiomyopathy”.

Diabetic cardiomyopathy was initially described by *Rubler et al*, and since then it was identified as a distinct clinical entity by clinicians and epidemiologists. *Rubler et al* recognized diabetic cardiomyopathy as diabetes mediated structural and functional changes of the heart, manifested independent of other risk factors such as hypertension and CAD (8). This concept was further consolidated by the Framingham study, which demonstrated that increased incidences of congestive heart failure in diabetic males (2.1:1) and females (5:1), independent of age, hypertension, CAD and hyperlipidemia (9). Further, many studies also have confirmed the increased risk of development of heart failure and increased mortality from myocardial infarctions (MI) in diabetics (10, 11). Diabetic cardiomyopathy may have a long subclinical period before the patient develops symptoms.

#### **2.1.4 Diabetes associated changes in the cardiac architecture**

Structural alterations in the diabetic heart have been reported in humans and experimental diabetic animal models. One of the major histopathological determinant of diabetic cardiomyopathy is the presence of interstitial or perivascular fibrosis, or both. The extent of fibrosis correlates to the heart weight. Also it was observed as the diabetes progress, the cardiomyocyte loss is significantly increased and it is replaced with fibrosis (12, 13). The exact mechanism of myocardial fibrosis associated with diabetes is yet to be understood. However, it was observed that glycation of collagen fibers are increased in the diabetic hearts (14).

Left ventricular (LV) hypertrophy is also considered as a hallmark for diabetic cardiomyopathy. MRI scanning demonstrates that hyperglycemia and insulin resistance is

strongly associated with increased LV mass and LV end diastolic volume regardless of the gender (15). Many signaling cascades have been reported to be altered in the diabetic cardiomyopathy, which enable the manifestation of LV hypertrophy. Many reports support the fact that certain cytokines secreted by the adipose tissue in obese may confer for the LV hypertrophy development. Leptin is such cytokine which develops cardiac hypertrophy in obese and directly induced hypertrophy in cultured cardiomyocytes (16). Further, resistin which is also an adipokine was found to induce hypertrophy in cardiomyocytes via MAPK and IRS-1 signaling pathway (17). Also, insulin resistance and hyperinsulinemia are associated directly correlates to LV hypertrophy (18). Increased LV mass directly contributes for the development of heart failure.

### **2.1.5 Functional changes in the diabetic heart**

There are many reports which suggest the development of dysfunctions in the in the heart in the course of diabetes.

Diabetic cardiomyopathy is characterized by diastolic dysfunction, which precedes the systolic dysfunction in humans and transgenic diabetic animal models. LV diastolic dysfunction was observed in 47-75% of asymptomatic normotensive Type 2 – diabetics with well controlled glycemic levels (19). Diastolic dysfunction directly correlates to the extent of triglyceride accumulation in cardiomyocytes in ob/ob transgenic diabetic mice. Further, these mice demonstrated impaired  $\text{Ca}^{2+}$  uptake and associated contractile dysfunction (20). Therefore, cardiac lipotoxicity and altered  $\text{Ca}^{2+}$  homeostasis have been suggested as possible mechanisms

responsible for the development of diastolic dysfunction associated with diabetes. Interestingly, diastolic dysfunction was detected in diabetic hearts in the absence of LV hypertrophy. This observation suggests that LV hypertrophy is not a prerequisite for the development of diastolic dysfunction in the diabetic heart (21).

In the presence of diastolic dysfunction, early diastolic LV filling is also reduced leading to prolonged relaxation of the myocardium. In diabetic transgenic animal hearts, the papillary muscles demonstrate a delayed relaxation pattern and reduced relaxation velocity. Further studies implicate that diastolic dysfunction may give rise to decoupling of the contractile apparatus in the absence of concomitant chamber wall stiffness (22).

LV systolic dysfunction is also observed in diabetic subjects, which usually after occurring of the diastolic dysfunction. However, some diabetic have normal LV systolic function under resting conditions, but may show abnormalities in the systole during exercise (23). Further, it was observed that diabetics have lesser cardiac output due to reduced stroke volume during supine exercise and this was independent of the duration of diabetes (24).

### **2.1.6 Cellular mechanisms that predispose the heart to diabetic cardiomyopathy**

#### **➤ Metabolic disturbances**

The heart requires a large supply of ATP to maintain its continuous mechanical work load. The main source of carbon substrate that is used to supply about 70% of this massive energy demand in normal adult healthy heart is fatty acids. Generally, glucose oxidation

accounts for about 30% of the total ATP produced by cardiomyocytes. Nevertheless, cardiomyocytes shows extensive metabolic flexibility by its ability to generate ATP from other substrates such as amino acids, ketones and lactate (25). This normal pattern of substrate utilization is altered in the diabetic heart, which leads to development of diabetic cardiomyopathy.

Multiple mechanisms are responsible for the disturbances in the substrate supply and utilization in the diabetic heart. The major metabolic derangement that occurs in the diabetic heart is increased utilization and supply of fatty acids in to cardiomyocytes, which is also associated with defective glycolysis and glucose oxidation.

The elevated levels of fatty acid uptake and oxidation are very well demonstrated in diabetic individuals and transgenic animal models (26, 27). Diabetes is characterized by elevated levels of circulating free fatty acid (FFA) and triglycerides (TG). Therefore, the changes that contribute to substrate utilization are due to increased delivery of FFA and TG to the myocardium. Despite the increased FA oxidation, the FA uptake exceeds the oxidation rates resulting in lipid accumulation leading to lipotoxicity in the heart (28, 29). The lipid intermediates such as ceramides that accumulate in the heart promote apoptosis of cardiomyocytes (30).

The decreased glucose utilization in the heart is observed in both diabetic animal models and humans. One of the factors that contribute to restricted glucose oxidation is the allosteric inhibition of glucose use in the presence of increased FA utilization (Randle cycle) (31). Another mechanism that suppress the glucose oxidation is slow rate of glucose uptake across the sarcolemma of the myocardium, which is attributed to the depleted levels of GLUT (glucose



transporter) 1 and 4 (32). Further, increased FFA uptake to cardiomyocytes exerts an inhibition on the pyruvate dehydrogenase complex, which also leads to reduced glucose oxidation (33).

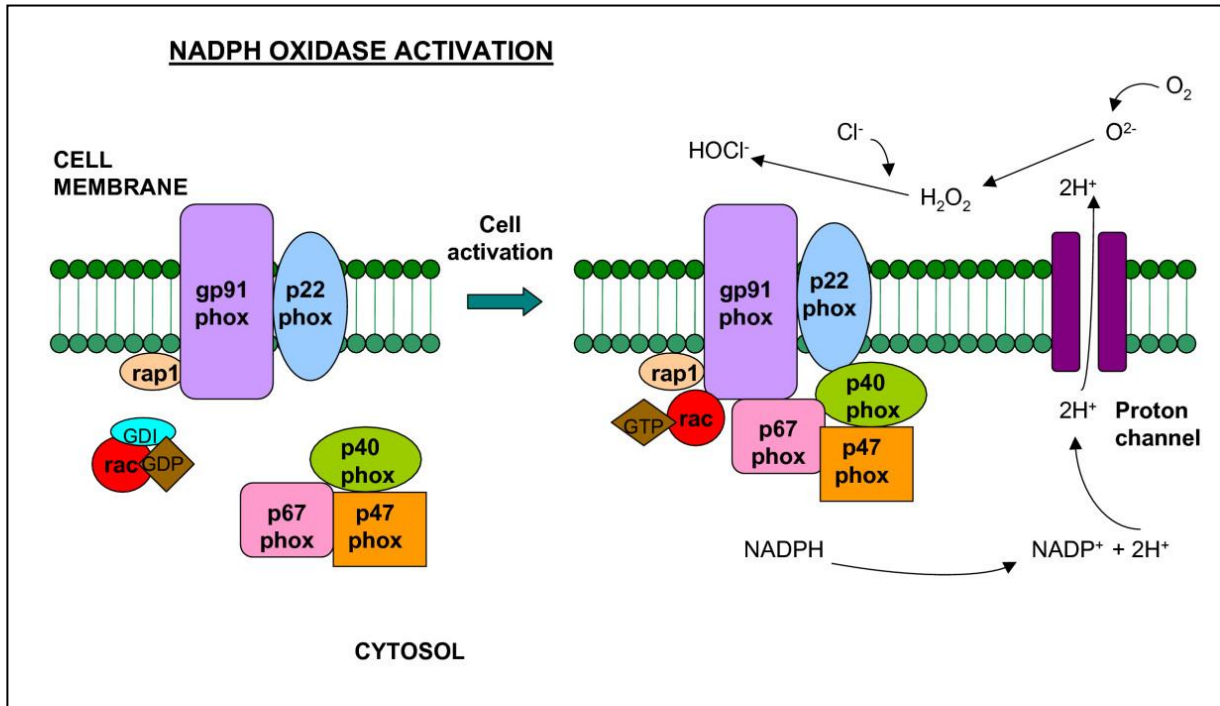
It is evident that these alterations of the substrate metabolism that occur in the diabetic heart leads to contractile dysfunction. It was observed that the rate of glycolysis from exogenous glucose is at 48% in the diabetic transgenic hearts compared to control and glucose oxidation was reduced by 16%. Over-expression of the GLUT4 in these the diabetic transgenic hearts normalize the contraction and improve the cardiac metabolism (34).

➤ Oxidative stress

It is well established that diabetic hearts have increased ROS production and undergo extensive oxidative stress in both human and animal models. Further, many published reports confirm the correlation between increased oxidative stress and the development of diabetic cardiomyopathy. Despite the definitive mechanism for increased ROS production in the diabetic heart is not fully understood, it is suggested that increased FA delivery to the cardiomyocytes may contribute to the oxidative stress. It is believed that increased FA levels in the myocytes may lead to decoupling of mitochondria, resulting in increased ROS production (35).

Even though mitochondria are responsible for producing majority of the total ROS produced in the cell, it is found that enzymatic systems that are present in the cells also contribute for the generation of ROS. NADPH (Nicotinamide Adenine Dinucleotide Phosphate) oxidase is one such enzymatic system which is capable of producing ROS under cellular stresses. NADPH oxidase consists of cluster of proteins and donates a single electron to molecular oxygen

(O<sub>2</sub>) to generate superoxide (O<sub>2</sub><sup>-</sup>). This reaction initiates an immune response against bacterial and fungal pathogens.



<http://www.medimmunol.com/content/5/1/4/figure/F1>

**Figure 2.2: NADPH oxidase enzymatic complex**

The membrane components of the enzymatic complex contain two subunits: p22phox and gp91phox which respectively produce the smaller and larger chain of the cytochrome-b<sub>558</sub>. Two cytosolic subunits: p67phox and p47phox; a p40phox accessory protein and a Rac-GTP binding protein then translocate to the cell membrane upon cell activation to form the NADPH oxidase complex which generates a respiratory burst. Superoxide can react to form hydrogen peroxide which participates in bacterial killing.

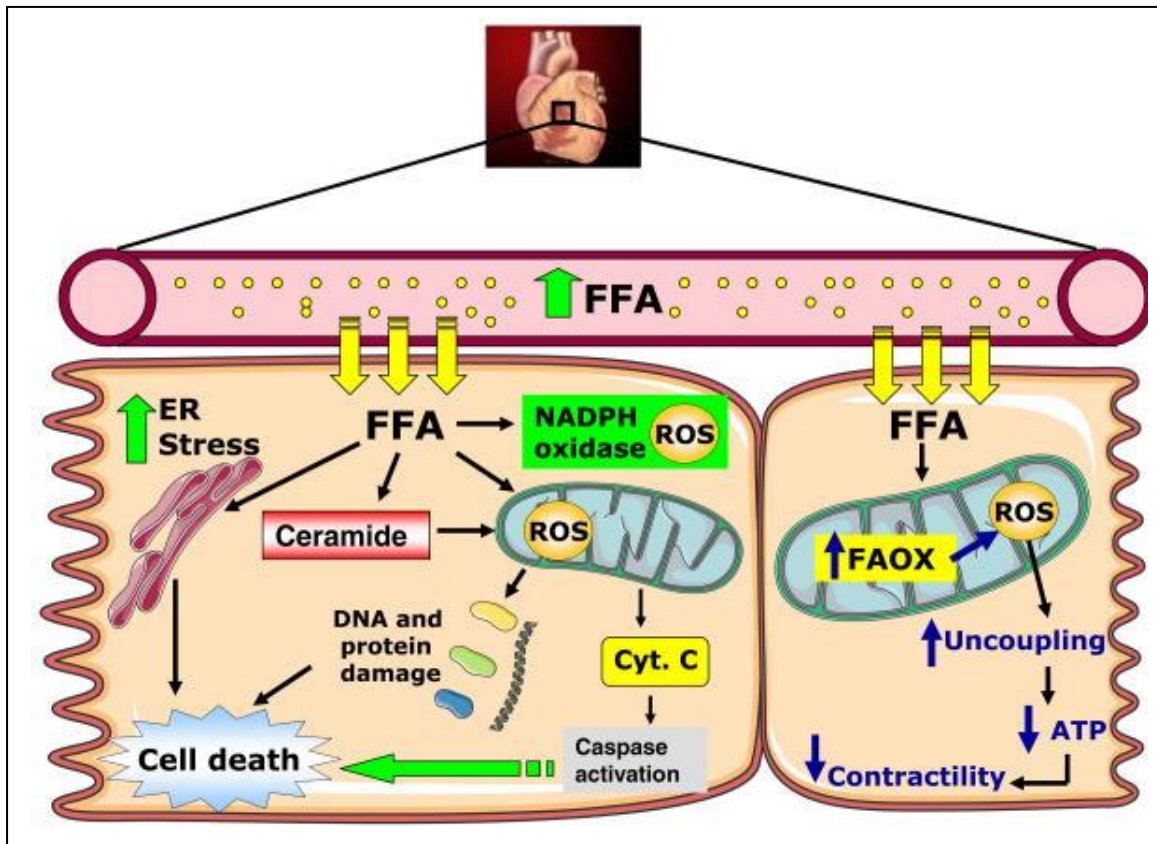
In the inactive state, a portion of this enzymatic complex remains bound to the membrane, while other components remain in soluble form in the cytosol. The membrane bound

section consists of gp91<sup>phox</sup>, which is a large glycosylated protein and a smaller adapter protein called p22<sup>phox</sup>. This p22<sup>phox</sup> protein has SH3 binding domains which offer sites for linkage for soluble components to bind when the complex is active (Figure 2.2).

The soluble components of the complex are comprised of p47<sup>phox</sup>, p67<sup>phox</sup> and p40<sup>phox</sup>. When the cell is stimulated p47<sup>phox</sup> subunit undergoes phosphorylation on several residues via PKC pathway, resulting in translocation of the soluble components to the bound complexes on the membrane. However, it was observed that several other kinases such as Akt, MAPK and c-Src are also capable of phosphorylating p47<sup>phox</sup> and promote the translocation of the soluble components to activate the enzymatic complex (36).

Despite exerting initial immune response, NADPH oxidase complex was found to be modulated in pathological conditions such as diabetes (37, 38). It was observed that factors such as angiotensin II (AT II), thrombin and TNF- $\alpha$  play a significant role in stimulating NADPH oxidase complex (39, 40). Further, elevated levels of G6PD (Glucose -6- phosphate dehydrogenase) found in the diabetic myocardium significantly induce NADPH oxidase mediated generation of O<sub>2</sub><sup>-</sup> (37).

Additionally, excess ROS also can react with other molecules such as nitric oxide to produce free radicals such as nitrotyrosine species in the diabetic myocardium (41). Also, it was observed that excess ROS is capable of altering the gene expression. Increased ROS in the diabetic myocardium switches the cardiac  $\alpha$ -MHC (Myosine Heavy Chain) expression to  $\beta$ -MHC, suggesting that the cardiomyocytes advent fetal metabolic reactions leading to cardiomyopathy. This transition of gene expression is mediated by ROS induced NF $\kappa$ B and antioxidant treatment was able to prevent this switch (42).



*Rev Endocr Metab Disord. 2010 Mar;11(1):31-9. doi: 10.1007/s11154-010-9131-7*

**Figure 2.3: Role of increased free FA in ROS production in the diabetic myocardium**

Increased FA uptake in cardiomyocytes *in vivo* precipitates cardiomyocyte dysfunction by multiple mechanisms including increased mitochondrial and cytosolic ROS generation and ER stress. FA-mediated ROS generation leads to uncoupling of mitochondria, which reduces mitochondrial ATP production. FFA: free fatty acids; ROS: reactive oxygen species; ER: endoplasmic reticulum; Cyt. C: cytochrome c; FAOX: Fatty acid oxidation.

➤ Mitochondrial dysfunction

Many published reports verify the contribution of mitochondrial dysfunction to the pathogenesis of diabetic cardiomyopathy. Diabetes is known to mediate structural and functional alterations in the mitochondria (43). Further, mitochondria in the diabetic myocardium have

reduced oxidative capacities and attenuated levels of protein expression of the oxidative phosphorylation components (44). These alterations can significantly impact the cardiac contractility because of reduced availability of ATP.

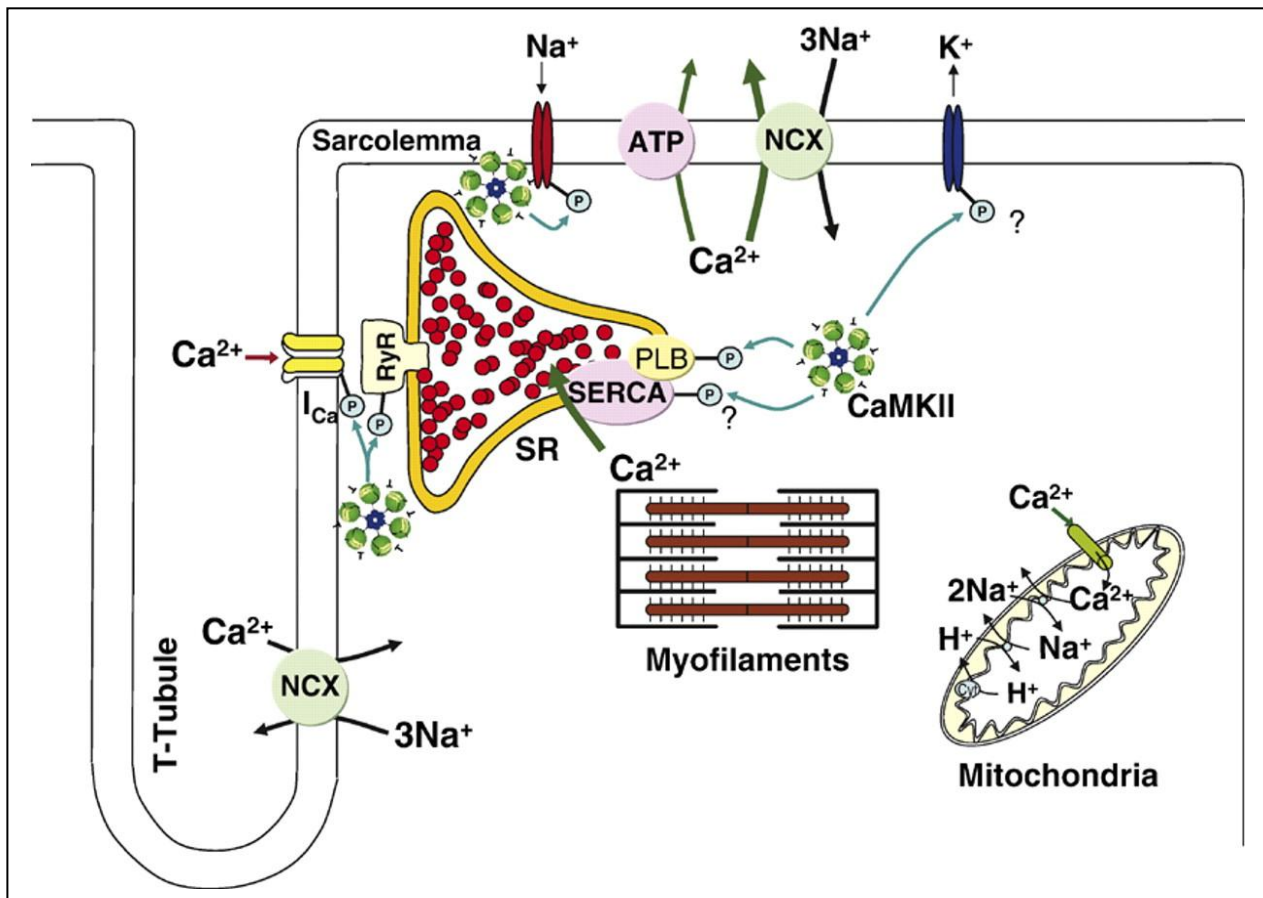
In the diabetic heart, the genes which regulate FA oxidation are upregulated. However, it was found that genes that regulate the components of the oxidative phosphorylation are suppressed in the diabetic myocardium. Therefore, the rate of oxidative phosphorylation in the mitochondria is unable to meet the rate of FA delivery, which leads to increased ROS production, which in turn uncouples the electron transport chain and suppress the production of ATP (44, 45) (Figure 2.3).

Further, it was observed that glutathione, which is capable of inducing antioxidant effects is reduced in the diabetic heart. Also, it was observed that hydrogen peroxide production is elevated in these hearts, and rotenone (a mitochondrial complex III inhibitor) can significantly ameliorate this effect. This indicates that diabetes mediated oxidative stress may alter the function of mitochondrial proteins, leading to mitochondrial dysfunction (46).

#### ➤ Impaired $\text{Ca}^{2+}$ homeostasis

Cardiac contractility is regulated by synchronization of many ion channels located in the sarcolemma of cardiomyocytes.  $\text{Ca}^{2+}$  channels and intracellular  $\text{Ca}^{2+}$  levels play a major role in cardiac contractility.  $\text{Ca}^{2+}$  influx through voltage dependent L-type  $\text{Ca}^{2+}$  channels induce membrane depolarization which stimulate the release of  $\text{Ca}^{2+}$  stored in the sarcoplasmic reticulum via ryanodine receptors. This phenomenon is called  $\text{Ca}^{2+}$  induced  $\text{Ca}^{2+}$  release. The

released  $\text{Ca}^{2+}$  then binds with troponin C which overcomes the inhibition exerted by troponin I on the contractile apparatus of the cardiomyocytes.  $\text{Ca}^{2+}$  binding to troponin C triggers sliding of thin filaments made of actin over thick myosine filaments resulting in cardiomyocyte contraction. The relaxation of cardiomyocytes are induced by  $\text{Ca}^{2+}$  reuptake by SERCA2a (sarcoplasmic reticulum  $\text{Ca}^{2+}$  pump) in to the sarcoplasmic reticulum. Further, sarcolemmal  $\text{Na}^+$  -  $\text{Ca}^{2+}$  exchanger and sarcolemmal  $\text{Ca}^{2+}$  ATP<sub>ase</sub> also play a role by promoting efflux of  $\text{Ca}^{2+}$  from the cytoplasm to extra cellular matrix (47) (Figure 2.4).



*Cardiovasc Res. 2007 Mar 1;73(4):631-40. Epub 2006 Nov 10.*

**Figure 2.4: Role of  $\text{Ca}^{2+}$  in cardiac contractility**

**The Ca<sup>2+</sup> influx through the Ca<sup>2+</sup> channels located in T-tubules, lead to Ca<sup>2+</sup> induced Ca<sup>2+</sup> release from sarcoplasmic reticulum. The released Ca<sup>2+</sup> will bind to the troponin and remove the inhibition on the contractile apparatus and promote contraction. The relaxation of the cardiomyocytes is induced by the Ca<sup>2+</sup> uptake by SERCA.**

It is well established that the diabetes mediated oxidative stress and abnormalities in the metabolism induce subcellular remodeling which exert adverse effects on Ca<sup>2+</sup> homeostasis. Diabetes has been found to reduce the activity of ATP<sub>ases</sub>, reduce the Ca<sup>2+</sup> uptake to the sarcoplasmic reticulum and reduce the activities of Na<sup>+</sup> - Ca<sup>2+</sup> exchanger and sarcolemmal Ca<sup>2+</sup> ATP<sub>ase</sub> (48-50). However, changes in the activity of L-type Ca<sup>2+</sup> channels were not observed in the diabetic myocardium. The reduced Ca<sup>2+</sup> uptake to the sarcoplasmic reticulum in the diabetic myocardium is attributed to decreased SERCA2a and ryanodine receptor protein levels. Also it was observed that diabetes increases total and nonphosphorylated phospholamban levels in the sarcoplasmic reticulum which suppress the function of SERCA2a (51). Suppression of Ca<sup>2+</sup> uptake in to the sarcoplasmic reticulum increases free Ca<sup>2+</sup> levels in the cytosol, resulting in desensitization of the myofilaments to Ca<sup>2+</sup> and suppresses the activity of the contractile apparatus (52). This may significantly affect the diastolic and systolic function of the heart and induce contractile abnormalities in the heart.

➤ Renin Angiotensin system (RAS)

The RAS in diabetic individuals and animal models is overly activated, and is found to play a significant role in manifesting diabetic cardiomyopathy (53). Traditionally RAS was

identified as a part of the endocrine system, which regulates the homeostasis of the salt and water. Therefore, RAS regulates the blood volume and also vascular resistance, which determine the cardiac output and vascular resistance, and significantly impact blood pressure.

Renin is the initiator of the RAS. It is secreted as a prohormone from juxtaglomerular apparatus and is converted to active renin in response to variety of stimuli. Once active renin is released in to blood, it converts circulating angiotensinogen, which is produced from the liver to angiotensin I. Then the enzyme angiotensin converting enzyme (ACE) cleaves angiotensin I to angiotensin II (AT II). ATII exerts its biological effects via two cell surface receptors named AT<sub>1</sub> and AT<sub>2</sub>, which are seven transmembrane G- protein coupled receptors. AT<sub>1</sub> is predominantly expressed in adult tissues while AT<sub>2</sub> is prominent in the fetal tissues (Figure 2.5).

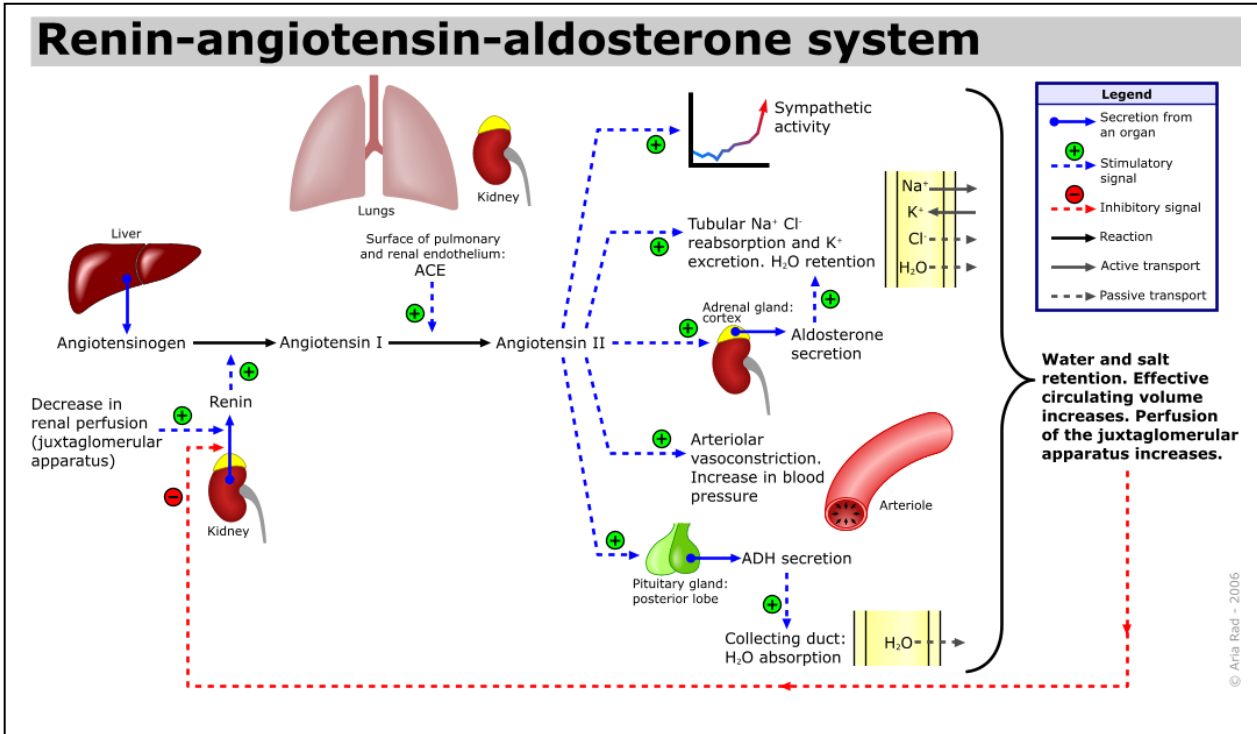
In addition to haemodynamic functions, RAS has been found to exert variety of biological effects on the tissues, such as inducing protein synthesis, proliferation and cellular hypertrophy thus influencing organ structure and function. RAS has been also implicated in manifesting insulin resistance, inflammation, producing ROS and also endothelial dysfunction. Also, RAS has been found to trigger cardiomyocyte hypertrophy and fibroblast proliferation, leading to LV hypertrophy, diastolic and systolic dysfunction, arrhythmias and cardiac failure (54).

It was observed that the AT II receptor gene and protein expression is elevated in the diabetic myocardium (55). Further, it was also reported that pharmacological blockage of RAS in the diabetic animals attenuated the cardiac dysfunction (56).

Other than the systemic RAS system, it was found that there are tissue bound RAS as well. Many reports emerged supporting the presence of a functional tissue RAS (tRAS) in the heart which elicits cardiac hypertrophy (57, 58). The presence of RAS components independent



of systemic RAS is established. The tRAS in the heart is capable of exerting mitogenic and apoptotic effects in the heart.



[http://commons.wikimedia.org/wiki/File:Renin-angiotensin-aldosterone\\_system.png](http://commons.wikimedia.org/wiki/File:Renin-angiotensin-aldosterone_system.png)

**Figure 2.5: Renin Angiotensin System (RAS)**

Angiotensinogen released from the liver is converted to angiotensin I and subsequently to angiotensin II via ACE. Angiotensin II is a potent peptide and mediates many responses that would promote water and salt retention. This would increase the blood volume and as a result, blood pressure.

Other than the systemic RAS system, it was found that there are tissue-bound RAS as well. Many reports emerged supporting the presence of a functional tissue RAS (tRAS) in the heart which elicits cardiac hypertrophy (57, 58). The presence of RAS components independent

of systemic RAS in the heart is established. The tRAS in the heart is capable of exerting mitogenic and apoptotic effects in the heart.

Many reports recently emerged confirming the overexpressed RAS system in diabetics. Some of these reports claim that the RAS over-activity may play a role in manifestation of complications associated with diabetes. Furthermore, large scale clinical trials have demonstrated that pharmacological inhibition of RAS can attenuate the development of these complications (59). Interestingly, work by *Singh et al* confirms the over activation of the tRAS system in the diabetic rat heart. Activation of tRAS in the heart has been found to increase the oxidative stress and cardiac fibrosis (60).

## **2.2 Diabetic cardiomyopathy and arrhythmias**

### **2.2.1 Cardiac arrhythmias**

The heart coordinates the atria and ventricles to act as a pump, and supply the blood throughout the body. Therefore, it is very crucial that the normal mechanical activity is maintained by modulating the electrical impulses in the heart. These electric impulses that regulate the heart function are generated intrinsically, however the autonomic nervous system also play a role in the regulation.

There are specialized cells in the heart, known as pacemakers which spontaneously generate the electrical impulses and coordinate the contractions between the atria and ventricles. The ability of generating a spontaneous electrical impulse by pacemakers is known as

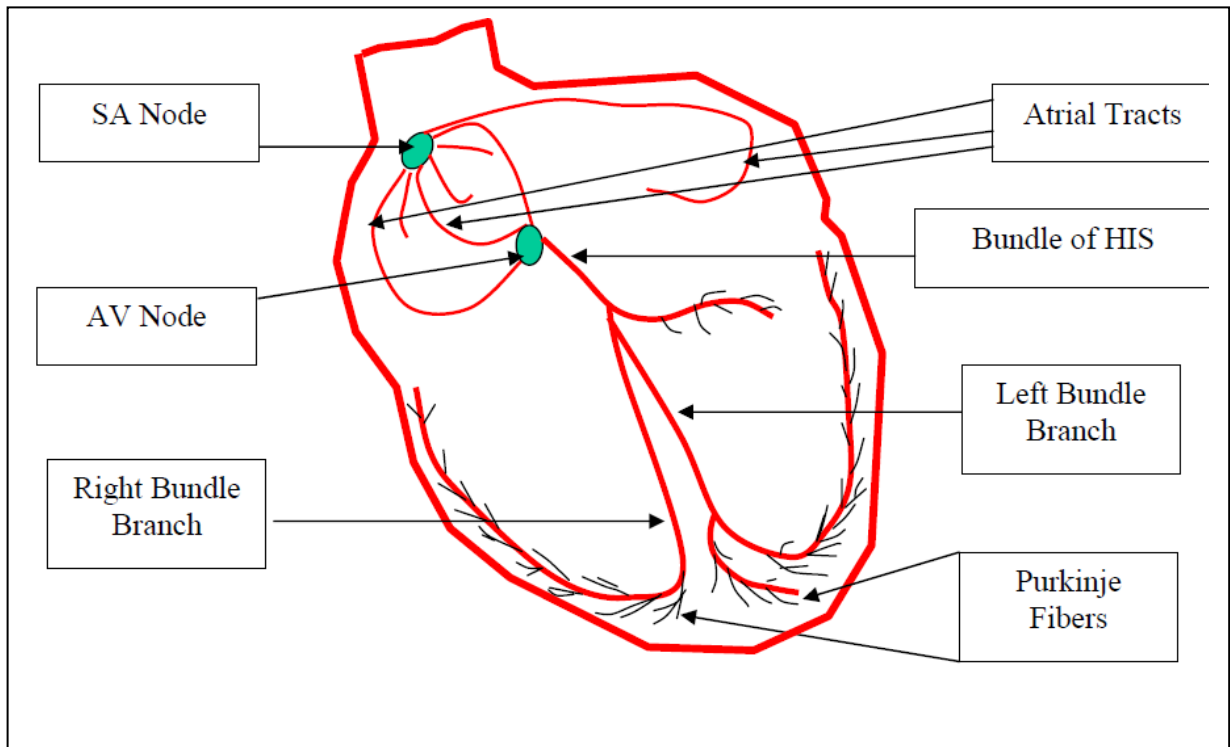
automaticity. The regions that are capable of inducing automaticity in the heart are SA node (sinoatrial), AV (atrio ventricular) node, bundle of His and Purkinje fiber network. SA node is the dominant pacemaker and suppresses the latent pacemaker tissues in the other regions of the heart. The signals generated by the SA node stimulate the atria to contract and travel to the AV node. Once the signal reaches the AV node, there is a delay and then the stimulus is transmitted through the bundle of His to Purkinje fibers to trigger the ventricular contraction (Figure 2.6). Despite the electrical properties of the heart is regulated diligently, under certain pathological conditions and circumstances this rhythm can fail, which gives rise to development of cardiac arrhythmias.

Cardiac arrhythmias are a group of conditions in which the electrical activity of the heart becomes irregular or faster or slower than the normal. Some of cardiac arrhythmias can be life threatening and manifested from many acquired and inherited diseases in the cardiovascular system. Abnormal electrophysiological properties that occur due to alterations in the cell surface ion channels and cell – cell communication induce arrhythmogenesis.

The cardiac arrhythmias that occur due to slow heartbeat are called bradyarrhythmias or bradycardia. The major complication that arises with bradycardia is the inability to maintain sufficient blood pressure to the brain. Therefore bradycardia can induce sudden loss of consciousness. Generally, bradycardia is manifested by death or disease in the pacemakers and the other cells specialized in conducting the electrochemical signals in the heart. Bradycardias can be effectively treated by placing artificial electronic pacemakers.

The arrhythmias that are induced by faster than normal heart beat are called tachyarrhythmias or tachycardia. These types of arrhythmias also fail to maintain blood pressure effectively and may lead to syncope. Some of the tachycardia types that arise in the ventricles are

the most dangerous, as it can cause sudden death. These tachycardia are known as ventricular tachycardia, tachycardia torsades de pointes ventricular fibrillation (61).



<http://www.cardiacnursing.ir/conducting%20system.htm>

**Figure 2.6: Electrical conduction system in the heart**

**The cardiac pacemakers spontaneously initiate the electrical conductance and coordinate the contraction of atrial and ventricular cardiomyocytes.**

### **2.2.2 Increased risk of development of arrhythmias with diabetes**

It is evident that diabetic cardiomyopathy increases the incidences of cardiac arrhythmias, including ventricular fibrillation and sudden death (62, 63). The risk factors that can induce

arrhythmias in diabetic heart are attributed to silent ischemia, slowed conduction, imbalance in autonomic nervous system, heterogeneous repolarization of the atria and ventricles and the myocardial damage and scar formation.

Diabetic individuals are highly susceptible for development of neuropathy. Therefore, the risk of autonomic nerves that innervate the heart and the blood vessels being damaged are high in the diabetics. It was found that 20% of the asymptomatic diabetics have abnormalities in the cardiac autonomic functions (64). Further, the defects in the baroreflex response and vascular stiffness induced by diabetes may aggravate the cardiac autonomic dysfunction (65). The Hoorn study implicate that the individuals with depressed autonomic function in the heart have approximately two fold increased mortality rates (66). Further, these patients are highly susceptible for ischemia because it causes an imbalance between the myocardial supply and demand.

Many studies have confirmed the increased risk for occurrence of atrial fibrillation in diabetic patients. This is more likely due to fibrosis in the atria, which delay the intra atrial conduction. Also, it was proved that increased sympathetic innervations in the diabetic heart promote the development of atrial fibrillation as well (67, 68). Some studies report that the risk of atrial fibrillation in diabetic heart increases with the existence of other cardiovascular pathologies such as hypertension, ischemia and atherosclerosis (69, 70). Further, the same studies claim that diabetes independently does not increase the development of atrial fibrillation. Nevertheless, the exact mechanism how diabetes elevate the risk of developing atrial fibrillation is not fully elucidated.

There is a positive association between diabetes and ventricular fibrillation leading to sudden cardiac death. Metabolic changes, electrolyte abnormalities, changes in the ion channels,

acute pressure overload are identified as causative factors for development of ventricular arrhythmias. Additionally, unequal ventricular repolarization, myocardial damage and scar formation in the diabetic heart also contribute to development of ventricular arrhythmias (71). It was identified that glucose intolerance, even at pre-diabetic stage predisposes the heart to variety of electrophysiological abnormalities which may compromise survival by increasing the risk of sudden cardiac death (72).

### **2.2.3 Electrocardiogram (ECG) of diabetics**

ECG is the most common and simplest test that is available to detect arrhythmias by measuring the heart's electrical activity over a period of time. The normal pattern of an ECG is shown below. The ECG wave illustrates the depolarization and repolarization that occurs during a cardiac cycle. The prominent peaks in ECG depict distinct stages of the cardiac cycle (Figure 2.7).

The baseline/ isoelectric line: indicates the resting membrane potential

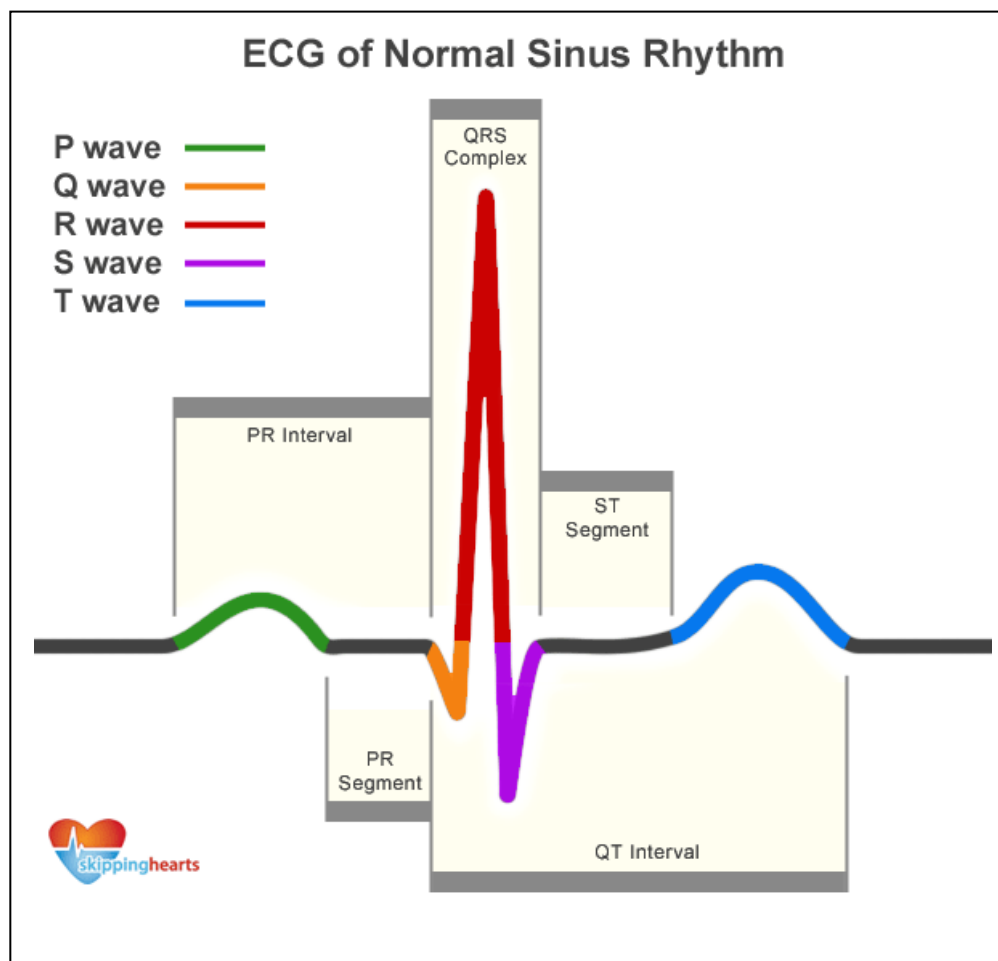
P wave: Atrial depolarization initiated by the SA node

QRS complex: Ventricular depolarization which precedes ventricular contraction

T wave: Ventricular repolarization

As mentioned above, the electrical impulse of a cardiac cycle is initiated by SA node. It is the predominant pacemaker in the heart because it has faster rate of spontaneous firing compared

to rest of the specialized tissues. However, in circumstances where the activity of SA node is reduced, other components of the electric conductive system can gain control (Figure 2.8).

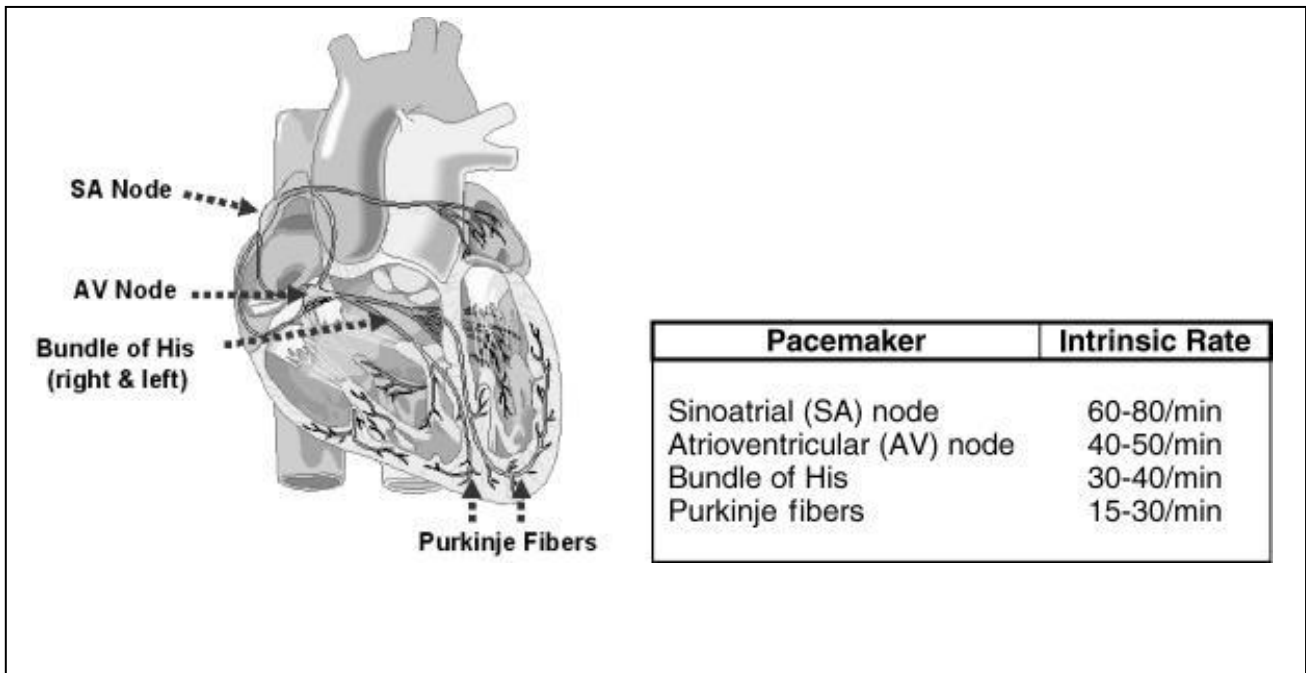


<http://research.vet.upenn.edu/tabid/4930/Default.aspx>

**Figure 2.7: Schematic representation of a normal ECG**

ECG contains prominent peaks which depicts distinct phase of the cardiac cycle. The alterations of the peaks and the duration of segments indicate cardiac arrhythmias.

The electrophysiological abnormalities that lead to these life threatening arrhythmias are evident by the electrocardiogram (ECG) of the diabetics (62). The ECG of diabetics show alterations in normal patterns, majority affecting the repolarization such as prolonged QT interval, QT dispersion and T wave lengthening (62, 73). QT interval is the measurement that is taken at the beginning of the QRS complex to the end of the T wave and illustrates ventricular depolarization and repolarization. However, clinically QT interval is recognized as an indicator of ventricular repolarization. Prolonged QT interval can induce QT dispersion, which leads to unequal repolarization within the ventricular cardiomyocytes setting up an electrical gradient which facilitates reentry circuits and develop arrhythmias. People with myocardial autonomic neuropathy also have prolonged QT interval.



*Anesth Prog. 2006 Summer;53(2):53-63; quiz 64*

**Figure 2.8: Conductive tissues and their normal firing rates**

The SA node is the primary pacemaker and has the fastest spontaneous firing rate compared to other cardiac pacemakers.



Interestingly, it was found that hyperinsulinemia mediated hypoglycemia in healthy individuals is capable of prolonging the QT interval and depress the T wave in ECG (74). Further, EURODIAB study implicate that increased HbA1c values are significantly increase the risk of developing a prolonged QT interval segment in the ECG (75). Therefore, these studies suggest that the glycemic levels have a profound effect on the electrical conduction system in the heart. Prolonged QT interval and depressed ST segment can be used as a predictor for mortality rates among type 2 diabetics (76).

Also, ECGs can assist to diagnose silent ischemia in diabetic patients. Usually, myocardial ischemia does not induce pain in patients with diabetes (77). Therefore, abnormalities in the resting ECG were found to be useful in diagnosing silent ischemia in asymptomatic diabetic patients (78).

During the early stages of diabetic cardiomyopathy, metabolic alterations and the diastolic dysfunction may be present even though the structural abnormalities may are absent. Therefore, it is suggested that the subtle changes seen in the ECG at this stage can be used to diagnose early stages of diabetic cardiomyopathy (21).

#### **2.2.4 ECG, action potential and ion currents**

ECG includes the total action potentials of all the cardiomyocytes in the atria and ventricles. The role of different ion channels involved in generating the cardiac action potential was identified in the attempts to understand the molecular basis of inherited cardiac arrhythmias. During this process, the genes encoding major ion channels were identified and sequenced. Also,

it was identified that these ion channels function as macromolecular complexes, which are assembled in a specific manner and specific sites in the membrane. Some arrhythmias are manifested due to gene mutations of these ion channels (79).

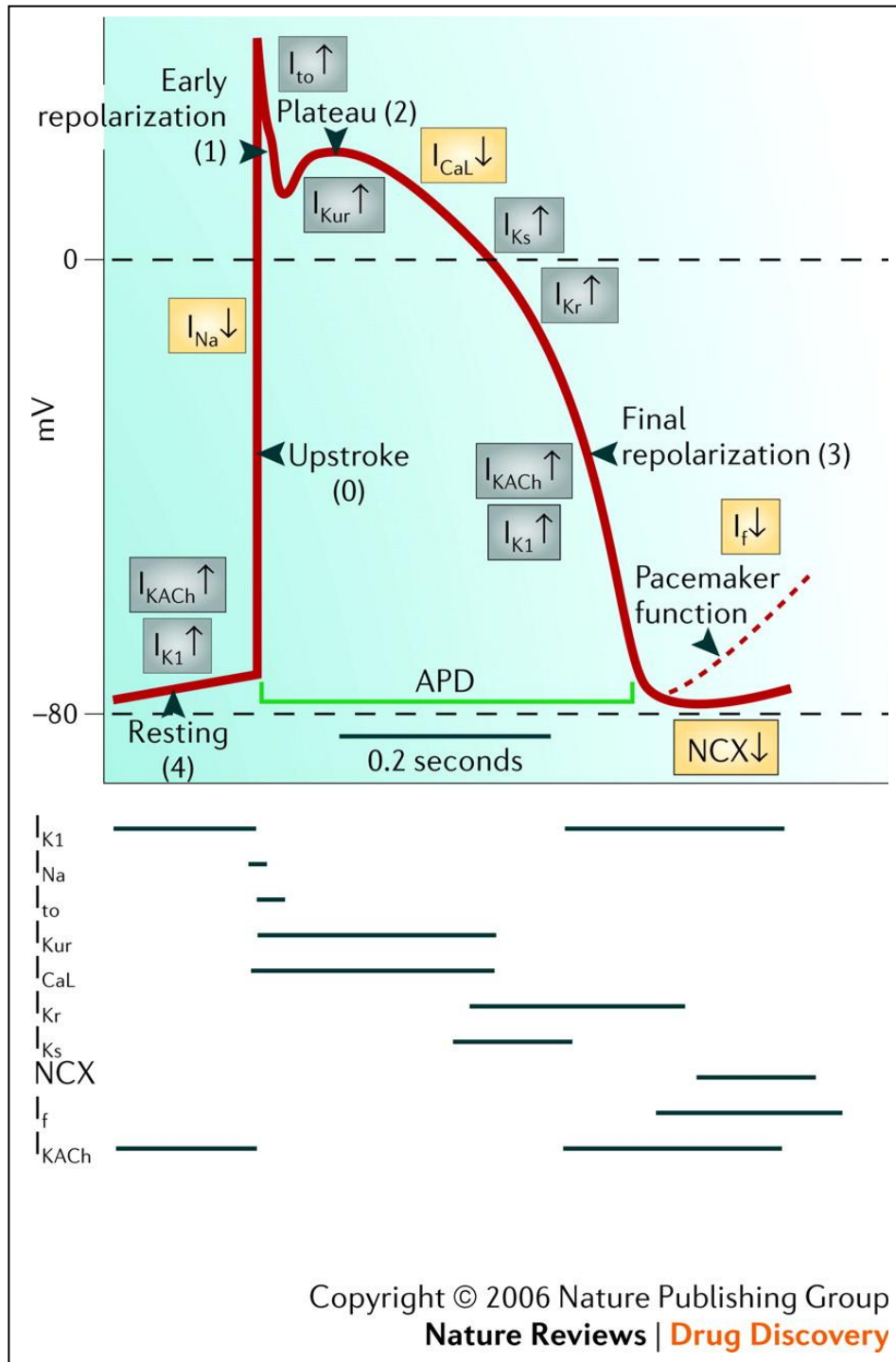
The cardiac action potential consists of depolarization and repolarization waves. These depolarization and repolarization waves of a single cardiomyocyte are mediated by  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{2+}$  currents. Membrane currents that are responsible for generating cardiac action potential are depicted in Figure 2.9. There are 5 phases during the propagation of a cardiac action potential (79).

- Phase 4: The resting action potential:

Approximately -90mV due to differences in the ionic concentration and conductance across the membrane. This phase illustrates the action potential when the cardiomyocyte is not being stimulated.

- Phase 0: Rapid depolarization

This current is produced by the fast  $\text{Na}^+$  current which results in large influx of  $\text{Na}^+$  in to the cardiomyocyte. This phase is reflected by the QRS complex of the ECG.



*Circ Arrhythm Electrophysiol.* 2009 Apr;2(2):185-94. Doi: 10.1161/CIRCEP.108.789081

**Figure 2.9: Cardiac action potential and the ion channels involved**

**Distinct ion channels regulate the currents of different phases of action potential**

- Phase 1: Rapid repolarization

This phase occurs with the inactivation of  $\text{Na}^+$  currents. The small downward deflection is due to activation of transient  $\text{K}^+$  currents which cause the  $\text{K}^+$  ions to leak outside the cells.

- Phase 2: Plateau phase

This is the longest phase of the action potential. This marks the phase of  $\text{Ca}^{2+}$  entry in to the cardiomyocyte and corresponds to the ST segment of the ECG.

- Phase 3: Rapid repolarization

This phase is mediated by outward potassium currents which bring back the membrane potential to the resting stage. Phase 3 correlates with the T wave in the ECG.

The action potential of the pacemakers is significantly different from the normal atrial and ventricular cardiomyocytes. The total action potentials of the cardiomyocytes in the ventricle are depicted by the QT interval in an ECG.

### **2.2.5 The ion channels and their properties**

Ion channels facilitate the movement of ions across lipophilic membranes with less activation energy. During the action potential, the permeability of the ion channels changes and

the ions move across the membrane according to an electro – chemical gradient. This electro – chemical gradient regulates the direction of ion movement. When the cations move in to the cell, it creates depolarizing currents, and the movement of cations out of the cell generates Repolarizing currents. The ion transporters maintain the cellular ion homeostasis at the expense of ATP hydrolysis.

The function of an ion channel is determined by ion permeation and gating. Ion permeation indicates the movement of ion through the channel. Ion channels are specific for a particular ion (eg:  $\text{Na}^+$  channels will allow permeation to  $\text{Na}^+$  ions). The selectivity for the ions are determined by the size, valency and the hydration energy (80).

Gating is based on the mechanisms involved in opening and closing of the ion channels. Based on the gating properties ion channels are divided in to two classes as follows.

- Voltage dependent ion channels – the most common method of channel opening. These channels change the conductance based on the membrane potential.
- Ligand dependent ion channels – Binding of a ligand is required to alter the conductance of the channel.

### **2.2.6 Sodium channels**

The cardiac sodium channels are voltage gated and comprised of  $\alpha$  and  $\beta$  subunits. The  $\alpha$  subunit of this channel is involved in generating the current while  $\beta$  subunit regulates the level of ion expression (81). These  $\text{Na}^+$  channels open less than 1ms during more than 99% of the depolarization wave. These channels play an important role during the phase 0 depolarization of

the cardiac action potential. Several inherited diseases are reported due to mutations in the cardiac Na<sup>+</sup> channel gene (SCN5A) (82).

### **2.2.7 Calcium channels**

Ca<sup>2+</sup> is the predominant ion involved in intracellular signaling. In the cardiomyocytes Ca<sup>2+</sup> plays a main role in excitation contraction coupling and also regulates the function of certain enzymes. Ca<sup>2+</sup> channels serves as the major gateway for the extracellular Ca<sup>2+</sup> to enter in to the cells. There are mainly two types of Ca<sup>2+</sup> channels in the cardiomyocytes.

- L-type (low threshold type) Ca<sup>2+</sup> channels
- T-type (transient) Ca<sup>2+</sup> channels

L-type Ca<sup>2+</sup> channels are present ubiquitously on all the types of cardiomyocytes. However, T-type Ca<sup>2+</sup> channels are found in pacemaker cells and also atrial cardiomyocytes. Generally, cardiac L-type Ca<sup>2+</sup> channel is comprised of  $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  subunits. The  $\alpha_{1c}$  subunit is made of cardiac specific Cav1.2 subunit. The  $\beta$  subunits are capable of increasing the channel expression and also regulate the kinetics (83).

### **2.2.8 Potassium channels**

Voltage gated K<sup>+</sup> channels (Kv) play a major role in regulating the repolarization of the action potential in the mammalian myocardium. There is a large diversity in the function and

electrophysiological properties of the cardiac Kv channels. There are three main classes of the currents created by the potassium channels.

- Voltage – gated channels – ( $I_{to}$ ,  $I_{Kur}$ ,  $I_{kr}$  and  $I_{ks}$ )
- Inward rectifiers channels – ( $I_{k1}$ ,  $I_{kAch}$ ,  $I_{kATP}$ )
- Background  $K^+$  currents – (TASK -1, TWIK – 1/2)

The expression of these different potassium channels can vary depending on the region. Therefore it accounts for regional differences of the action potential in the atria and ventricles.  $K^+$  currents are highly regulated in the cardiomyocytes.

As other types of voltage gated ion channels,  $K^+$  channels are also constructed of  $\alpha$  and  $\beta$  subunits. Further, ancillary proteins such as Kv-channel associated protein (KChAP) and Kv channel interacting proteins (KChiP) are required to maintain the proper function of the channel.

There are various types of  $\alpha$  and  $\beta$  subfamilies involved in constructing the cardiac potassium channels. The cardiac  $\alpha$  subunits include KvN.x (N=1 to 4), the HERG channel and KvLQT1. The KvN.x subunits assemble as hetero multimers to form the core of the ion channel. However HERG and KvLQT1 found to be assemble as homotetramers. Therefore,  $\alpha$  subunits are involved in generating the voltage gated  $K^+$  currents in the cardiomyocytes. The ancillary subunits are required to increase the channel activity and also capable of regulating the channel kinetics.

$I_{Kur}$ ,  $I_{kr}$  and  $I_{ks}$  are slow and play a main role during cardiac repolarization by facilitating outward  $K^+$  currents during phase 3 of action potential.  $I_{Kur}$  is highly expressed in atrial cardiomyocytes. It was found that  $I_{kr}$  is more expressed in the left atrium and ventricular

endocardium. One of the major causes for manifestation of inherited arrhythmias are the mutation in the genes that encode cardiac  $K^+$  channels.

### **2.2.9 Transient outward potassium currents ( $I_{to}$ )**

Based on the time and voltage gated properties, the Kv currents are categorized in to two broad classifications (84).

1. Transient outward potassium current ( $I_{to}$ )
2. Delayed, outwardly rectifying potassium current ( $I_k$ )

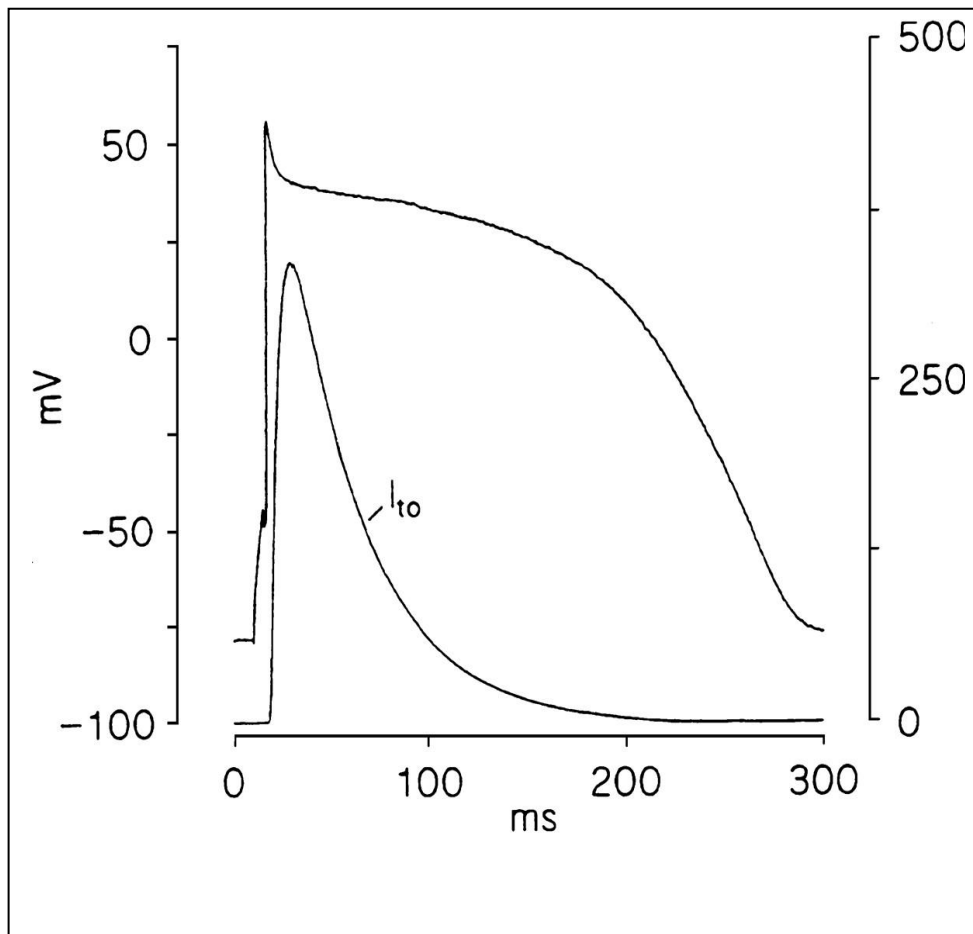
In this chapter the main focus will be on the  $I_{to}$  current.  $I_{to}$  plays a major role in regulating the rapid repolarization during phase 1 of the action potential and is known to rapidly activate and deactivate in the myocardium (85) (Figure 2.10) . The  $I_{to}$  current has been reported to be prominent in ventricles of many species, including rodents and humans (86).

$I_{to}$  have the capability to impact the L-type  $Ca^{2+}$  channels because its involvement in regulating the early repolarization. Therefore, these  $K^+$  currents significantly affect the excitation – contraction coupling and repolarization in cardiomyocytes. Further, it was identified that the changes in  $I_{to}$  can alter the delayed rectifier currents. Therefore, changes in the  $I_{to}$  can alter the duration of action potential and also increase the risk for development of arrhythmias.

Attenuation of  $I_{to}$  in the hypertrophied heart has reported to induce prolonged action potential and generate arrhythmias (87). This was further consolidated by another study which demonstrates that the attenuation of  $I_{to}$  prolong the QT interval in the mouse heart (88). Further,



it was observed that diabetes can significantly suppress the  $I_{to}$  current in the diabetic myocardium, thus reduce the repolarization rate of the ventricular cardiomyocytes (89). Further studies indicated that diabetes can impair the recovery of  $I_{to}$  in the myocardium (90).



*Patel et al; J Physiol 569.1 (2005) pp 7–39*

**Figure 2.10:  $I_{to}$  current corresponding to cardiac action potential**

**Conventional whole cell patch clamp view of  $I_{to}$ : Theoretically predicted kinetic behavior of  $I_{to,fast}$  during a ventricular action potential.**

### 2.2.10 Molecular components of $I_{to}$

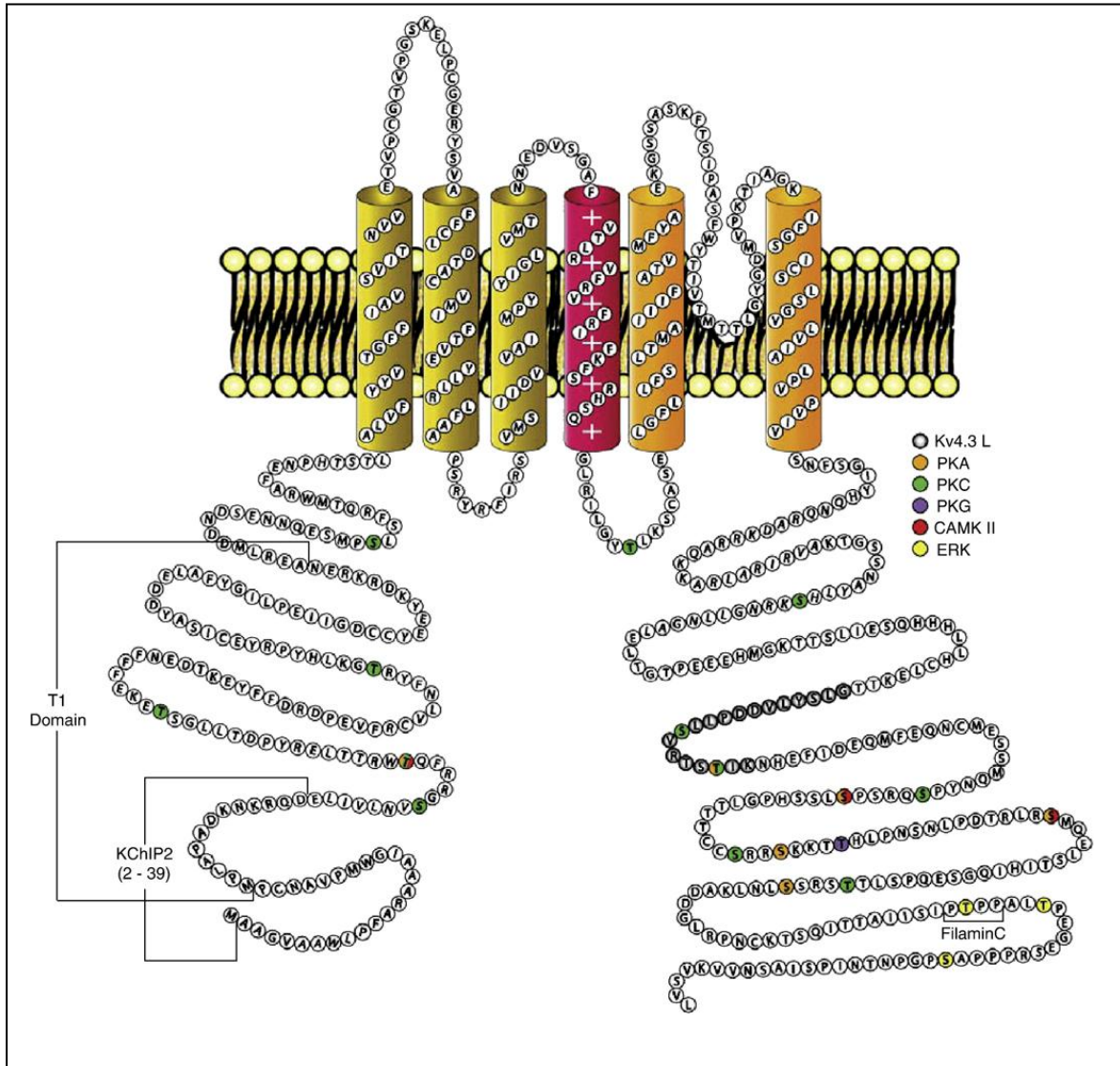
To further understand the regulatory aspects of the  $I_{to}$  current, it is necessary to gain an insight of the molecular components which compose the Kv/  $I_{to}$  channels. There are two main subunits that comprise the Kv channels.

1.  $\alpha$  subunit – principle protein required for the formation of the ion channel
2.  $\beta$  subunit – regulate the properties of the principle subunit

The Kv channels are tetramers; therefore assembly of 4  $\alpha$  subunits are required for the proper formation and the function of the ion channel. Each of this  $\alpha$  subunit has six transmembrane segments, including the pore formation between S5 and S6 segment, and voltage sensitivity at S4 segment which provides the specificity for the  $K^+$  (91, 92). Cardiac Kv channels are composed of various types of  $\alpha$  subunits with different kinetic properties, and these subunits can assemble as homotetramers or heterotetramers (92).

The  $\alpha$  subunits of Kv channel have been classified to nine families. These families are named starting from Kv1.x to Kv9.x. In the heart, Kv1.4, KV1.7, Kv4.2 and Kv4.3 are substantially expressed. Out of these the most important Kv  $\alpha$  subunits which underlie  $I_{to}$  channels in humans and rodents are Kv 4.3 and Kv 4.2 (93-95). Once these subunits are translated, they are accumulated in the endoplasmic reticulum and subsequently undergo further post-translational modifications in the Golgi bodies before they are translocated to the sarcolemma (96) (Figure 2.11). The assembly of 4  $\alpha$  subunits is sufficient to create a  $K^+$  current, however the level of expression and biophysical properties are determined by the  $\beta$  subunits,

KChIP and KChAP proteins. The  $\beta$  subunits have conserved core region and has oxido reductase properties with its active site positioned to interact with the voltage sensor of the  $K^+$  channels (97) (Figure 2.12).



<http://www.ncbi.nlm.nih.gov/pubmed/19619557>

**Figure 2.11: Schematic representation of Kv 4.3 channel**

**The linear amino acid sequence of human Kv 4.3 illustrated**

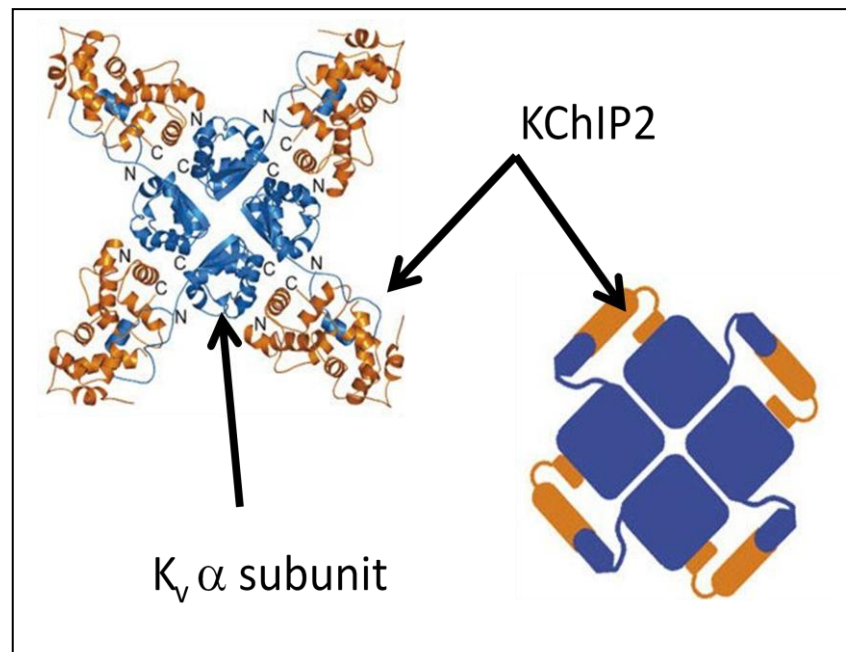
KChAP proteins increase the cell surface expression but do not affect the properties of the channels. Previous publications have demonstrated co-immunoprecipitation of Kv4.3 with KChAP. This indicates that KChAP may have an important regulatory role in the heart. KChIP proteins binds with  $\text{Ca}^{2+}$  that interact with Kv4-  $\alpha$  subunits and control the density, kinetics and recovery of the ion channels. KChIP proteins are categorized in to KChIP1, KChIP2 and KChIP3. However, KChIP2 has been shown to have tissue specific expression in the heart (98). Therefore, only cytoplasmic protein KChIP2, has been found to be an essential component of myocardial  $I_{to}$  (99).

Recently published reports implicate that insulin stimulate the production of  $I_{to}$  protein components. Further, disruption of actin filament network in the cardiomyocytes adversely affected the  $I_{to}$  current, indicating the necessity of the cellular cytoskeleton for trafficking the newly synthesized channel components to the sarcolemma (100).

### **2.2.11 Regulation of $I_{to}$**

The  $I_{to}$  current is regulated by many endocrine and paracrine systems. Specifically,  $I_{to}$  was found to be modulated by endothelin – 1,  $\alpha 1$  – adrenergic agonists and AT II. A rapid reduction of  $I_{to}$  was observed in the isolated adult rat cardiomyocytes, rabbit atrial cardiomyocytes with the treatment of  $\alpha$  – adrenergic agonists via PKC dependent and independent pathways (101, 102). The treatment of neonatal cardiomyocytes with phenylepinephrine and endothelin-1 treatment also induced a reduction of the  $I_{to}$  current and this was attributed to the reduction of Kv 4.2 subunit in the cells (103, 104). Similarly, a reduction of  $I_{to}$  was observed in the myocardium with AT II. It was observed that incubation of canine

ventricular cardiomyocytes with AT II significantly reduced the  $I_{to}$  current by replacing the Kv4.3 with subunits that have slow kinetic properties. Interestingly, treatment with AT II receptor blocker (Losartan) converted the slow  $I_{to}$  current to faster  $I_{to}$  current, which is regulated by Kv4.3 and Kv 4.2 (105). Also, it is understood that the properties of  $I_{to}$  can be changed dramatically without any changes in the sub unit expression.



*Nature 419, 35-42(5 September 2002)*

**Figure 2.12: The assembly of Kv  $\alpha$  subunits**

**Four  $\alpha$  subunits assembly to form the functional channels which facilitate  $K^+$  currents across the membrane.**

**The KChIP2 subunits are required for the translocation of the alpha subunits on to the membrane.**

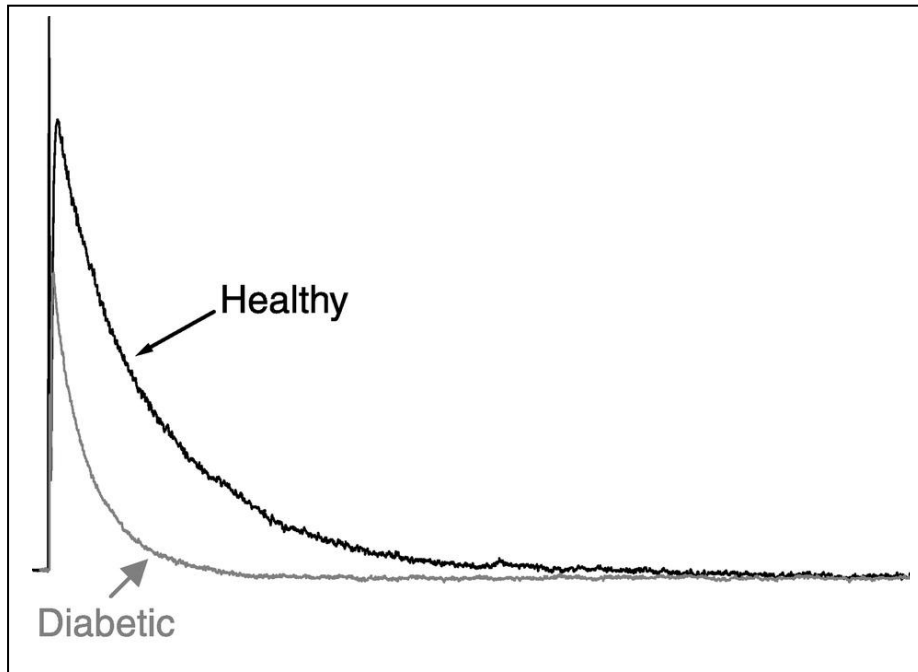
Fatty acids such as arachidonic acid were found to have a profound effect on  $I_{to}$  density and prolong the action potential in ventricular cardiomyocytes. Further, it was implicated that the arachidonic acid suppresses the  $I_{to}$  via PKC dependent pathway (106).

### 2.2.12 Diabetes and $I_{to}$

Many published reports confirm the effects of diabetes on the  $I_{to}$  current in the heart. Diabetes was found to impact  $I_{to}$  in two ways (Figure 2.13).

- Diabetes can accelerate the inactivation kinetics
- Diabetes slow the recovery of  $I_{to}$  current from inactivation

The work by *Shimoni et al* suggests that diabetic cardiomyocytes have slower recovery kinetics (90). Further, it was demonstrated that diabetes can also impact adversely on the expression of  $I_{to}$  channels by attenuating the expression of the channel forming subunits which can cause a reduction in total potassium current amplitude (107-109). The work by *Qin et al* demonstrates that gene expression of Kv 4.3 and Kv 4.2 are significantly reduced in rats with Streptozotocin induced diabetes (animal model of Type 1 DM) (Figure 2.15). Also, diabetes was found to increase the expression of Kv1.4 proteins which have slow kinetic properties and slow recovery rates compared to Kv 4.2 and 4.3 (107). How diabetes alters the expression of channel forming subunits in the myocardium is not fully elucidated.



*Can J Physiol Pharmacol.* 2009 Feb;87(2):77-83. doi: 10.1139/Y08-106

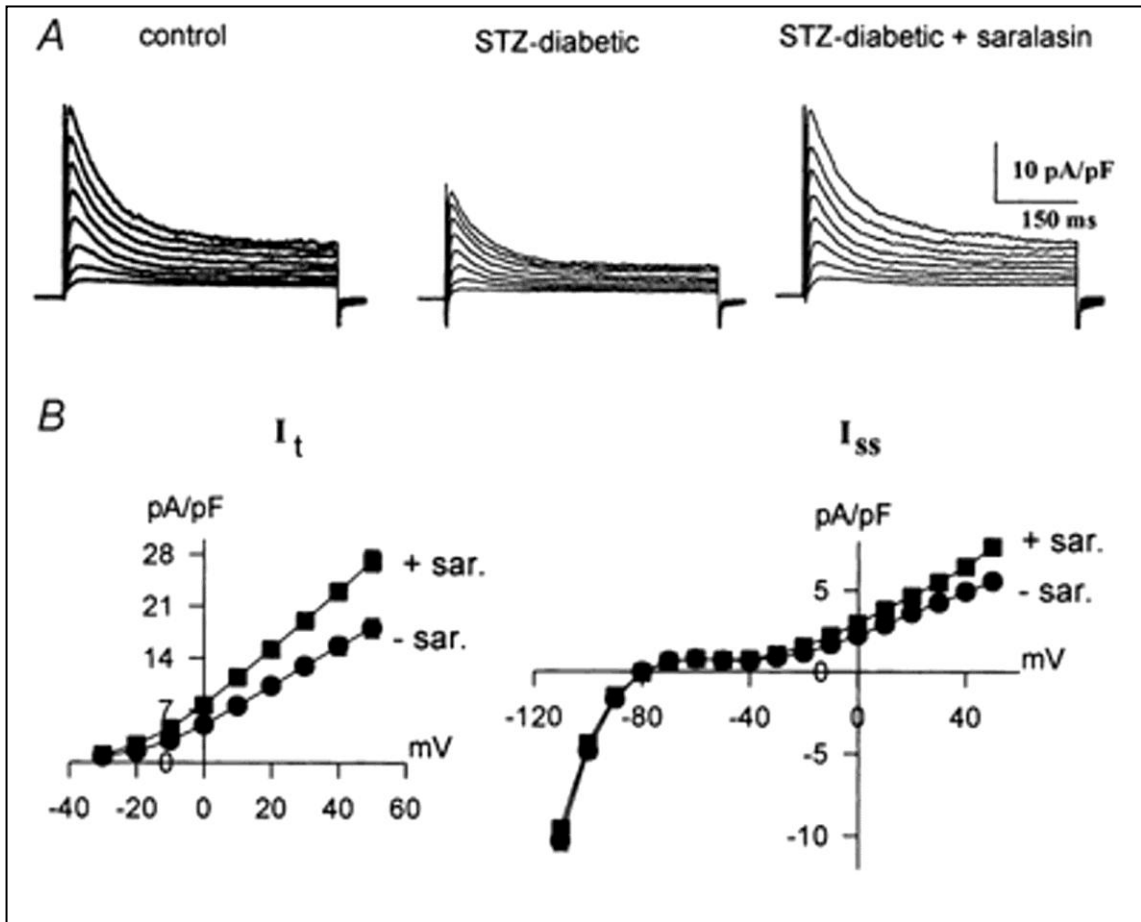
**Figure 2.13:  $I_{to}$  recordings in healthy and diabetic rat cardiomyocyte**

$I_{to}$  recordings elicited in a diabetic and a healthy (non diabetic) rat myocytes with similar cell size. In the diabetic cell  $I_{to}$  amplitude is reduced approximately 40%, and the current decays faster because of a faster channel inactivation.

Interestingly, it was found that long term incubation with insulin can reverse the  $I_{to}$  amplitude in isolated diabetic cardiomyocytes (89). MAPK appears to be involved in the insulin mediated stimulatory effects of  $I_{to}$ . Further, the pharmacological agents which can increase the glucose utilization have the capability of normalizing the  $I_{to}$  current (110).

Previous publications reported that treatment of diabetic rats with AT II receptor blocker reverse the downregulated  $I_{to}$  current in the heart (Figure 2.14). This study and other studies support that AT II, which is upregulated during diabetes, can have a profound effect the expression of  $I_{to}$  current density and also it's pore forming subunits (109, 111). Therefore, the

enhanced expression of AT II during diabetes exerts a significant impact on the molecular components of  $I_{to}$  and thus leads to alterations in ionic currents in the heart and altered ECG (Figure 2.14). Work by Zhang et al suggests that AT II treatment attenuate the Kv4.3 gene expression in cardiomyocytes.



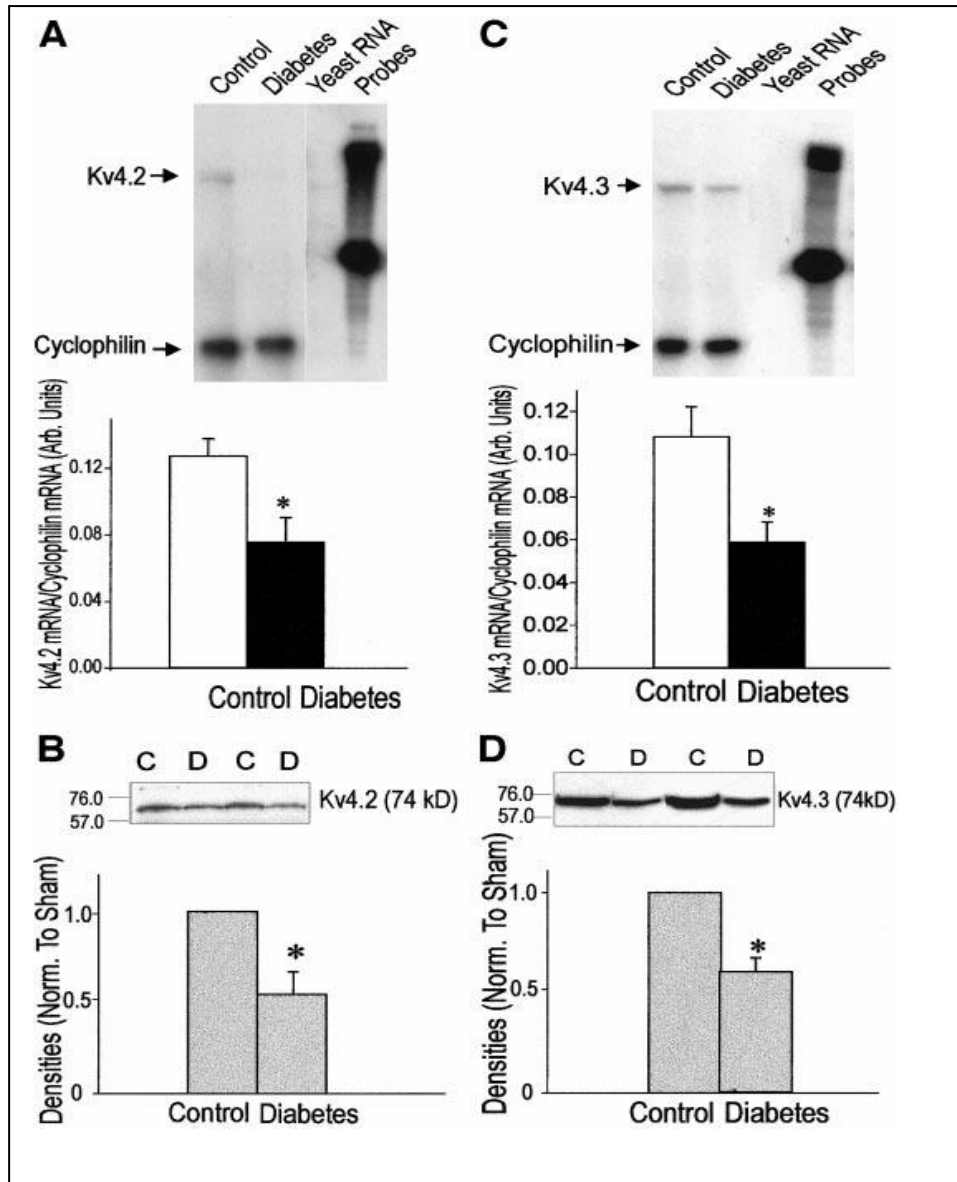
*J Physiol.* 2001 Nov 15;537 (Pt 1):83-92

**Figure 2.14: The effects of AT II blockers on  $I_{to}$  in the diabetic heart**

(A) superimposed current traces obtained in response to 500 ms depolarizing pulses given from a holding potential of  $-80$  mV to potentials ranging from  $-20$  to  $+50$  mV. Examples are given for cells obtained from a control rat (left), and from an STZ-diabetic rat in the absence of (centre) or 7 h after incubation with  $1 \mu\text{M}$  saralasin (AT II receptor blocker) (right). (B) current-voltage relationships for the peak outward current ( $I_t$ , left) and the sustained current ( $I_{ss}$ , right) in cells from STZ-diabetic rats in the absence ( $\bullet$ ,  $n = 24$ ) and



presence (■,  $n = 23$ ) of saralasin (6–9 h incubation). Mean current density values ( $\pm$  S.E.M.) are plotted against membrane potential.

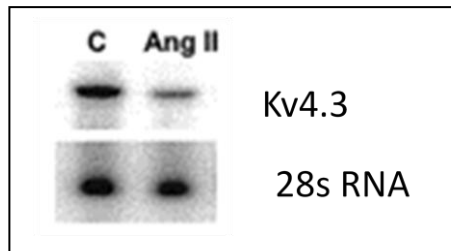


*Qin et al; BBRC Vol 283, 549-553 2001*

**Figure 2.15: The attenuation of gene expression of Kv4.2 & 4.3 under diabetes**

(A & B) Comparison of cardiac Kv4.2 gene expression (A) and western blot analysis of Kv4.2 by westerns (B) in the LV of sham (control) and diabetic experimental groups. The gene and the protein expression of the Kv4.2 is attenuated in the diabetic animals. (C & D) Comparison of cardiac Kv4.3 gene and protein

expression in the sham (control) and diabetic groups. Kv 4.3 gene and protein expression is also reduced in the diabetic hearts.



*Zhang T et al. Circulation Research 2001;88:476-482*

**Figure 2.16: AT II mediated down regulation of Kv4.3 gene in cardiomyocytes**

Neonatal cardiomyocytes were treated with AT II (100nM) for 8 hours. The gene expression of Kv4.3 is significantly attenuated in AT II treated cardiomyocytes.

## 2.3 Peroxisome proliferator activated receptors and the heart

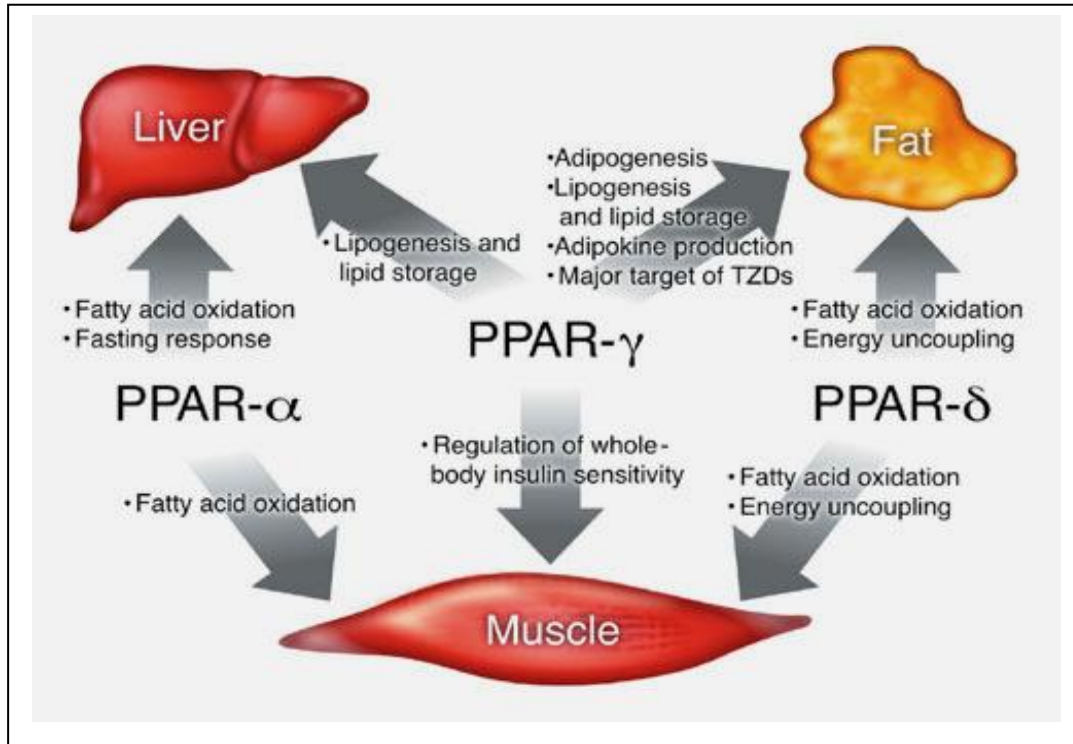
### 2.3.1 PPARs (Peroxisome proliferator activated receptors)

PPARs are ligand activated transcription factors which belong to within the broad nuclear receptor superfamily. There are three types of PPARs encoded by distinct genes.

1. PPAR  $\alpha$
2. PPAR  $\beta/\delta$

### 3. PPAR $\gamma$

These three receptors have distinct tissue expression patterns, and also specific and overlapping functions (112) (Figure 2.17).



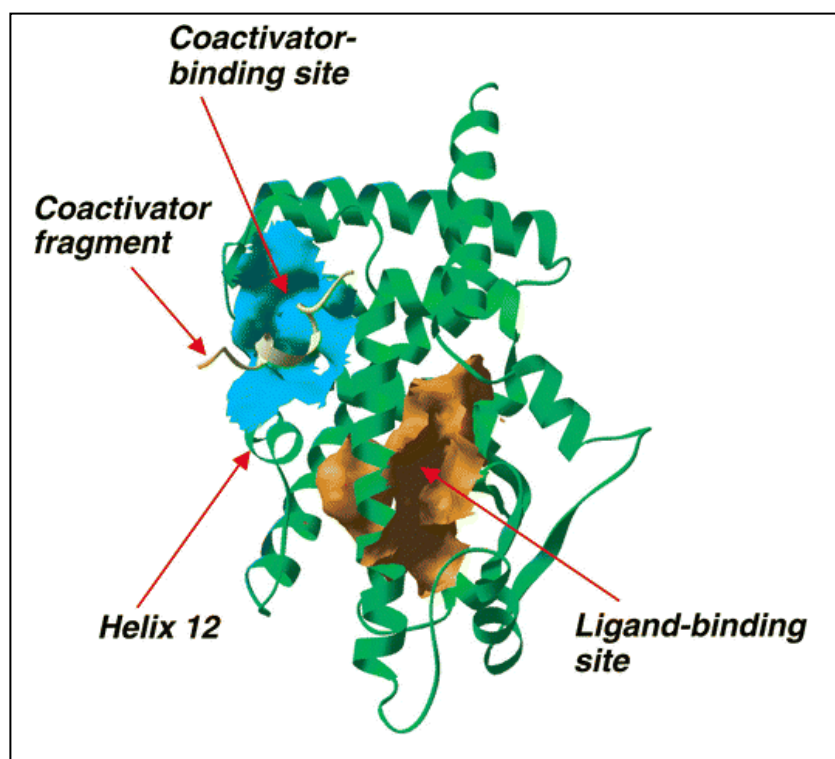
*Nat Med.* 2004 Apr;10(4):355-61

**Figure 2.17: PPAR subtypes, their distribution and functions**

The three PPAR isoforms regulate lipid and glucose homeostasis through their coordinated activities in liver, muscle and adipose tissue.

PPARs form heterodimers with Retinoid X Receptors (RXR) and bind to specific DNA sequence elements known as PPRE (Peroxisome Proliferator Response Element) to regulate the target gene expression. Once the ligand is bound, the conformation of the PPAR is altered, which stabilized and create a binding cleft that facilitate the recruitment of co-activators.

The first type of PPAR that was cloned is PPAR $\alpha$ , and since then extensive research was conducted on PPARs. Variety of FA and their derivatives which includes prostaglandins, eicosonids have found to be ligands for PPARs. Further, PPARs has been implicated to play a crucial role in sensing nutrient levels and in modulating their metabolism. Other than these functions, recent publications verify myriad of other roles regulated by PPARs. All the three PPARs can regulate cellular differentiation and claimed to take part in pathophysiology of carcinogenesis (113).



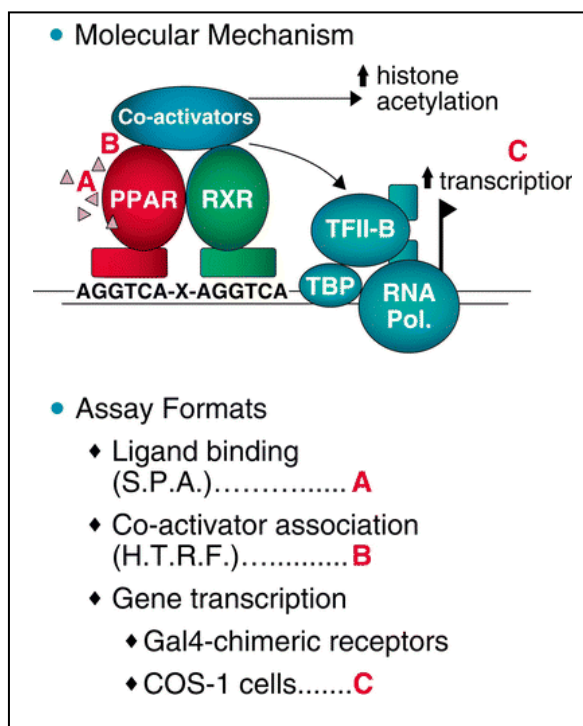
*Annu Rev Med. 2002;53:409-35*

**Figure 2.18: Crystal structure of PPAR $\gamma$  LBD**

X-ray crystal structure of PPAR $\gamma$  ligand binding domain. Several key  $\alpha$ -helices are shown along with the relative location of key functional regions.

Like any other nuclear receptors, PPARs also contain functional domains (114). Amongst the three PPARs, the DNA binding domain (DBD) and the ligand binding domain (LBD) are highly conserved (Figure 2.18). Compared to other nuclear receptors, the PPARs have a large LBD (115). This large LBD pocket allows the PPARs to bind with a broad range of natural and synthetic ligands. The C-terminus of the LBD consists of ligand dependent activation domain AF-2, which is involved in constructing the co-activator binding pocket of the receptor (115).

PPARs require binding of RXR and form heterodimers to exert the transcriptional activity. RXR also exists in three forms, such as RXR $\alpha$ ,  $\beta$  and  $\gamma$  (116). However, specific roles of different isoforms of RXR within the PPAR: RXR complex have not been identified up to date (Figure 2.19).



*Annu Rev Med. 2002;53:409-35*

**Figure 2.19: Mechanisms of transcriptional activation of PPAR isoforms**

Mechanism of transcriptional activation by PPAR isoforms. Selected molecular components are shown relative to assay formats that can be used to characterize compound activities.

Once the PPAR: RXR complex is formed, it binds to the PPRE regions of the target genes to exert its transcriptional effects. PPRES consists of direct repeat elements which consists of two hexanucleotides with the consensus sequence AGGTCA separated by a single nucleotide spacer. *Cis* elements adjacent to the PPRES core site regulate the binding selectivity of these response elements (117).

### **2.3.2 Co-activators of PPAR isomers**

Co-activators and repressors mediate the activation or repression of the transcription of the nuclear receptors respectively. Co-activators interact with nuclear receptors in a ligand dependent manner (118). The co-activators of PPARs include CBP/ p300 have histone acetylase activity that can remodel histones (119). Also, DRIP/ TRAP complex such as PPAR binding protein (PBP)/ TRAP220 form a bridge between the nuclear receptor and facilitate the transcription initiation machinery (120). Molecular function of PGC-1 which is another co-activator of PPAR is not fully elucidated. In summary, it is hypothesized that the co-activators with histone acetylase properties remodel the histones and expose the chromatin structure near the regulatory region of the gene. Then the complexes such as DRIP/ TRAP are recruited and provide a direct link to the basal transcription machinery, which results in initiation of the transcription (113).

### 2.3.3 PPAR $\alpha$ and heart

PPAR  $\alpha$  is highly expressed in metabolically active tissues such as liver, kidney, heart, brown fat, skeletal muscles and vascular endothelial and smooth muscle cells (121-124). Diverse class of pharmacological compounds, including hypolipidemic fibrates acts through PPAR  $\alpha$  receptors (125). Further, PPAR  $\alpha$  plays an important role in modulating cellular uptake, activation and  $\beta$  – oxidation of FA. PPAR  $\alpha$  regulates the gene expression of FATP (FA transport protein) which is a FA transporter (126). Also PPAR  $\alpha$  activation induces the gene expression of many enzymes that are involved in  $\beta$  – oxidation pathway. These enzymes are acetyl – CoA synthase, enoyl – CoA hydratase and keto – acyl – CoA thiolase (127-129). Carnitine palmitoyltransferase I (CPT I), which catalyzes the translocation of active FA in to the inner membrane of the mitochondria is also strongly induced by PPAR  $\alpha$  activity (130).

PPAR  $\alpha$  is abundantly expressed in the heart and plays a critical role in myocardial energy and lipid metabolism. PPAR  $\alpha$  regulate myriad of genes involved in fatty acid uptake and oxidation in cardiomyocytes. As PPAR  $\alpha$  plays a vital role in cardiac energetic, it is identified as an important determinant of myocardial energy production. The activity of the PPAR  $\alpha$  is altered during various cardiac pathologies (131). PPAR  $\alpha$  activity is known to be attenuated in pathological hypertrophied heart, which leads to diminished fatty acid utilization and increased glucose oxidation (132).

However, the diabetic heart demonstrates a chronic activation of PPAR  $\alpha$  pathway which is ensued by lipid accumulation, which is a feature of diabetic cardiomyopathy. It was also observed that cardiac specific over-expression of PPAR  $\alpha$  produce similar phenotype to that of the diabetic heart (133).

### 2.3.4 PPAR $\beta/\delta$ and heart

In contrast to PPAR  $\alpha$  and PPAR  $\gamma$ , the function of PPAR  $\delta$  in the heart is not extensively explored. PPAR  $\delta$  is ubiquitously expressed in the body, therefore initially it was perceived to be involved in regulating a “housekeeping” role (134). However, it was identified as an important mediator in fatty acid metabolism in cardiomyocytes (135). More specifically, PPAR  $\delta$  was shown to play a vital role in fatty acid oxidation via activating oxidative genes in isolated neonatal and adult cardiomyocytes (136). Further, cardiac specific deletion of PPAR  $\delta$  induces cardiac hypertrophy, lipotoxicity and heart failure in mice (137).

### 2.3.5 PPAR $\gamma$ and heart

PPAR  $\gamma$  plays a major role in adipocytes development and differentiation, thus its role in adipose tissue was extensively characterized (138) (Figure 2.20). It interacts directly with the *cis* element that regulates adipocytes specific expression of FA binding protein aP2 (139). This aP2 protein belongs to a family of intracellular FA binding proteins (FABP) and is involved in shuttling free FA through the cytosol (140). Further, it was found that PPAR  $\gamma$  regulates many genes involved in lipid metabolism in adipocytes, such as LPL, PEPCCK and acyl – CoA – synthase (127, 141, 142). PPAR  $\gamma$  also regulates the expression of CD36 and FATP-1 which are involved in lipid uptake (126).

PPAR  $\gamma$  also exerts a profound effect on insulin sensitivity in the body. It has been found to regulate several genes that affect insulin action. Previous publications demonstrate that PPAR

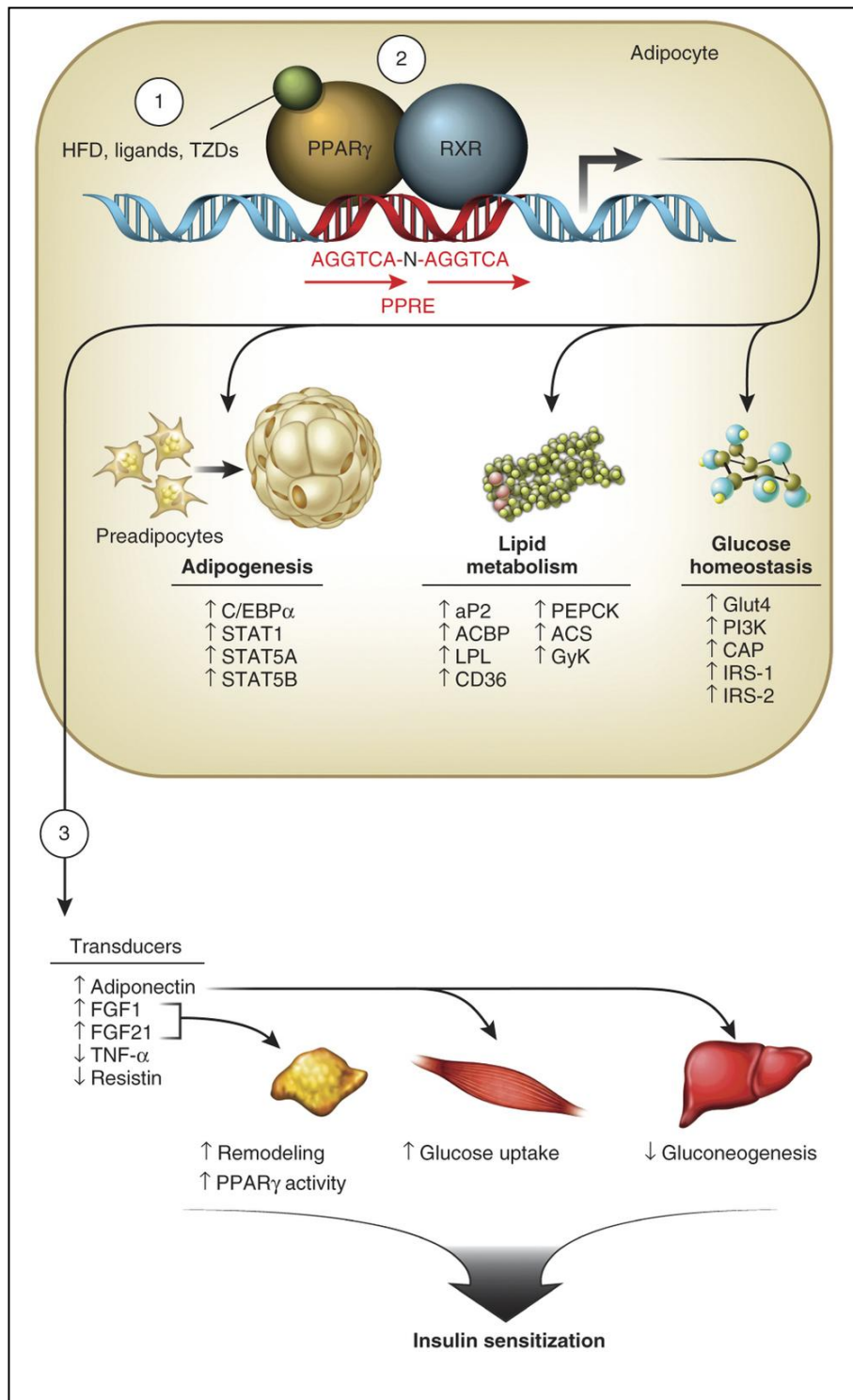


$\gamma$  can induce the expression of IRS-2 (insulin receptor substrate – 2) protein that is integral for insulin signaling in insulin sensitive tissues (143). PPAR  $\gamma$  agonist treatment significantly inhibited the expression of TNF –  $\alpha$  in adipose tissues of obese rats. TNF –  $\alpha$  is a pro-inflammatory cytokine that is expressed in the adipose tissue and is known to induce insulin resistance and suppress insulin signal transduction (144).

Additionally, PPAR  $\gamma$  involves in regulating the genes that modulate glucose homeostasis. PPAR  $\gamma$  is a known inducer of glucose transporter type 4 (Glut4). This protein is required to uptake systemic glucose in to insulin sensitive tissues such as adipose tissue, skeletal muscles, liver and also heart (145).

### **2.3.6 Thiazolidinediones**

Pharmacological agonists of PPAR  $\gamma$ , which are known as thiazolidinediones (TZD) are promising therapeutic agents to overcome the insulin resistance associated with Type 2- DM. TZDs exerts potent antidiabetic effects by lowering hyperglycemia, hyperinsulinemia and hypertriglyceridemia. Increased circulating FA levels are known to induce insulin resistance. The ability of TZDs to sequester the free FA to adipose tissue also contributes to improve the whole body insulin sensitivity (146). Also, the increase uptake of FA and enhanced adipogenesis induced by TZDs induce weight gain. Therefore, use of TZDs in the treatment of Type 2-DM has become controversial. Further TZDs promotes peripheral edema and predispose the heart to failure in patients with existing cardiovascular diseases (147). Bone loss and higher risk for fractures are other side effects reported with the use of TZDs (148).



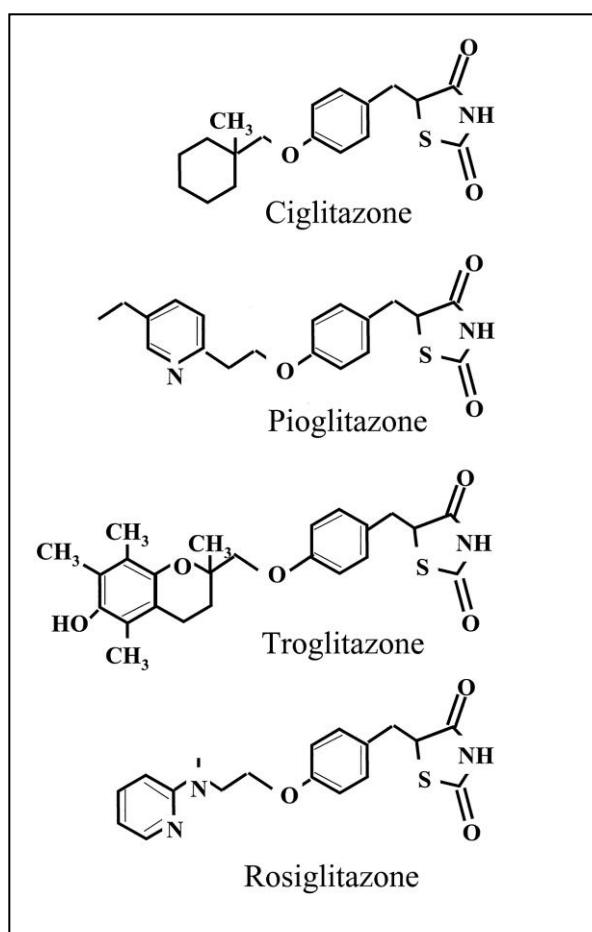
*Nat Med.* 2013 May;19(5):557-66. doi: 10.1038/nm.3159. Epub 2013 May 7

**Figure 2.20: Role of PPAR  $\gamma$  in the adipose tissue**

PPAR  $\gamma$  has multiple roles in adipose tissue. High fat diet, ligands or TZDs activate PPAR $\gamma$ -RXR functional heterodimers and maintain metabolic homeostasis through direct regulation of genes harboring PPAR

response elements (PPREs) involved in adipocyte differentiation, lipid metabolism and glucose homeostasis, as well as the expression of adipose secreted factors that act as transducers for PPAR  $\gamma$ .

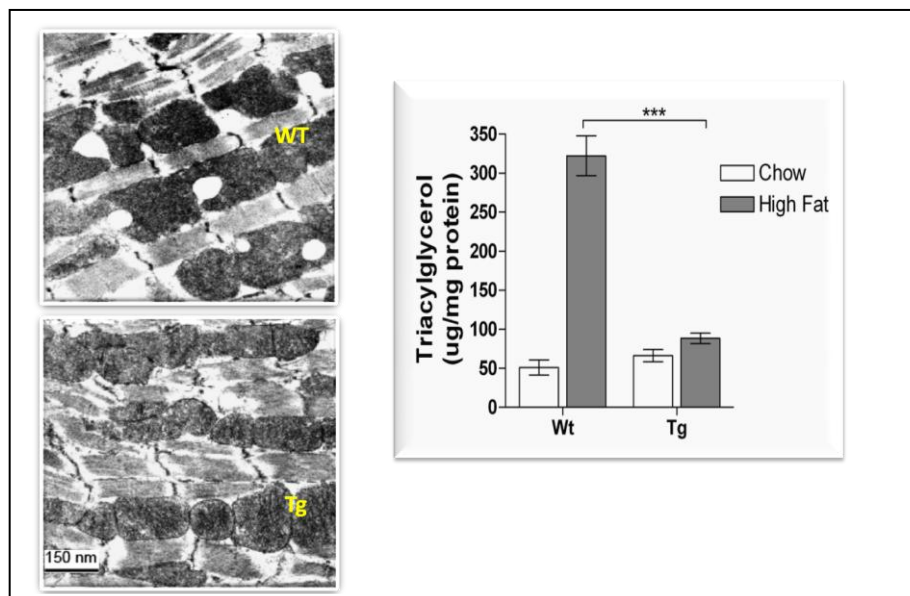
There are many synthetic PPAR  $\gamma$  ligands which belong to TZD family (Figure 2.21). Out of these ligands, Pioglitazone and Rosiglitazone is available in the USA market as antidiabetic agents, however wide use of these drugs are restricted and under the scrutiny of FDA (Food and Drug Administration).



*Drugs Fut 2006, 31(10): 875*

**Figure 2.21: Chemical structures of TZDs**

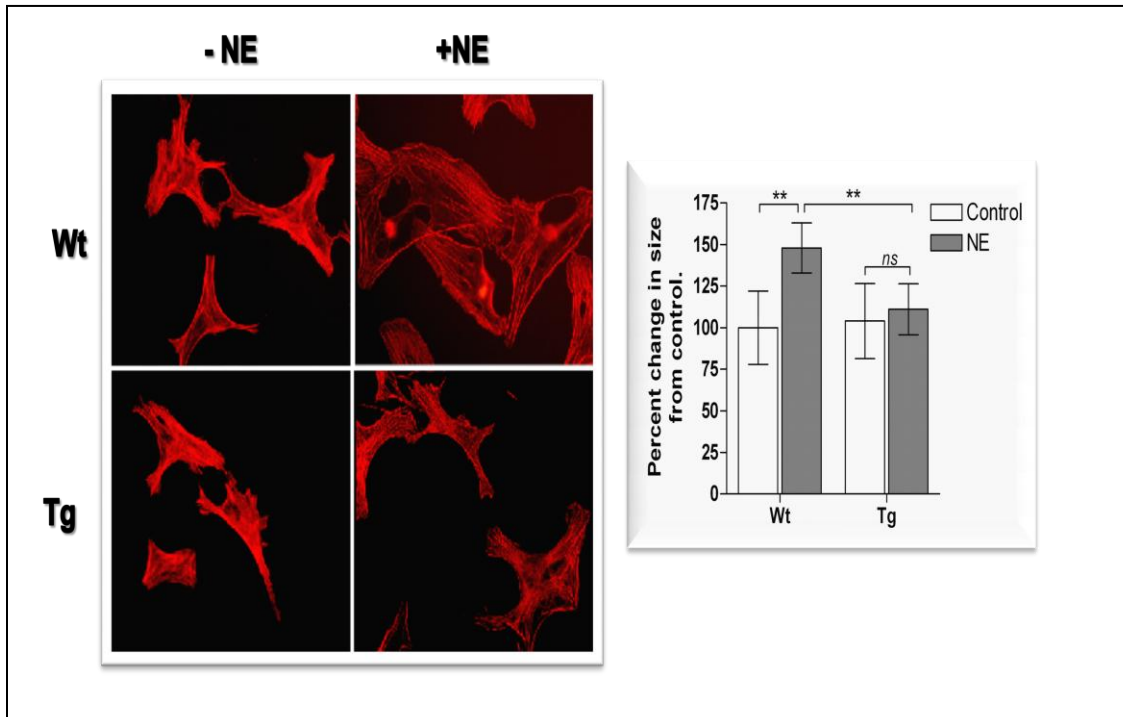
PPAR  $\gamma$  is expressed in the heart and it is understood that the most of the cardiac effects of PPAR mediated by indirect mechanisms. Despite the many unwanted side effects exerted by PPAR  $\gamma$  in the heart, many reports support the beneficial effects of these compounds in variety of cardiovascular pathologies. PPAR  $\gamma$  agonists are capable of reducing the infarction when the hearts are subjected to ischemia/ reperfusion (I/R) in animal models (149, 150). Also, PPAR  $\gamma$  agonists can inhibit the development of cardiac hypertrophy in pressure overload animal models (151). The mechanisms responsible for cardiac effects of PPAR  $\gamma$  agonists are attributed to lipid and glucose metabolism and anti-inflammatory properties. Also, transgenic mice with constitutively active PPAR  $\gamma$  (CA-PPAR  $\gamma$ ) have been shown to be protected against triglyceride (TG) accumulation in cardiomyocytes with high fat diet (Figure 2.22). Interestingly, it was also observed that the hearts of these transgenic mice are protected against hypertrophy induced by adrenergic stimulation (152) (Figure 2.23).



Amin et al; *Am J Physiol Heart Circ Physiol* 299: H690–H698, 2010

**Figure 2.22: CA-PPAR $\gamma$  protects cardiomyocytes against TG accumulation**

The electron microscopy data indicates the deposition of TG globules within the cardiomyocytes in high fat diet mice. This effect was rescued in transgenic mice with PPAR  $\gamma$  over-expression in myocytes.



*Amin et al; Am J Physiol Heart Circ Physiol 299: H690–H698, 2010*

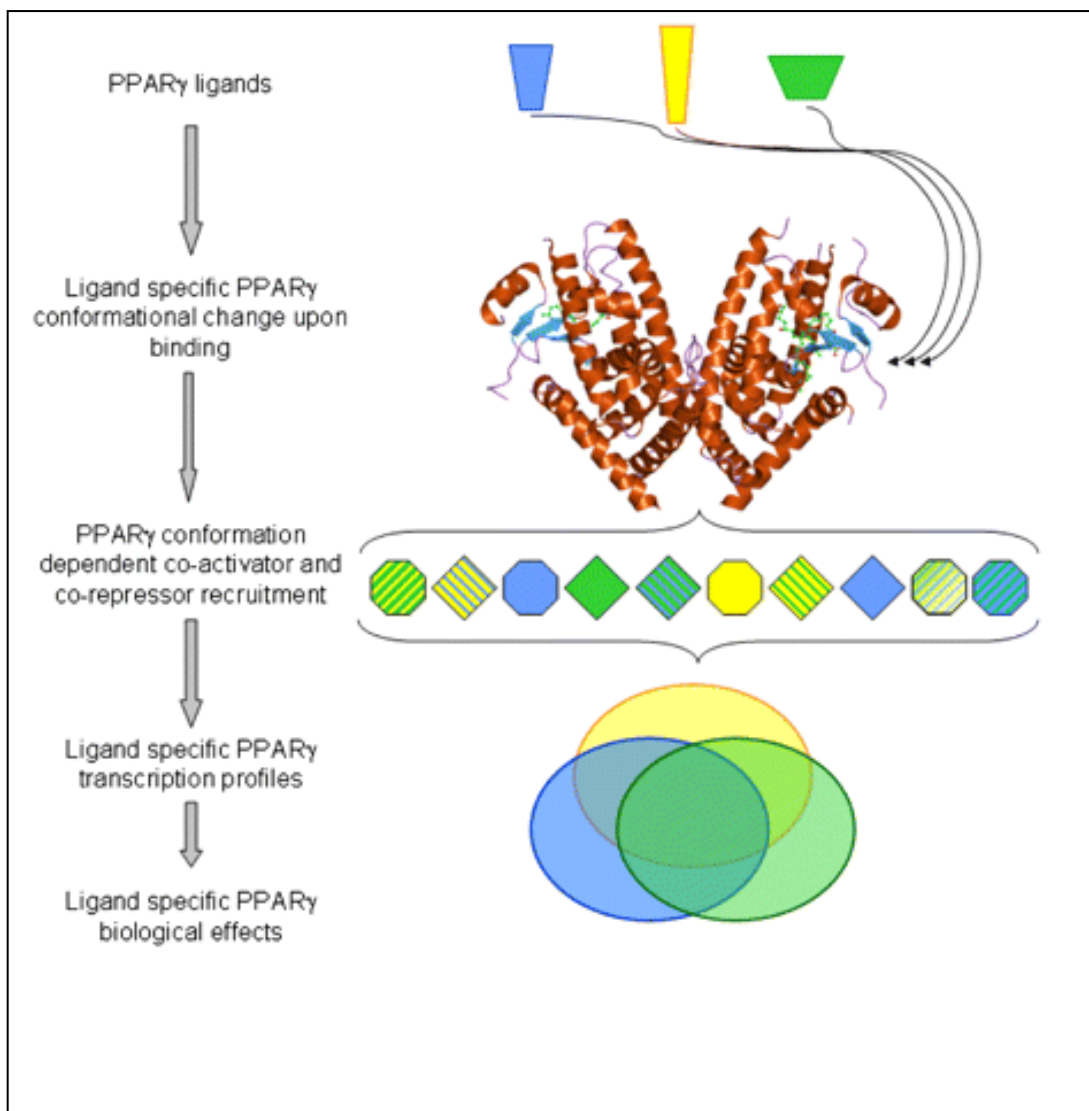
**Figure 2.23: CA-PPAR $\gamma$  protects against hypertrophy induced by adrenergic stimulation**

Fluorescence micrographs of phalloidin -stained transgenic primary neonatal cardiomyocytes. Cardiomyocytes isolated from transgenic PPAR  $\gamma$  over expressed mice were resistant to hypertrophy by adrenergic stimulation.

**2.3.7 Selective PPAR  $\gamma$  modulators**

Amongst the TZDs rosiglitazone is considered as a full PPAR  $\gamma$  agonist. Recently, few studies published on the aspect of constructing new PPAR  $\gamma$  agonist molecules which possess

antidiabetic effects without the adverse effects reported with conventional TZDs. The strategy that is utilized in constructing such drug molecule is achieving selective agonism for the PPAR  $\gamma$  receptor. These compounds are collectively named as selective PPAR  $\gamma$  modulators (SPPARMs).



*PPAR Res. 2007;2007:32696*

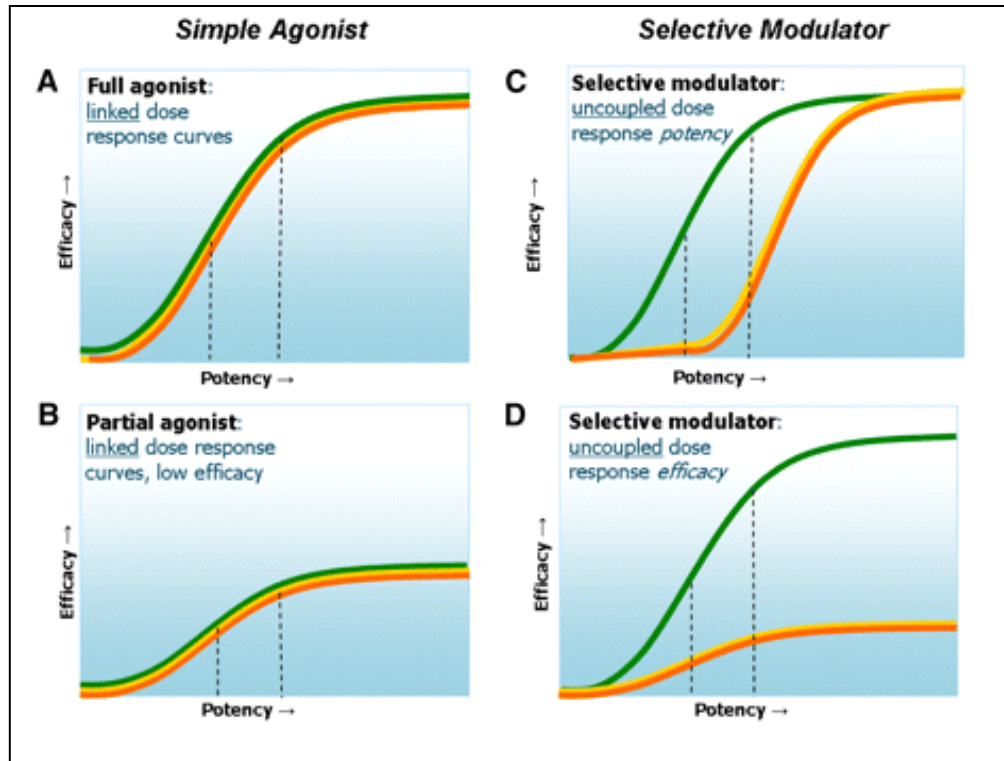
**Figure 2.24: Differential binding of regulators produces distinct gene expression patterns**

**Ligands bind to LBD of PPAR  $\gamma$  in various ways leading to distinct transcription patterns.**

SPPARMs are constructed based on the concept the conformation state of PPAR  $\gamma$  is vital to its activity. This is because the conformation of the receptor determines the affinity for co-repressors and co-activators (153). Different ligands for PPAR  $\gamma$  bind to the LBD in different ways giving rise to slightly different interactions with its regulator proteins. These differential bindings can lead to distinct transcription patterns (153) (Figure 2.24).

PPAR  $\gamma$  produces variety of responses due to numerous potential ligand binding conformations and the large number of responsive genes. Full PPAR  $\gamma$  agonists such as rosiglitazone have dose response relations for multiple activities linked. In the case of partial agonists, they show the same activation patterns of a full agonist, but has a lower activity compared with a full agonist. Unlike a partial agonist, SPPARMs have dose – response relations for various activities uncoupled from each other. An ideal SPPARM should have potent insulin sensitizing activities with less effects on adiposity, loss of bone mineral density, fluid retention and congestive heart failure (153).

As PPAR  $\gamma$  LBD domain possesses a large binding pocket. Therefore, it offers a large number of potential contact points for variety of endogenous ligands to bind in different combinations. Full agonists such as rosiglitazone, forms tight hydrogen bonds with AF-2 (activation function) domain of the LBD, which promotes recruitment of regulator proteins and induce high maximal activity. Similar to rosiglitazone, all the other small full PPAR  $\gamma$  agonists have common binding mode, in which the acidic head groups bind with three amino acid residues (Y 473, H 449 and H323) within the LBD. A partial agonist that binds to the same binding pocket but devoid of interactions with AF-2 would result in weak recruitment of regulator proteins and low maximal efficacy (154).



*PPAR Res. 2007;2007:32696*

**Figure 2.25: Dose response curves for agonists**

As PPAR  $\gamma$  LBD domain possesses a large binding pocket. Therefore, it offers a large number of potential contact points for variety of endogenous ligands to bind in different combinations. Full agonists such as rosiglitazone, forms tight hydrogen bonds with AF-2 (activation function) domain of the LBD, which promotes recruitment of regulator proteins and induce high maximal activity. Similar to rosiglitazone, all the other small full PPAR  $\gamma$  agonists have common binding mode, in which the acidic head groups bind with three amino acid residues (Y 473, H 449 and H323) within the LBD. A partial agonist that binds to the same binding pocket but devoid of interactions with AF-2 would result in weak recruitment of regulator proteins and low maximal efficacy (154) (Figure 2.25).



However, a SPPARM bind to the LBD in such a way that leads to differential binding of co-activators or repressors to the complex. This property gives the ability to produce tissue and promoter selective gene expression. The first SPPARM that was published in the literature was GW0072. Unlike full PPAR  $\gamma$  agonists, it was observed that this compound does not directly interact with the AF-2 helix. This resulted in differential biological responses with less impact on the adipose tissue. There are several SPPARMs have been identified, which requires to be characterized at the molecular level before going forward with clinical trials.

### **2.3.8 The role of PPAR $\gamma$ agonists in the I/R injury**

As mentioned earlier in the chapter, cardiovascular diseases are the major causative factor for the mortality among diabetics. Further, diabetics sustain extensive cardiac damage when subjected to I/R injury compared to a non diabetic individual. Higher mortality rates from acute myocardial infarctions among diabetics have been reported in numerous studies (155).

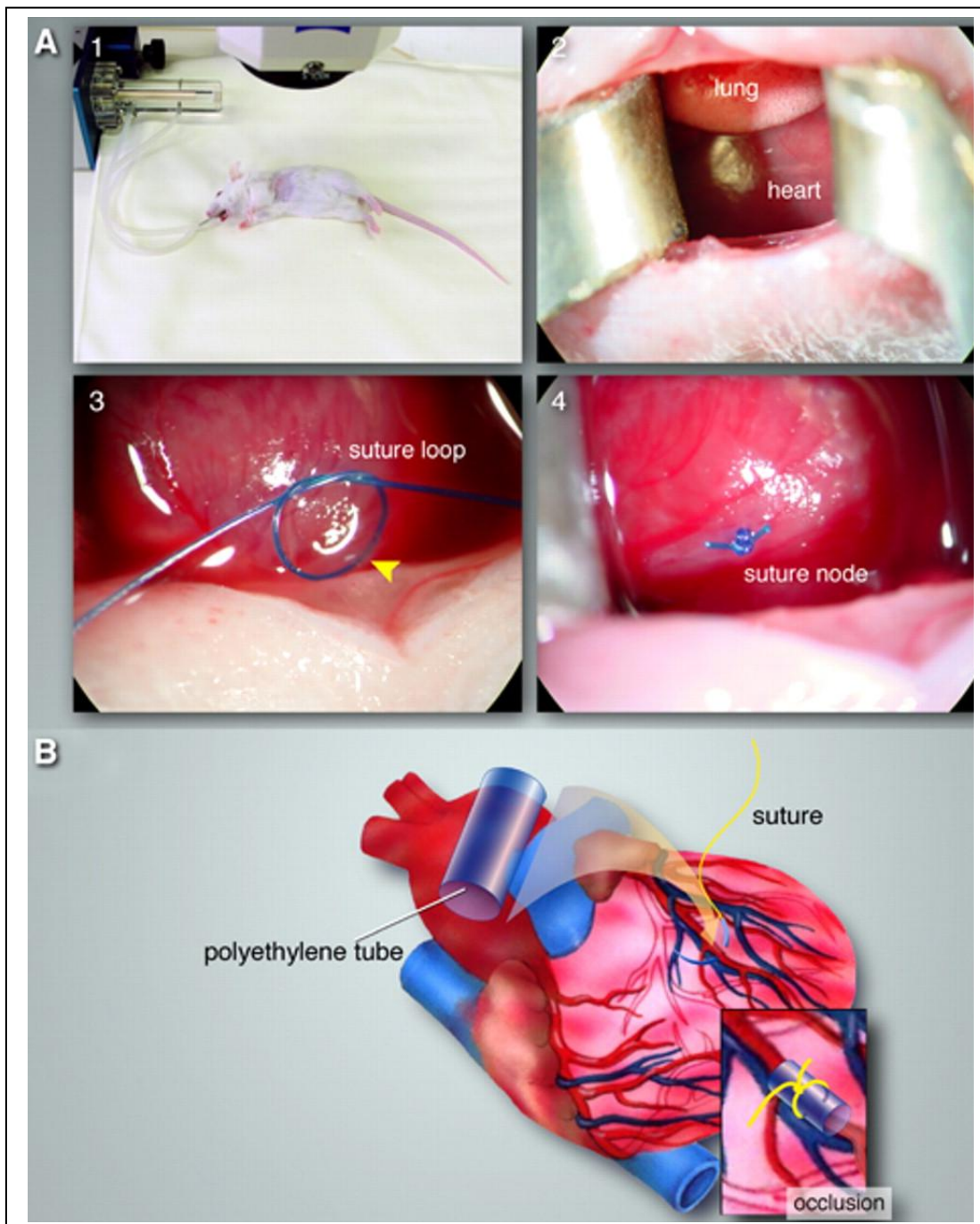
Many published reports verify that the treatment with TZDs improve the response to ischemia reperfusion injury in animal models. Majority of these studies claimed the protective effects exerted by TZDs were due to their anti-inflammatory properties (150, 156). Additionally, rosi treatment in Zucker fatty rats rescued the insulin resistance and elevated the GLUT4 levels in the myocardium, which facilitated the glucose entry during I/R insult. This prevented the ATP loss during the insult and improved the recovery of contractile dysfunction (157).

Majority of the aforementioned and published studies induce I/R in rodents. The laboratory rodents offer the advantages such as easy to handle, require less space for housing and also economically feasible. Further, the mouse strains offer the possibility of creating over-

expressed or knockdown transgenic animal models, which enable to study the particular role of a signaling molecule in the response to I/R.

Greater number of studies utilized surgical methods to induce I/R stress in animal models. According to the duration and extent of the obstruction to the blood flow, these surgical models are classified to myocardial infarction (MI), I/R injury and chronic ischemia. There are various surgical techniques available to induce MI and I/R injury. Most common method used for many studies, including the work conducted in our lab is the ligation of the left anterior descending coronary (LAD) artery. In this model, mice is anesthetized and ventilated by orotracheal intubation or by tracheotomy. Then a thoracotomy is conducted to expose the heart for LAD ligation. This method usually produces an infarction in the LV. In this model, the degree of the LV impairment is usually directly proportional to the extent of myocardial loss (158) (Figure 2.26).

PPAR  $\gamma$  is a transcription factor. Therefore, the treatment of PPAR  $\gamma$  prior to induction of I/R injury may be due to upregulation of target genes which can precondition the heart. Interestingly, we **observed that the PPAR  $\gamma$  treatment elevate the expression of hypoxia inducible factor – 1 $\alpha$  (HIF-1 $\alpha$ ) protein in response to I/R injury in the diabetic heart.** HIF-1 $\alpha$  is considered as the master transcription factor involved in upregulating myriad of genes in cells/ tissues that offer protection against hypoxia/ ischemia. Previous publications have demonstrated that PPAR  $\gamma$  treatment induces the HIF-1 $\alpha$  expression in response to hypoxia in breast cancer cell line and the adipose tissue (159, 160). Therefore, we postulated that TZDs can elevate the HIF-1 $\alpha$  response in tissues, therefore PPAR  $\gamma$  – HIF-1 $\alpha$  signaling to be a potential mechanism which offers protection against I/R injury in the heart.



*Cardiovasc Res.* 2007 Apr 1;74(1):29-38. Epub 2006 Nov 23

**Figure 2.26: LAD ligation in a mouse**

### 2.3.9 HIF-1 $\alpha$ and the ischemia response in the heart

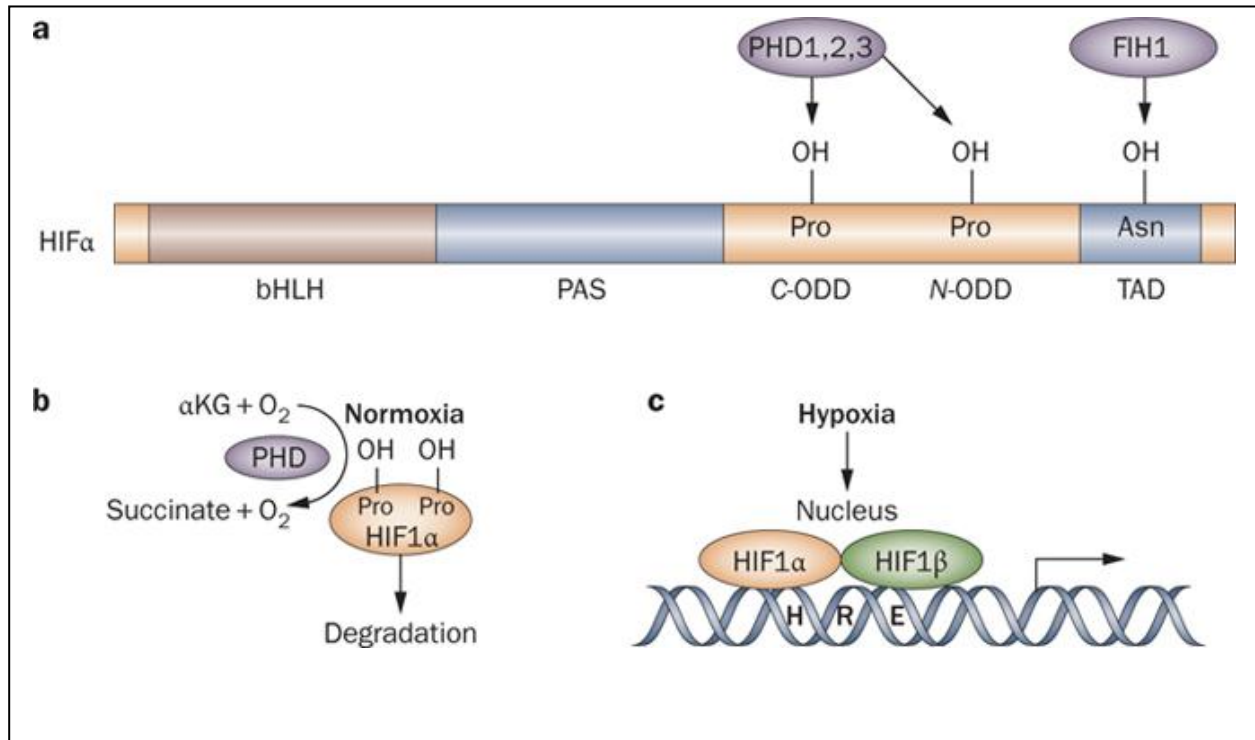
The functions of the organs, tissues and cells are restricted when the oxygen levels drop below a certain threshold value. Generally, hypoxia constitutes when the oxygen partial pressure is reduced more than 40mmHg in the arterial blood (161). As mentioned above, HIF-1 $\alpha$  is a key regulator which regulates the induction of many genes that facilitate adaptation and survival of cells and the organisms from normoxia to hypoxia. The functions of HIF-1 $\alpha$  have been extensively studied and more than 100 of genes were identified to be directly regulated by this transcription factor (162). Despite HIF-1 $\alpha$  being protective against hypoxia in healthy tissues, it is correlated to poor prognosis in cancer because it facilitates tumor growth (163).

The HIF transcription factor is a heterodimer comprised of HIF-1 $\alpha$  subunit and the constitutively expressed HIF-1 $\beta$  subunit. So far, three isoforms of HIF-1 $\alpha$  are characterized and they are referred as HIF-1 $\alpha$ , HIF-2 $\alpha$  and HIF-3 $\alpha$ . Out of these three isoforms, HIF-1 $\alpha$  and HIF-2 $\alpha$  are well characterized (164). Unlike HIF-1 $\beta$  which is constitutively expressed in the nucleus, HIF-1 $\alpha$  expression is tightly regulated under normoxic conditions. The HIF-1  $\alpha$  and  $\beta$  heterodimer recognizes and binds to the consensus sequence 5'-(A/G)CGTG-3' named as hypoxia response elements (HRE) to activate the transcriptional activity of target genes (165).

This strict coordination of HIF-1 $\alpha$  expression in normoxic and hypoxic environments are regulated by PHDs (Prolyl hydroxylase) and pVHL, which is an E3 ubiquitin ligase (Von Hippel – Lindau tumor suppressor protein).

In the presence of oxygen, PHDs post-transcriptionally modifies HIF-1 $\alpha$  by hydroxylating at specific proline residues (Pro402 and Pro564) located in its oxygen dependent degradation (ODD) domain. The hydroxylation of these specific proline residues targets the HIF-

HIF-1 $\alpha$  for pVHL mediated proteasomal degradation. This reaction requires oxygen, 2-oxoglutarate and ascorbate (166) (Figure 2.27).



*Nature Reviews Gastroenterology and Hepatology* 7, 281-287 (May 2010)

**Figure 2.27: Mechanisms of HIF-1  $\alpha$  stabilization**

(a) The PHDs can hydroxylase the prolines in the ODD. (b) Depiction of HIF hydroxylation by  $\alpha$  – ketoglutarate, O<sub>2</sub> and PHD in normoxia. (c) During hypoxia, HIF-1  $\alpha$  subunit binds with the HIF –  $\beta$  subunit within the nucleus to become transcriptionally active.

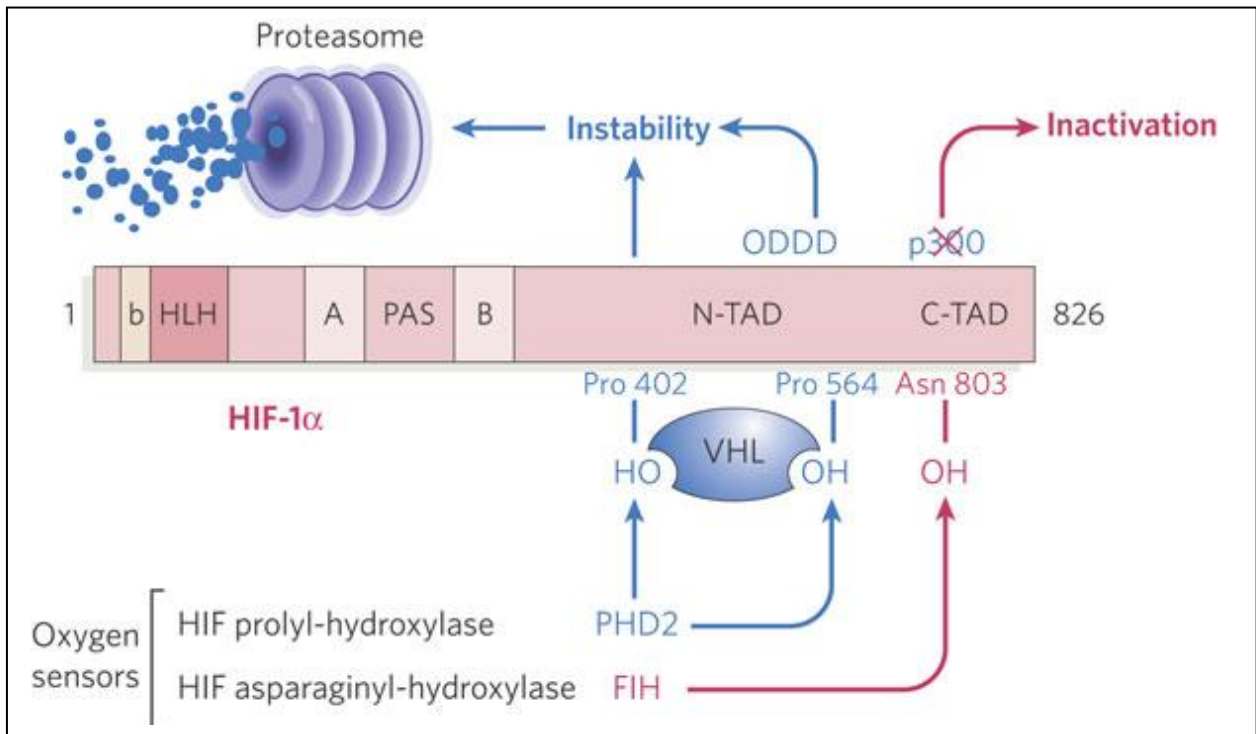
Under hypoxic conditions, PHD activity is inhibited because of the limited oxygen levels in the cell. Therefore, HIF-1 $\alpha$  gets stabilized and accumulated in the cells. Also, under hypoxic conditions, the mitochondria increases the ROS production which oxidize Fe<sup>2+</sup> to Fe<sup>3+</sup>. Fe<sup>2+</sup> is a cofactor required for the function of PHD. Therefore, this reaction will further inhibit PHD activity, leading to stabilization of HIF-1 $\alpha$  (167). Therefore, hypoxia can be chemically

mimicked by iron chelation by utilizing compounds such as desferrioxamine (DFO), use of 2-oxoglutarate analogs such as dimethylxalylglycine (DMOG) or substitution of  $\text{Fe}^{2+}$  by metal ions such as cobalt (168).

The ODD domain of the HIF-1 $\alpha$  consists of two portions: N-terminal ODD and C-terminal ODD, and both these domains can interact with pVHL independently (169). Further, two transactivation domains (TAD) were identified in HIF-1 $\alpha$ . These transactivation domains are referred to as N-terminal TAD and C-terminal TAD. The N-terminal TAD overlaps with the C-terminal ODD (170). In addition to the regulation of HIF-1 $\alpha$  by proline hydroxylation mentioned above, stabilization of HIF-1 $\alpha$  is also regulated by a hydroxylation reaction (at Asn 803) mediated by asparaginyl hydroxylase known as factor inhibiting HIF (FIH). This modification prevents interaction of HIF-1 $\alpha$  C-TAD with other cofactors such as CBP/ p300 (171).

This co-activators that bind to HIF-1 $\alpha$  stabilizes the transcription initiation complex containing RNA polymerase II. Also, these co-activators have histone acetyl transferase activity which remodels chromatin and expose the DNA for polymerase to access and transcribe it into RNA. The work by *Kung et al* describes that p300 binds to the CH1 domain located in the C-TAD of HIF-1 $\alpha$  (172). N-TAD transactivates less effectively than C-TAD, however, C-TAD and N-TAD function together synergistically (173) (Figure 2.28).

The genes regulated by HIF-1 $\alpha$  and consequences of HIF-1 $\alpha$  activation varies from one cell type to another. Currently, there are myriad of genes found to be regulated by HIF-1 $\alpha$ . So far, the best characterized processes of HIF-1 $\alpha$  regulation are erythropoiesis, angiogenesis and glucose uptake. Activation of all these biological pathways under a hypoxic insult exerts adaptive response in the cells and promotes survival (174).



*Nature* **441**, 437-443(25 May 2006)

**Figure 2.28: Different HIF-1 $\alpha$  domains**

### 2.3.10 Alternative mechanisms stabilizing HIF-1 $\alpha$ under normoxic conditions

Certain growth factors and cytokines are known to stabilize HIF-1 $\alpha$  under normoxic conditions. These include insulin and insulin like growth factors 1 and 2. It was found that insulin treatment in cell culture leads to stabilization of HIF-1 $\alpha$  and augments HIF-1 $\alpha$  mediated gene transcription. Further it was demonstrated that insulin mediated stabilization of HIF-1 is via Akt/ PI3K pathway (175). Nitric oxide (NO) also has been found to stabilize HIF-1 $\alpha$  under normoxic conditions.

### 2.3.11 Hyperglycemia and HIF-1 $\alpha$ signaling pathway

Recent published reports provide ample evidence that the glucose levels in the cells affects the expression and activation of HIF-1 $\alpha$  in response to hypoxia. It has been found that the relationship between glucose and HIF-1 $\alpha$  can be two ways. HIF can profoundly regulate the expression of most of the enzymes involved in glycolysis and GLUT1 and GLUT3 expression which mediates glucose uptake in the cells. In contrast, glucose uptake and glycolysis can adversely affect the stability and activation (176, 177). Even though glucose alone cannot regulate HIF-1 $\alpha$  in the absence of hypoxia, normoglycemic levels are vital for HIF-1 $\alpha$  protein and activation in response to hypoxia (176).

Many studies support the presence of reduced levels of HIF-1 $\alpha$  in the cells or tissues obtained from diabetic individuals and animal models. This observation was further consolidated in cells cultured in medium containing high glucose. Therefore, it is understood that hyperglycemia is responsible for compromised HIF-1 $\alpha$  protein expression and impaired transactivation function in response to hypoxia in diabetic tissues. Even though exact mechanism how diabetes impair HIF-1 $\alpha$  is not fully elucidated, some recent publications suggest few possible pathways which may help to explain this inhibition (178).

- The negative effects of methylglyoxal on HIF-1 $\alpha$

Methylglyoxal (MG) is a highly reactive by product formed in many metabolic pathways. The production of MG is increased in hyperglycemic conditions. The main adverse effect of MG is that it forms stable adducts primarily with arginine residues of intracellular proteins, and



induce the formation of advanced glycation end products (AGE) which alters the normal functions (179). In a hyperglycemic setting, ROS production is increased which inhibit the glycolytic enzyme glyceraldehyde – 3 – phosphate dehydrogenase (GAPDH) and subsequent upstream triose phosphate glycolytic metabolite accumulation, which leads to production of increased MG levels (179). Generally, the MG that is formed in metabolic reactions undergo catabolism via a rate limiting enzyme called glyoxalase I (GLO I). The accumulation of MG under diabetes or hyperglycemic conditions inhibits HIF-1 $\alpha$  activity and stability in response to ischemia (180).

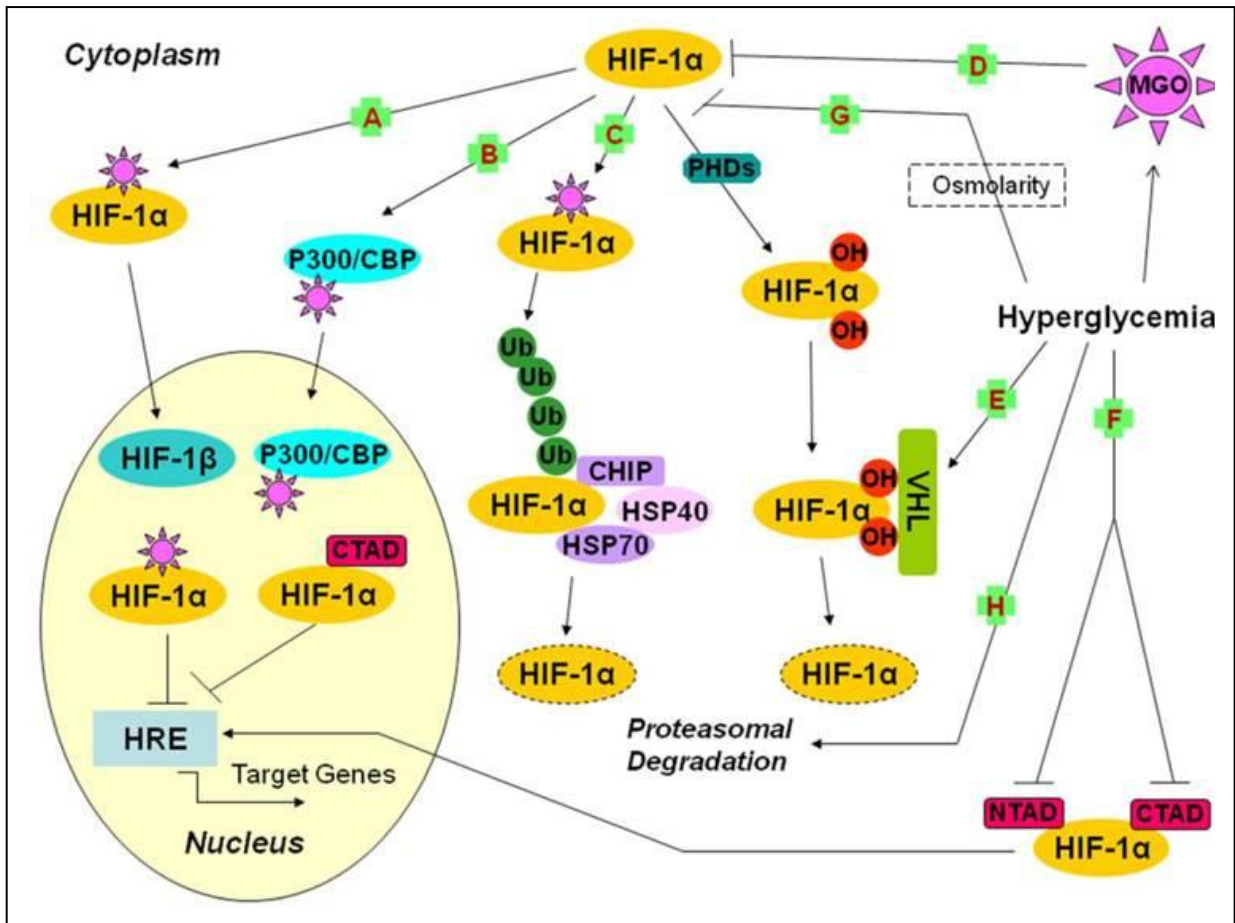
One mechanism which explains the reduced HIF-1 $\alpha$  activity in presence of MG is that it induces a covalent modification of HIF-1 at arginine 17 and arginine 23 of the bHLH domain which decreases the heterodimers formation of HIF-1  $\alpha$  and  $\beta$ , thus inhibits the binding of the complex to HRE of the target genes (180). Additionally, it was also found that MG forms a covalent interaction with p300, which prevents its binding to the C-TAD of HIF. This was responsible for impaired transcriptional activation of HIF-1 $\alpha$  (181). Also, it was demonstrated that MG caused its increasing association with heat shock protein 40/70 (Hsp 40/70), which in turn recruits heat shock cognate protein 70 (Hsc – 70) interacting protein (CHIP), which is a ubiquitin ligase leading to proteasomal degradation. It is also demonstrated that this reaction does not require hydroxylation of proline residues on HIF-1 $\alpha$  (182) (Figure 2.29).

➤ The role of ROS in HIF-1 $\alpha$  pathway

Hyperglycemia induces ROS production, which also is capable of modulating HIF-1 $\alpha$  activity. ROS has been found activate PHD and induce post translational modification in the

presence of Fe and increases ubiquitin proteasomal activity (183). Another mechanism that is suggested is reaction of ROS with NO and altering the signaling mechanisms induced by NO in the biological systems (184). NO is identified as a HIF-1 $\alpha$  inducer (185).

Additionally, Rac1, which belongs to a small GTP<sub>ase</sub> of the RHO family also found to play a significant role in determining intracellular redox status. Further, it also induces HIF-1 $\alpha$  protein and transcriptional activity in response to hypoxia. Therefore, increased ROS levels in diabetes elevate ROS levels which can suppress Rac1 activity, leading to repressed HIF-1 $\alpha$  expression (186).



*Int J Med Sci 2013; 10(10):1412-1421*

**Figure 2.29: Hyperglycemia and HIF-1 $\alpha$  signaling**

**The covalent modification of p300 leads to inhibition of the interaction of c-TAD and p300, which decrease the transactivation ability of HIF-1 $\alpha$ .**

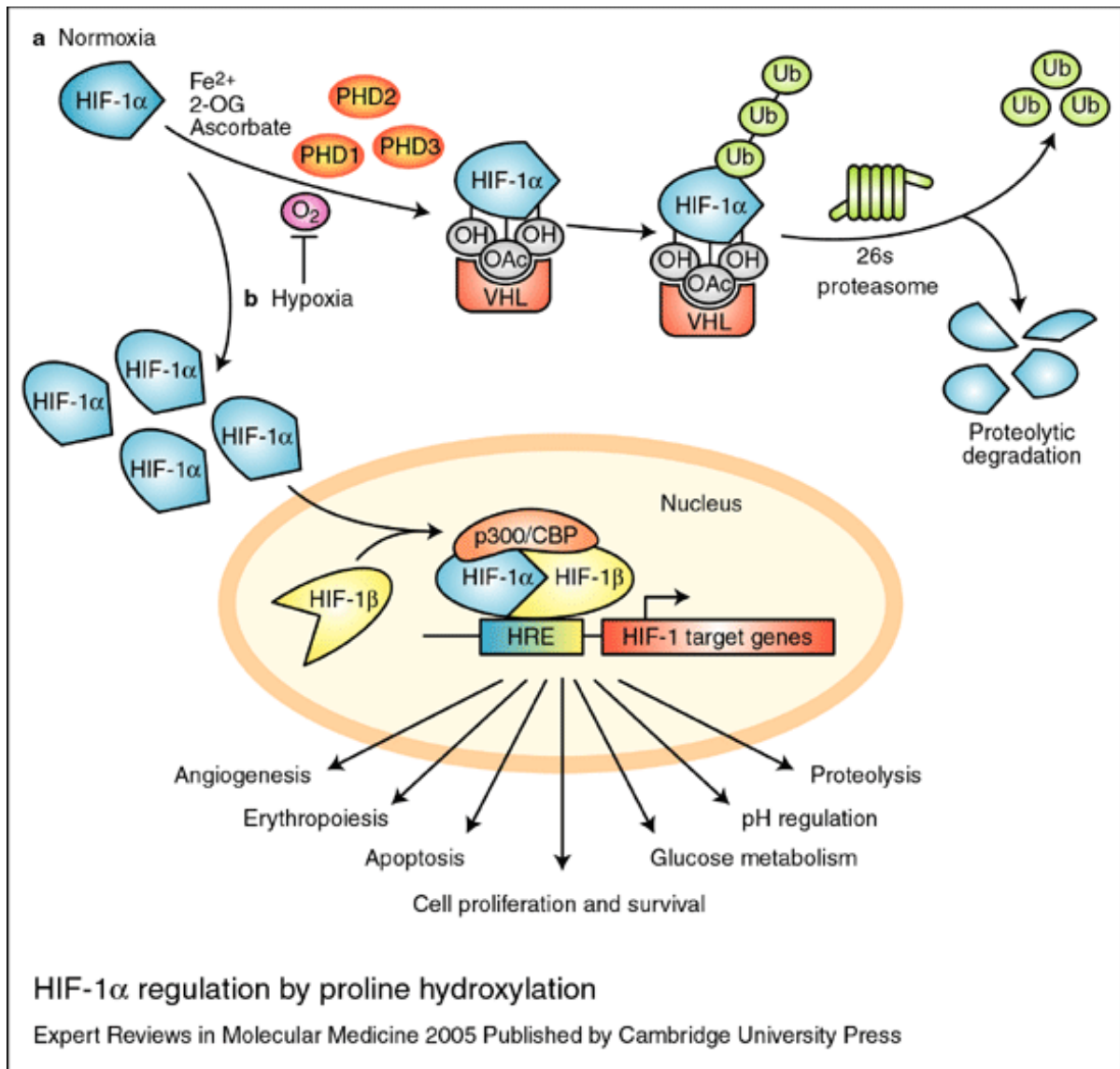
- Increased activity of pVHL

Previous publications report that hyperglycemia increase the activity of pVHL machinery leading to increased HIF-1 degradation in fibroblasts. Further, transactivation of C-TAD and N-TAD was also inhibited by high glucose levels (187).

### **2.3.12 Genes regulated by HIF- 1 $\alpha$**

The HIF-1 $\alpha$  mediated regulation of genes that modulate angiogenesis, glucose uptake and metabolism are well established. VEGF (vascular endothelial growth factor), which regulates angiogenesis is the most prominent gene regulated by HIF-1 $\alpha$  in vascular biology. Also, many genes that regulate glucose uptake and glycolysis were identified as HIF-1 $\alpha$  target genes (188) (Figure 2.30). Up to date, it was found that HIF-1 $\alpha$  regulates more than 100 genes. Our lab found new two targets which are regulated by HIF-1 $\alpha$  in cardiomyocytes in response to hypoxia. These new targets are

- **Adiponectin**
- **Fratxin**



*Expert Reviews in Molecular Medicine: Vol. 7; Issue 6; 15 April 2005*

**Figure 2.30: The cellular processes regulated by HIF-1 $\alpha$**

There, HIF-1 $\alpha$  dimerises with HIF-1 $\beta$ , binds to hypoxia-response elements (HREs) within the promoters of target genes and recruits transcriptional co-activators such as p300/CBP for full transcriptional activity. A range of cell functions are regulated by the target genes, as indicated.

### 2.3.13 Adiponectin

Many studies have emerged highlighting the importance of adiponectin in human cardiac pathologies as well as metabolic diseases. These cardiac pathologies such as coronary artery disease, hypertension and myocardial infarction have been correlated to attenuated circulating adiponectin levels (189-191). Furthermore, hypoadiponectemia is associated with obesity and diabetes (192-194), indicating its correlation with systemic insulin sensitivity and the ability to directly regulate whole body energy metabolism, including the heart. In healthy lean humans, circulating adiponectin levels range from 2-30 mg/L (195). Adiponectin has attracted much attention lately because of its cardioprotective effects, which are attributed to its anti-inflammatory, insulin sensitizing and antiatherogenic properties (196-198).

Adiponectin is a protein hormone consisting 247 amino acids and is found in chromosome 3q27, which is considered to be a locus that is highly susceptible for the development of metabolic syndrome and coronary heart disease (199-201). Interestingly, single nucleotide polymorphisms in the adiponectin gene have been reported in humans. Some of these polymorphisms markedly reduce plasma adiponectin levels and predispose the carriers to insulin resistance (202). I164T polymorphism (isoleucine is substituted by threonine at the 164<sup>th</sup> position) that is reported to be a causative factor for hypoadiponectemia and the development of type 2 diabetes. Furthermore, individuals with this mutation are highly susceptible for the development of hypertension and coronary artery disease, suggesting the protective role of adiponectin against development of heart disease in humans (203, 204). Various other polymorphisms in the adiponectin gene were reported in different ethnic groups

that were strongly associated with the development of insulin resistance and increased risk for cardiovascular diseases (202, 205-207).

### **2.3.14 Cardiac derived adiponectin**

Adiponectin, once thought of being exclusively secreted by adipose tissue has now been found to be secreted by human and murine cardiomyocytes as well. The levels of adiponectin produced by cardiomyocytes are relatively low in comparison to amounts produced by adipose tissue; therefore its contribution towards the circulating levels of adiponectin is minimal. However, adiponectin produced by cardiomyocytes can directly regulate cardiac metabolism by AMPK activation. These effects are found to be sequestered via cardiac AdipoR1 and AdipoR2, indicating cardiac derived adiponectin to act via an autocrine/ paracrine mechanism (208). Further work by *Amin et al* has shown that the adiponectin produced by cardiomyocytes is multimeric in form and is functional (209). Cardiac derived adiponectin and cardiac adiponectin receptor expression is significantly reduced in human hearts with moderate to severe dilated cardiomyopathy (DCM). This down regulation of cardiac derived adiponectin was found to be functionally significant and may play a significant role in the pathogenesis of heart failure in humans (210). Furthermore, the exact molecular signaling pathway of adiponectin (systemic and cardiac derived) mediated cardioprotection in humans is not known.

### **2.3.15 Adiponectin and the diabetic heart**

Cardiovascular complications are the leading cause for morbidity and mortality in diabetics (211). Decreased adiponectin levels are observed in patients with type II diabetes (193) and many studies support the predictive nature of low plasma adiponectin for the future development of diabetes (212-214). Furthermore, it was shown that increased plasma adiponectin levels significantly increases insulin sensitivity independent of percentage of body fat and obesity in human subjects (215).

Cardiac sensitivity for adiponectin is altered during the progression of type I diabetes. At the initial stages of the disease both exogenous and endogenous adiponectin fails to protect the heart from cardiac damage sustained from I/R injury, which may be due to significant reduction in AdipoR1 expression in the heart at this stage. The levels of AdipoR1 are restored in the heart as type I diabetes progresses. However, during the late stages of type 1 diabetes, the systemic levels of adiponectin are attenuated and therefore, the cardiomyocytes are more susceptible to damage from I/R injury (99).

The significance of cardioprotection from adiponectin in the diabetic heart is illustrated in db/db mice that were subjected to TAC. The db/db mice developed greater left ventricular posterior wall thickness and increased intraventricular septum when compared to the sham mice. The maladaptive response to TAC observed in db/db mice was ameliorated by adenovirus mediated supplementation of adiponectin (216). Furthermore, short term adiponectin treatment can significantly ameliorate the contractile dysfunction associated with elevated endoplasmic reticulum (ER) stress in db/db cardiomyocytes. Adiponectin treatment significantly improved calcium handling and cardiomyocyte contraction in db/db cardiomyocytes (217).

In contrast to hypertrophy and heart failure, myocardial energy substrate utilization shifts more towards fatty acid oxidation from glucose oxidation in obesity and diabetes (218). In diabetic/ obese heart, fatty acid uptake is elevated to a level that cannot be met by the rate of fatty acid oxidation, leading to lipid accumulation and lipotoxicity (26, 219). One of the contributing factors for cardiac contractile dysfunction associated with diabetes and obesity is lipotoxicity (219). Therefore, adiponectin can exert beneficial effects on the diabetic/ obese heart by increasing the efficiency of fatty acid oxidation and utilization and attenuate lipotoxicity. It was observed that adiponectin-AMPK signaling was able to enhance the insulin mediated phosphorylation of Akt, thus stimulate the glucose uptake in primary adult cardiomyocytes (220). Therefore, adiponectin can offer a profound protection against the adverse cardiac events associated with metabolic derangement in the diabetic/ obese heart.

**Currently we have preliminary data indicating the presence of HRE sites in the adiponectin promoter. The data we gathered is currently being formulated in to a manuscript.**



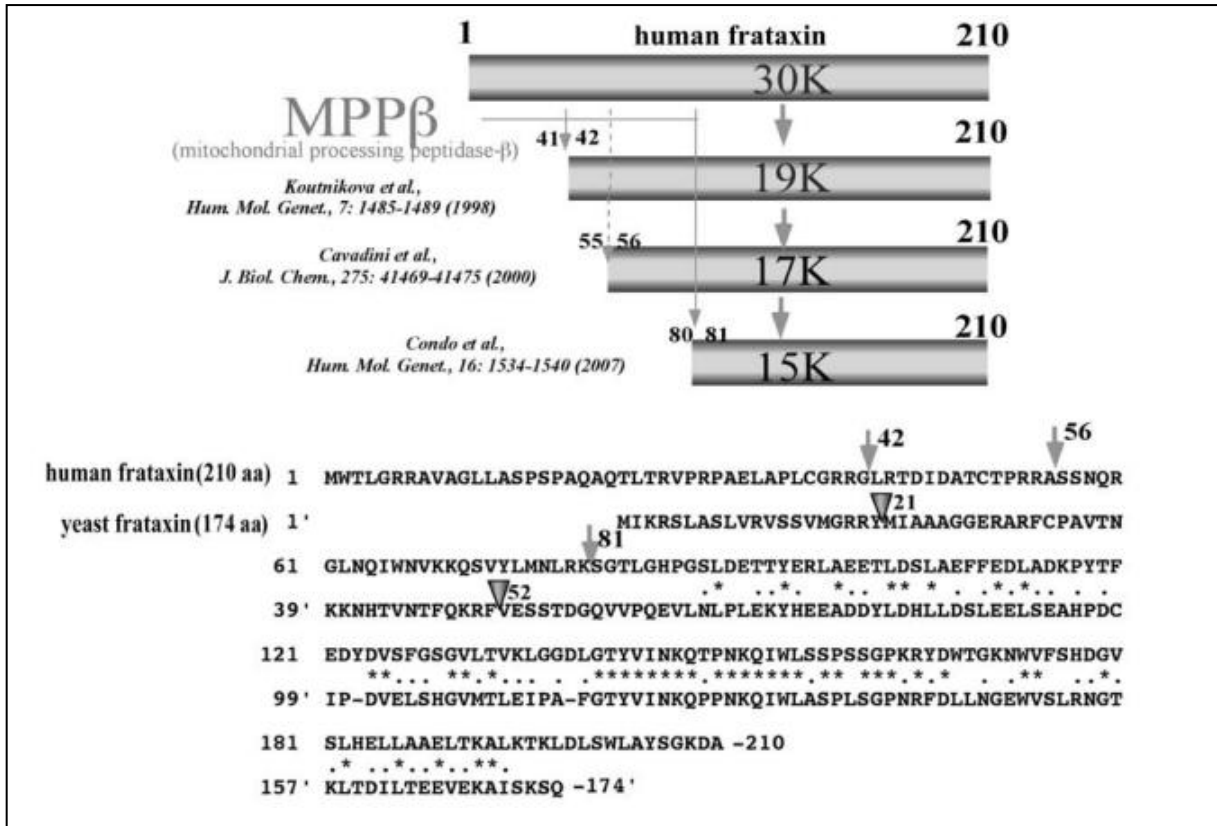
### **2.3.16 Frataxin**

Frataxin is a nuclear encoded mitochondrial protein. It is highly conserved protein found in both eukaryotes and prokaryotes. The exact function of this protein remains controversial, however, it is identified to play a role in iron homeostasis by acting as a chaperone during Fe-S cluster production in the cells. These Fe-S clusters get incorporated to the active site and facilitate the normal functions of enzymes that are required for oxidative phosphorylation. Further it was also identified as a factor that can reduce ROS production, thus control oxidative stress in the cells. Additionally, some reports claim that it acts as an iron storage protein during conditions of iron overload. Frataxin is primarily located in the mitochondria. Also, frataxin mRNA was abundant in tissues with high metabolic rate such as heart, neurons, kidney and liver (221).

Friedreich's ataxia is an autosomal recessive inherited genetic disorder that results in inability to produce frataxin protein. This leads to disruption of cellular homeostasis and severe cardio and neuro complications (222). This disorder is manifested due to extensive GAA repeats found in the first intron of the frataxin gene, which adopts abnormal DNA structures and impair frataxin transcriptions (223). It was also found that the length of the GAA expansion is proportional to the degree of frataxin deficiency in FRDA individuals. Cardiomyopathy arises in patients with larger expansion, and it does not depend on the duration of the disease. However, insulin resistance is usually observed in the later stages of the disease (224).

Frataxin is synthesized as a precursor polypeptide which requires to be proteolytically cleaved to become the mature form. The human frataxin is 210 amino acids long and is targeted to the mitochondria via its N-terminal transit sequence where it undergoes two step proteolytic

digestion mediated by mitochondrial processing peptidase (MPP). The mature form of frataxin is found in the matrix of the mitochondria (225) (Figure 2.31).



*Antioxid Redox Signal. 2008 Jun;10(6):997-1030. doi: 10.1089/ars.2007*

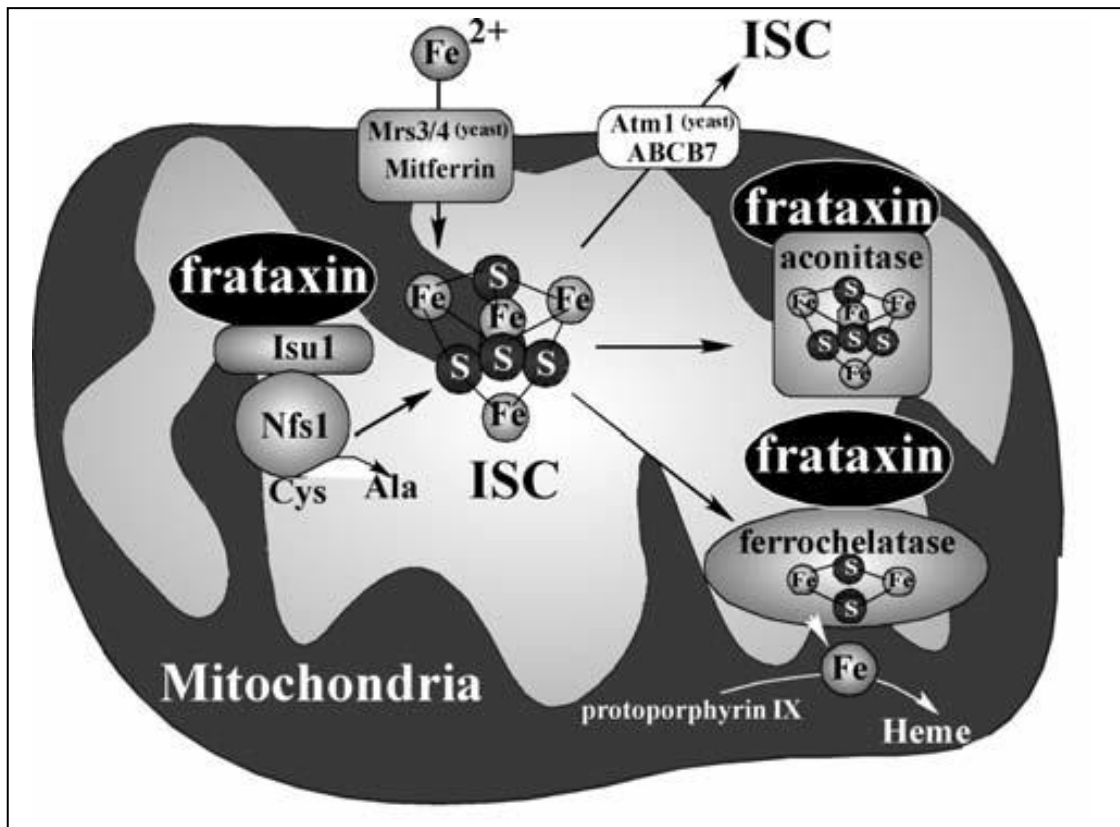
**Figure 2.31: Maturation of frataxin**

Protein processing of human frataxin by mitochondrial protein peptidase-β (MPP-β) is summarized (*top*). The comparison of amino acid sequences between human and yeast frataxin and their cleavage sites by MPP-β is shown (*bottom*).

### 2.3.17 Frataxin, heme synthesis and Fe-S cluster biogenesis

Many published reports confirm that frataxin is involved in heme synthesis. Downregulation of frataxin is shown to reduce the expression of proteins which regulate heme

biosynthesis pathway in both yeasts and humans. Heart cells obtained from frataxin deficient mice demonstrated that downregulation of iron sulfur scaffold protein Iscu, mitochondrial coproporphrinogen oxidase and ferrochelatase which are involved in heme synthesis. Frataxin interacts with ferrochelatase and facilitate the insertion of  $\text{Fe}^{2+}$  in to protoporphyrin IX to construct heme in the cells (226).



*Antioxid Redox Signal.* 2008 Jun;10(6):997-1030. doi: 10.1089/ars.2007

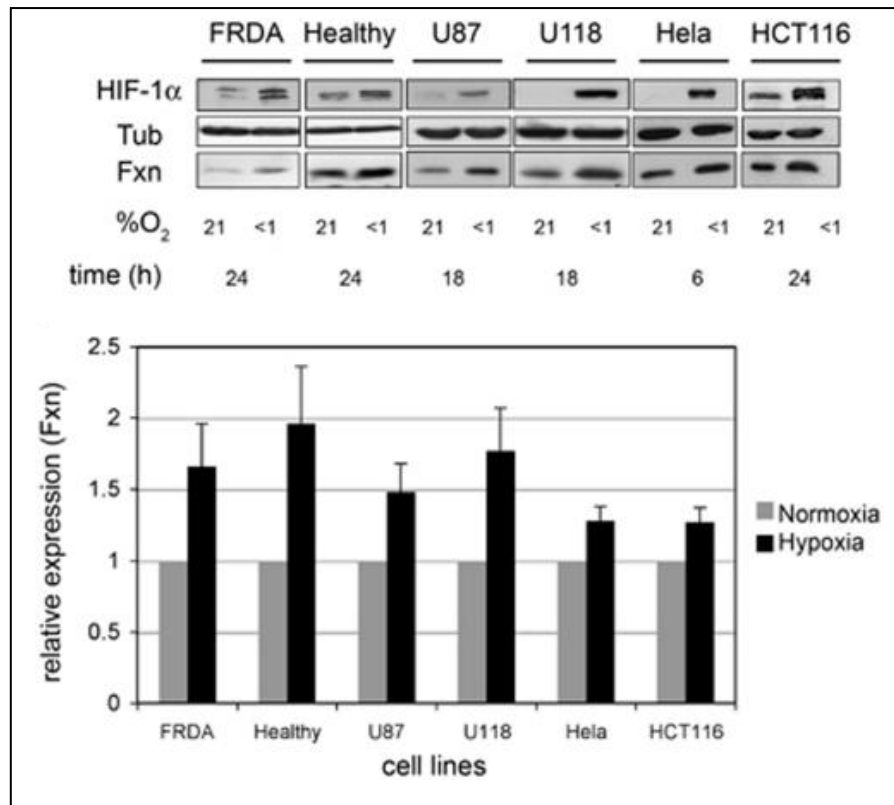
**Figure 2.31: The role of frataxin in heme formation and Fe-S cluster synthesis**

Frataxin interacts with a complex of the ISC scaffold protein Isu1 and the cysteine desulfurase Nfs1, which release sulfur from cysteine. Frataxin also interacts with ferrochelatase and facilitates the heme synthesis through ferrochelatase-mediated insertion of ferrous iron into protoporphyrin IX. Frataxin protects aconitase activity by restoring the aconitase 4Fe-4S cluster.

Further, the role played by frataxin in synthesis of Fe-S clusters is well established. Fe-S clusters are essential cofactors for functioning of aconitase in the Krebs cycle and complexes I & III in the electron transport chain. Therefore, frataxin plays a pivotal role in oxidative phosphorylation. Further Fe-S clusters are required to maintain the normal function of other proteins which regulate purine metabolism, amino acid and heme biosynthesis pathways. Frataxin acts as a chaperone for Fe<sup>2+</sup> and interacts with ISCU scaffold protein and Nfs1 to synthesis Fe-S clusters (Figure 2.31).

### **2.3.18 Frataxin and hypoxia**

Not many literatures are found which reports the role of frataxin played in hypoxia/ ischemia. However, *Guccini et al* reports that frataxin expression is upregulated in several tumor cell lines in response to hypoxic stress. Further, they demonstrate that the frataxin expression is elevated in human tumors in response to hypoxic stress and suggests that frataxin may play a role in tumor cell survival and progression (227) (Figure 2.33).



*Cell Death Dis. 2011 Feb 24;2:e123. doi: 10.1038/cdis.2011.5*

**Figure 2.33: Frataxin expression is increased in response to hypoxia in tumor cell lines**

(a) FRDA patient-derived B cells (FRDA) and respective control cells (Healthy), human glioblastoma U87, U118, colon carcinoma HCT116 and HeLa cells were placed for different time exposure (24 h for FRDA, healthy control-derived B cells and HCT116, 18 h for U87, U118 and 6 h for HeLa) into hypoxic chamber and frataxin (Fxn), tubulin (Tub) and HIF-1 $\alpha$  expression was analyzed by western blot. Data are representative of 6, 11, 4, 7, 5 and 3 independent experiments for FRDA, Healthy, U87, U118, HeLa and HCT116 respectively. (b) Densitometric quantification of frataxin upregulation. Frataxin expression was normalized with tubulin and frataxin expression in normoxia set to one. Data represent the mean $\pm$ 1 S.E.M. from the different independent experiments performed for each cell line described in a. *P*-values were calculated with Student's *t*-test and were statistically significant (*P*<0.05) for all cell lines analyzed

### **3. PPAR $\gamma$ Activation improves the Molecular and Functional Remodeling of $I_{to}$ by Angiotensin II**

#### **Abstract**

Patients with diabetes have been shown to have significantly altered renin-angiotensin system (RAS). Recently it has been determined that hyperglycemic conditions induce an increase in angiotensin II expression; specifically by cardiomyocytes. Altered RAS has also been shown to be associated with an increase in oxidative stress and cardiac contractile dysfunction leading to the development of cardiac hypertrophy. The transient outward potassium current ( $I_{to}$ ) in cardiac myocytes is mainly mediated by members of the Kv4 subfamily of voltage gated potassium channels and has been shown to be altered during the development of cardiac hypertrophy. However it is not clear as to how AT II affects the pore forming complex at the cell membrane and thus directly affects the Ito current, which is the focus of the current study. The current study examines the protective effect of PPAR-gamma ( $\gamma$ ) ligands on cardiomyocyte Ito by preventing NADPH Oxidase activation and the ensuing ROS formation. Furthermore short term PPAR $\gamma$  activation in diabetic leptin deficient db-/db- mice displayed an improvement in the membrane association of the Kv4 subfamily of voltage gated potassium channel members. These findings demonstrate that PPAR- $\gamma$  agonist, have the potential to reduce cardiomyocyte specific contractile dysfunction associated with NADPH Oxidase mediated ROS formation.

## Introduction

This chapter discusses how diabetes impairs the expression of outward potassium currents ( $I_{to}$ ) channels in cardiomyocytes. Further, the role played by PPAR  $\gamma$  agonism in rescuing the cardiomyocytes from this diabetes mediated adverse effect will be also discussed in this chapter. The work in this chapter had been already published in current pharmaceutical design journal (Current Pharmaceutical Design 2013;19(27):4839-47).

Diabetes Mellitus (DM) is at epidemic proportions in United States and is associated with increased risk of cardiovascular events which represent a major public health problem. One of the major complications that arise with DM is diabetic cardiomyopathy, which is recognized as a myocardial disease manifested independent of hypertension, coronary artery disease (CAD) and other cardiac pathologies (22). Diabetic cardiomyopathy can be fatal as it significantly increases the incidences of cardiac arrhythmias, including ventricular fibrillation (63). The causative factors for these life threatening arrhythmias are electrophysiological abnormalities which are shown by altered electrocardiograms (ECG). Some of these alterations in the ECG of the type I and type II diabetics include prolonged QT interval and increased QT dispersion (73). The QT interval in an ECG reflects the duration of ventricular depolarization and repolarization and prolonged QT interval is associated with sudden death (228). The QT interval varies between different leads and this range of intervals is referred to as QT dispersion. Increased QT dispersion is associated with increased cardiovascular morbidity and mortality (229). The reasons for QT abnormalities in diabetes are not completely understood, although cardiac potassium currents have been suspected to be involved.

Findings from a recent study indicate that attenuation of transient outward potassium current ( $I_{to}$ ) markedly prolonged the QT interval in mouse heart (88).  $I_{to}$  is activated and deactivated rapidly in the myocardium and is known to play a major role in regulating the rapid polarization during phase 1 of the action potential (84, 85). Diabetes induces suppression of  $I_{to}$  in rodents which result in decreased repolarization rate (89). It was also reported that diabetes can suppress the recovery of  $I_{to}$  in the myocardium (90). The main channel forming sub-units of  $I_{to}$  are Kv 4.3, Kv 4.2 and Kv 1.4 which differ in their kinetic properties. Additional ancillary subunits such as Kv channel interacting proteins (KChIP) are involved in regulation of kinetics of the main channel forming subunits of  $I_{to}$  (230). Diabetes also impacts the expression of the  $I_{to}$  channels by attenuating the expression of the channel forming sub units which can cause a reduction in total potassium current amplitude (107, 108). How diabetes induces alterations in the  $I_{to}$  channel sub units are not fully known. However, it was observed that angiotensin II (ATII), which is upregulated during diabetes, can profoundly affect the expression of molecular components of the  $I_{to}$  (111).

AT II is a peptide hormone and an integral part of Renin-Angiotensin system (RAS), and is known to induce many biological actions in the heart including hypertrophy. RAS which was initially identified as a circulating hormonal system involved in regulating blood pressure. However, many reports emerged supporting the presence of a functional tissue RAS (tRAS) in the heart which has the capability of producing cardiac hypertrophy (58, 231). This intracellular ATII can increase the oxidative stress and apoptosis in the cardiomyocytes. Recently, a significant increase of cardiac tRAS was reported in diabetic rat heart (60). Therefore, the enhanced expression of ATII during diabetes may exert a significant impact on the molecular component of  $I_{to}$  and thus lead to alterations in ionic currents in the heart and altered ECG.



Therapies that can improve the  $I_{to}$  may exert a profound protection against development of life threatening cardiac arrhythmias in the diabetic heart.

In this study, we demonstrate that treatment of Rosiglitazone in cardiac myocytes can ameliorate the AT II induced alterations in pore forming units of  $I_{to}$ . Given the prominent role of PPAR $\gamma$  in regulating cardiac metabolism and its regulatory role in modulating inflammatory response, we hypothesized that PPAR $\gamma$  may be involved in the protective mechanism against ATII mediated down regulation of the  $I_{to}$ . Our results underscore the influence of changes on  $I_{to}$  density by AT II and that PPAR $\gamma$  improves the alteration of the pore forming subunits of  $I_{to}$  from the cell membrane. Our findings in the current study indicate that PPAR $\gamma$  may rescue this effect by preventing ATII induced ROS formation. Furthermore it has been previously shown that both diabetes (db/db mouse model) as well as the infusion of ATII into normal mice for 5 days down regulates the  $I_{to}$ , however the exact signaling mechanism to this remains unclear (232, 233). In the current study we demonstrate the mechanism by which  $I_{to}$  is down regulated by AT II and diabetes in cardiomyocytes. The findings from this study better portray as to how diabetes associated stress such as ATII influences the down regulation of  $I_{to}$  and the potential development of contractile dysfunction.

Given the prominent role of PPAR $\gamma$  in regulating cardiac metabolism and its regulatory role in modulating inflammatory response, we hypothesized that PPAR $\gamma$  may be involved in the protective mechanism against AT II mediated down regulation of the outward  $I_{to}$  density. Our results underscore the influence of changes on  $I_{to}$  density by AT II and that PPAR $\gamma$  improves the alteration of the pore forming subunits of  $I_{to}$  from the cell membrane. Our findings in the current study indicate that PPAR $\gamma$  may rescue this effect by preventing ATII induced ROS formation. Furthermore it has been previously shown that both diabetes (db/db mouse model) as well as the

infusion of AT II into normal mice for 5 days down regulates the outward density ( $I_{to}$ ), however the exact signaling mechanism to this remains unclear (232, 233). In the current study we demonstrate the mechanism by which  $I_{to}$  is down regulated by AT II and diabetes in cardiomyocytes.

## **Material and methods**

### **Animals:**

All animal procedures were reviewed and approved by the Auburn University Institutional Animal Care and Use Committee. 10 weeks old male db/db and C57bl/6 mice were purchased from Jackson Laboratories. Mice were given an oral dose of 10mg/kg of rosiglitazone for 7 days. Cardiomyocytes were isolated from male adult Sprague Dawley rats (8 weeks old) (Charles Rivers). All animals were housed on a 12 hour light: 12 hour dark cycle and provided standard laboratory chow and water *ad libitum*.

### **Oral glucose tolerance test:**

Mice were starved (fasted) for 16-18 hours (overnight) prior to the glucose tolerance test and were given free access to water during this period. The mice were orally challenged with a 1.5mg D-glucose/ kg body weight glucose load. Blood was collected from the tail vein at time intervals: 0, 15, 30, 60, 90, 120 minutes and glucose was quantified using a One Touch Ultra glucometer.

### **Cardiomyocyte isolation:**

Single ventricular cells were isolated according the methods established previously (233). Briefly the rat was anesthetized with sodium pentobarbital (50mg/kg IP) and the heart was then

rapidly excised and attached to a Langendorff perfusion apparatus. The heart was then retrogradely perfused for 5 minutes with calcium free oxygenated Tyrode's solution. This was followed by enzymatic digestion in Tyrode's solution containing 0.3mg/ml Collagenase (Type II, 371 U/mg) and 30uM Calcium chloride for 10- 15 minutes. The ventricles were then removed from the heart and minced in to small pieces in Kraft Bruhe (KB) solution. Cells were filtered and slowly acclimated in 15 minute intervals acclimated to small increases in calcium until a 1 mM final concentration in KB solution.

### **Electrophysiology recordings:**

Isolated cardiomyocytes were exposed to AT II (100nM; Sigma-Aldrich) with or without rosiglitazone (10 $\mu$ M) for 4 hours. Isolated cardiomyocytes were then transferred to a chamber containing 0.5 ml of 2 mM/L Ca<sup>2+</sup> Tyrode's solution mounted on an inverted microscope (Nikon). Myocytes are perfused (2-3 ml/min) with 2 mM/L Ca<sup>2+</sup> Tyrode's solution. Single quiescent ventricular myocytes with clear striation were chosen for patch clamping. Membrane currents were recorded using whole cell patch-clamp method (234) by use of an Axopatch amplifier (Axon, CA) and analyzed by pClamp software (Axon Instruments, CA). Glass recording pipettes were pulled using a glass puller (Narishige, Japan) and fire polished (Narashige, Japan). To record  $I_{to}$ , pipettes were filled with intracellular solution (in mM/L) (80 L-aspartic acid, 50 KCl, 10 KH<sub>2</sub>PO<sub>4</sub>, 1 MgSO<sub>4</sub>, 5 HEPES, 3 ATPNa, 10 EGTA, pH adjusted to 7.4 with KOH). The extra cellular mediums were standard Tyrode's solution containing Co<sup>2+</sup> to eliminate a Ca<sup>2+</sup> current ( $I_{ca}$ ) or a Na<sup>+</sup> free Co<sup>2+</sup> to eliminate the tetrodotoxin (TTX) sensitive Na<sup>+</sup> current, the Na<sup>+</sup> activated K<sup>+</sup> current.  $I_{to}$  was recorded in the presence of external

tetraethylammonium chloride (TEA-Cl, 50 mM). The resting membrane potential was set to  $-60$  mV. In order to measure the currents, test pulses between  $-30$  mV to  $+50$  mV were applied to in  $10$  mV increments at a frequency of  $0.1$  Hz. Correct values were obtained by subtracting the TEA-resistant  $I_{ss}$ . The bath solution was composed of (in mmol/L):  $50$  TEA-Cl,  $87$  NaCl,  $4$  KCl,  $1$  MgCl<sub>2</sub>,  $10$  HEPES,  $0.5$  CaCl<sub>2</sub>,  $10$  glucose, pH adjusted to  $7.4$  with NaOH. The pipette solution for K<sup>+</sup> current recording with (in mmol/L):  $80$  L-aspartic acid,  $50$  KCl,  $10$  KH<sub>2</sub>PO<sub>4</sub>,  $1$  MgSO<sub>4</sub>,  $5$  HEPES,  $3$  ATPNa,  $10$  EGTA, pH adjusted to  $7.4$  with KOH.

### **AT II and reactive oxygen species:**

The membrane-permeable probe 5-(6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H<sub>2</sub>DCFDA) (Molecular Probes, Eugene, OR) enters cardiomyocytes and produces a fluorescent signal after intracellular oxidation by ROS such as hydrogen peroxide and hydroxyl radical (235). Intracellular oxidant stress was monitored by measuring changes in fluorescence resulting from intracellular probe oxidation. Isolated myocytes were loaded with  $1$   $\mu$ mol/l CM-H<sub>2</sub>DCFDA (Molecular Probes, Eugene, OR) for  $30$  min. After myocytes were washed, fluorescence intensity from individual cells was measured using an excitation wavelength of  $485$  nm and an emission wavelength of  $530$  nm. Cells were sampled at random in each preparation using a flow cytometer. Fluorescence was calibrated with InSpeck microspheres (Molecular Probes) and was used as internal standards. Calibration curves were generated, and cell brightness was measured. The intensities of cells and beads were calibrated by subtraction of the background, and the final result for comparison was assessed by ratio of myocyte fluorescent intensities divided by intensities of internal fluorescent beads. The cardiomyocytes from each

group were incubated at room temperature separately in KB Solution with normal glucose concentration as control (5.5 mmol/l), AT II (100 nmol/l), and normal glucose plus hydrogen peroxide as a positive control (H<sub>2</sub>O<sub>2</sub>, 40 μmol/l) data not shown. Treatment groups included AT II with or without rosiglitazone (10μM), or Candesartan (0.1 μmol/l), Apocyanin (0.1 μmol/l), N-acetylcystein (0.1μmol/l). All reagents were purchased from Sigma-Aldrich (St Louis, USA). The culture conditions were initiated 30 min before CM-H<sub>2</sub>DCFDA was added to the medium. As a positive control 0.5 M hydrogen peroxide was used after an incubation time of 30 min. Cells were harvested, washed three times with Tyrode's buffer and analyzed (3 x 10<sup>5</sup> cells/sample) on a flow cytometer (Accuri Cytometers. Ann Arbor, MI) equipped with a 488 nm argon-ion laser and a 525 ± 10 nm band pass emission filter.

#### **Gene analysis: quantitative RT-PCR:**

RNA was isolated from db/db and C57/bl hearts using Rneasy mini kit (Quiagen). Two-step qRT-PCR was carried out using the iScript cDNA synthesis kit (Bio-Rad Laboratories). Gene expression studies were carried out utilizing Absolute QPCR SYBER Green Mix (Abgene) and an ICycler real-time PCR detection system (BIO-RAD). Real-time PCR data were analyzed using the  $\Delta\Delta C_t$  method with beta-actin gene serving as reference.

#### **Western analysis:**

Pierce Sub-Cellular fractionation kit was used to isolate the membrane and cytoplasmic components of the isolated adult rat cardiomyocytes. Protein lysates were reduced using LDS

Sample buffer (Thermo Scientific) with DTT and heated 90<sup>0</sup>C for 5 minutes. The reduced proteins were resolved in SDS Protein gels; 4-20% (Thermo-Scientific) and transferred to a Polyvinylidene fluoride membrane (Millipore, USA). Blots were then blocked (5% milk in TBS-Tween (0.1% Tween)), washed and incubated in primary antibody (1:1000) in blocking solution overnight. Primary antibodies were purchased from; Na,K – ATPase (Cell signaling- Danvers, MA), P47-phox (Santa Cruz, CA), Kv4.3; UC Davis/ NINDS/ NIMH Neuro Mab Facility – Anti Kv4.3 K<sup>+</sup> channel, clone 75/41; Anti- KChIP2 K<sup>+</sup> Channel, Clone K60/ 73; Anti-Kv 4.2 K<sup>+</sup> Channel, clone K57/1, Alpha tubulin; Developmental Studies Hybridoma Bank at the University of Iowa. Blots were washed in TBS-T and exposed to secondary anti-mouse/rabbit HRP conjugated antibodies in blocking solution (1:2000 dilution) for 1 hour. After extensive washing, the blots were analyzed by utilizing ECL reagents; Super signal Femto/ SuperSignal West Pico (Pierce) and imaging using a BioRad Gel Doc system. Protein Bands were analyzed by the BioRad software and standardized using the Na,K – ATPase antibody for the membrane fractions as well as  $\alpha$ -tubulin. Data were then averaged and statistical analysis based upon 5 individual experiments were then analyzed using the GraphPad (Prism) 5.02 Software using the standard error means.

### **QT- Interval measurements:**

ECG recordings were performed on anesthetized animals from each treatment were using a physiologic data acquisition system (Biopac, Santa Barbara, CA). QT intervals obtained by measuring the duration (ms) between the start of the QRS complex to the end of the T wave.

**Statistical analysis:**

Unless indicated otherwise, data from four to six independent experiments are shown  $\pm$  SE. Statistical significance among the groups were tested with t-test. A *P* value of  $\leq 0.05$  was considered significant. For statistical calculations, GraphPad-Prism version 5.02 was utilized. Analysis of flow cytometry histograms was done with the free software WinMDI ver. 2.8 (Scripps Research Institute Cytometry Software page at <http://facs.scripps.edu/software.html>)



## Results

### **PPAR- $\gamma$ activation protects against the AT II mediated depression of $I_{to}$**

To explore the consequence of PPAR $\gamma$  activation upon AT II effects on cardiomyocyte  $I_{to}$ , we incubated isolated adult rat cardiomyocyte with AT II (100nM) with or without PPAR $\gamma$  agonist (rosiglitazone, 10 $\mu$ M) for 4 hours and then recorded  $I_{to}$ . As shown in (Figure 3.1), treatment of cells with AT II down regulated the  $I_{to}$  in isolated cardiomyocytes. This approach is supported by previous findings that show AT II infusion in to rodent models down regulates Kv 4.3 mRNA, which is a key component of the membrane pore forming complex of  $I_{to}$ . Furthermore, we also observed that PPAR $\gamma$  agonist improved the attenuated peak  $I_{to}$  density mediated by AT II.

### **PPAR $\gamma$ activation improves AT II mediated attenuation of membrane associated densities of potassium channel components associated with $I_{to}$**

To better understand the down regulation of  $I_{to}$  by AT II, we measured the membrane association of the pore forming components of  $I_{to}$  by western analysis. We found significant alterations in membrane localization of Kv 4.3, 4.2 subunits. The AT II mediated changes in the localization of the components of  $I_{to}$  were rescued by rosiglitazone treatment (Figures 3.2 A & B). Further, rosiglitazone improved the AT II induced alteration of KChIP2 in the membrane fraction (Figure 3.2C). KChIP2 regulates the docking of several molecular components of  $I_{to}$ .

The increase in reactive oxygen species (ROS) formation down regulates molecular components of  $I_{to}$  (236). Therefore, we hypothesized that PPAR $\gamma$  exerts beneficial effects on localization of molecular components of  $I_{to}$  by inhibiting AT II mediated ROS formation. Our data indicate that a significant induction of ROS formation by AT II, as measured by flow cytometry from isolated rat cardiomyocytes (Figure 3.3A). In addition, rosiglitazone prevented this AT II mediated ROS formation. To implicate AT II as the direct mediator of ROS formation, we incubated cardiomyocytes with a known AT II inhibitor; Candesarten in the presence of AT II and found a significant reduction in ROS formation. Previously utilization of ACE inhibitors in diabetic and AT II infused animal models resulted in protection against the increase in NADPH oxidase expression in vasculature (237, 238). Increased NADPH oxidase results in formation of ROS. To implicate NADPH oxidase as the central mediator in AT II mediated ROS formation, we applied Apocyanin (NADPH Oxidase Inhibitor) and found attenuated AT II mediated ROS formation (Fig. 3.3 B). To further validate that NADPH oxidase induced the ROS formation by AT II in cardiomyocytes, we utilized a generic ROS inhibitor; N-acetyl-cysteine (NAC) and found that it did not inhibit AT II mediated ROS formation (Figure 3.3 C). Furthermore, the membrane association between p47-Phox and regulatory components of NADPH Oxidase are required for ROS formation (239). We confirm that AT II induces an increase in membrane association of p47-Phox (>70%) by western analysis (Figure 3.4). In addition, rosiglitazone (10 $\mu$ M) attenuated the increase in AT II- induced membrane association of p47-Phox (Fig. 4) (>60%).

**PPAR $\gamma$  activation protects against the increase in AT II receptor expression and improves the QT interval in the diabetic heart.**

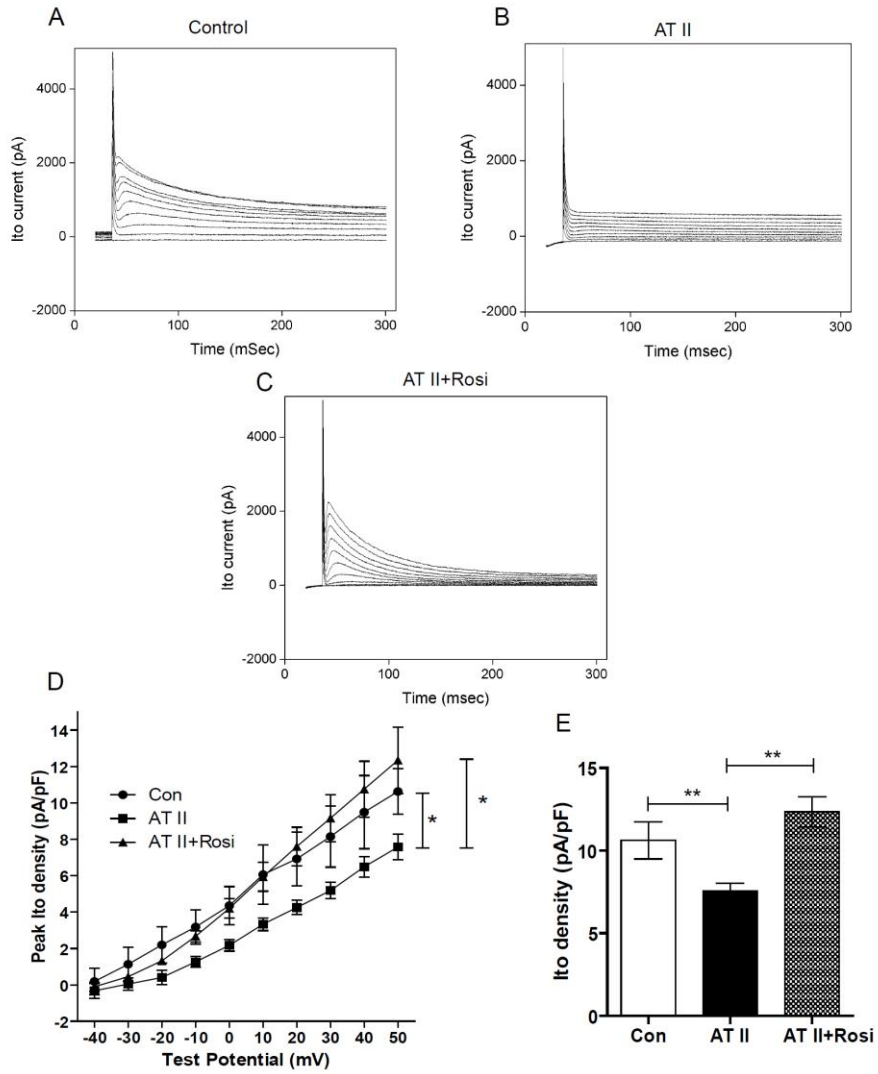
Rosiglitazone treatment improved the QT interval in the diabetic hearts (Figure 3.6). Based on this important functional outcome, we explored a potential mechanism by which diabetes may diminish  $I_{to}$  in left ventricular cardiomyocytes. AT II and its receptors have been reported to increase in the diabetic heart and vasculature in response to hyperglycemia (60, 240). We also have found an increase in gene expression of both AT II receptors Ia and Ib in diabetic (db/db mice) hearts which were ameliorated by rosiglitazone treatment (10 mg/kg) (Figures 3.5 A and B). These findings may be due to the improved circulating glucose levels after rosiglitazone treatment as demonstrated by the oral glucose tolerance test results (Figure 3.5 C). The consequences of the findings from Figure 3.5A and B may explain the how the diabetic heart was measured to have prolonged relaxation as determined by measuring the QT interval.

**Activation of PPAR $\gamma$  improves the membrane association of the pore forming components associated with  $I_{to}$  in diabetic hearts.**

We observed that the pore forming components of  $I_{to}$  in hearts from diabetic mice were dramatically attenuated in membrane association (Figures 3.7 A-C). We believe that this outcome is due to the increase in AT II and its receptors as demonstrated by ours as well as previous reports (60, 240). Interestingly, rosiglitazone treatment improved the membrane association of the pore forming components of  $I_{to}$ , as demonstrated by western analysis (Figures 3.7 A-C). Furthermore, as demonstrated in our isolated cardiomyocyte system, AT II induces an

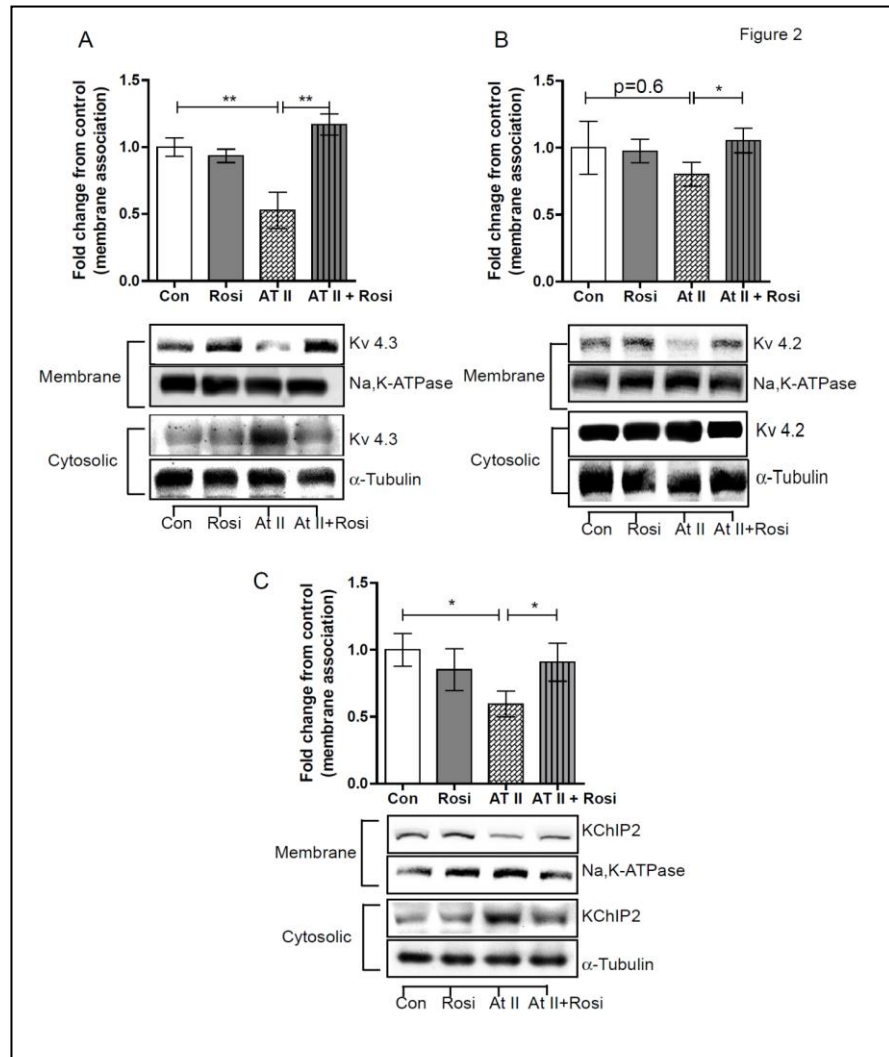
increase in membrane association of p47-phox (Figure 3.4). However others have suggested that the elevated ROS levels in the diabetic (db/db) heart maybe due to lipid overload and mitochondrial dysfunction (26). In our study we found an increase in p47-phox membrane association in the diabetic mouse heart which may also contribute to increased levels of ROS formation. Further that rosiglitazone treatment ameliorated this effect (Figure 3.7 D).

## Figures and Figure Legends



**Figure 3.1: PPAR  $\gamma$  activation protects cardiomyocytes from AT II mediated  $I_{to}$  dysfunction.** Representative traces of  $I_{to}$  were recorded in the ventricular cardiomyocytes from control (A), AT II treated (100 nM) (B) and AT II (100 nM) + Rosiglitazone (10  $\mu$ M) (C). Averaged peak  $I_{to}$  current-voltage relationships were measured in treated cardiomyocytes. Peak

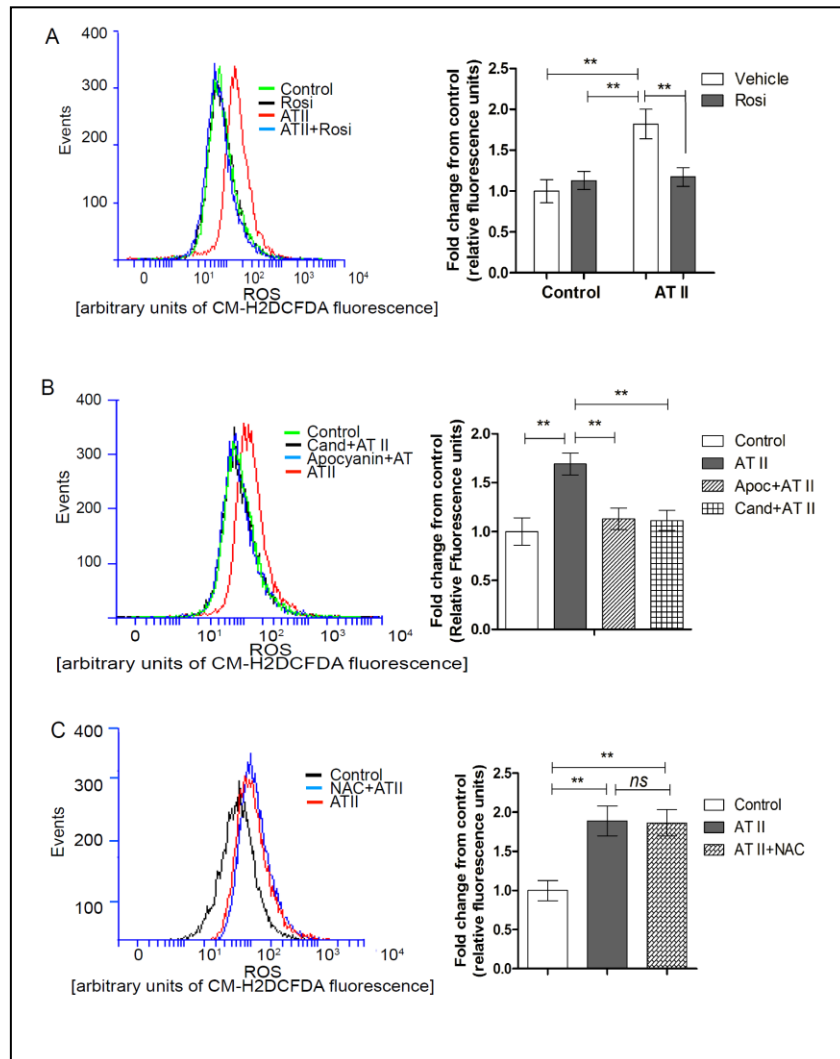
$I_{to}$  density vs test potential is graphically represented (**D**). Activation of PPAR $\gamma$  by rosiglitazone improved the  $I_{to}$  density (**E**). Cardiomyocytes were isolated and treated for 4 hours with and without rosiglitazone and or AT II as mentioned in the material section. Currents were elicited by the test potentials ranging from -30 to +50mV from a holding potential of -60mV. Test potentials were changed with 10mV increments at 6 second intervals. Current values in D and E were expressed as current density by dividing the peak current with cell capacitance. Each value represents means +/- SEM. Ten cells from 3 different rats each was used to attain the data. \*;P<0.05, \*\*; P<0.005.



**Figure 3.2: PPAR  $\gamma$  activation improves attenuated levels of membrane associated densities of potassium channel members related with  $I_{t_0}$  by AT II.**

Isolated adult rat cardiomyocytes treated with AT II for 4 hours showed an alteration in membrane localization of the pore the forming components of  $I_{t_0}$  (A) Kv4.3 (B) Kv4.2 (C) KCHIP2. However, rosiglitazone prevented AT II mediated effect as demonstrated by western analysis. Sodium-Potassium ATPase was used as a standardizing marker for the membrane

fraction. Data are mean  $\pm$  SEM from 4 independent experiments. \*;  $P < 0.05$ , \*\*;  $P < 0.005$ , *ns* = not significant.

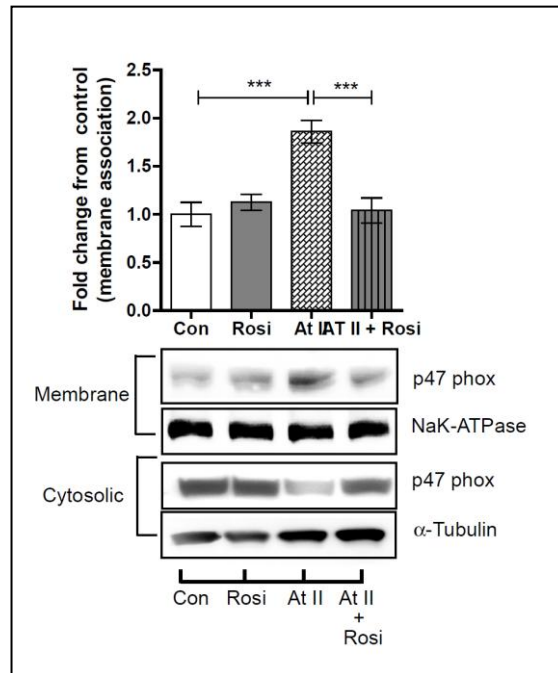


**Figure 3.3: PPAR  $\gamma$  prevents AT II induced reactive oxygen species formation.**

AT II (100nM) induces ROS formation which is prevented by PPAR $\gamma$  activation (Rosiglitazone (10 $\mu$ M) in isolated adult rat cardiomyocytes as measured by flow-cytometry using CM-

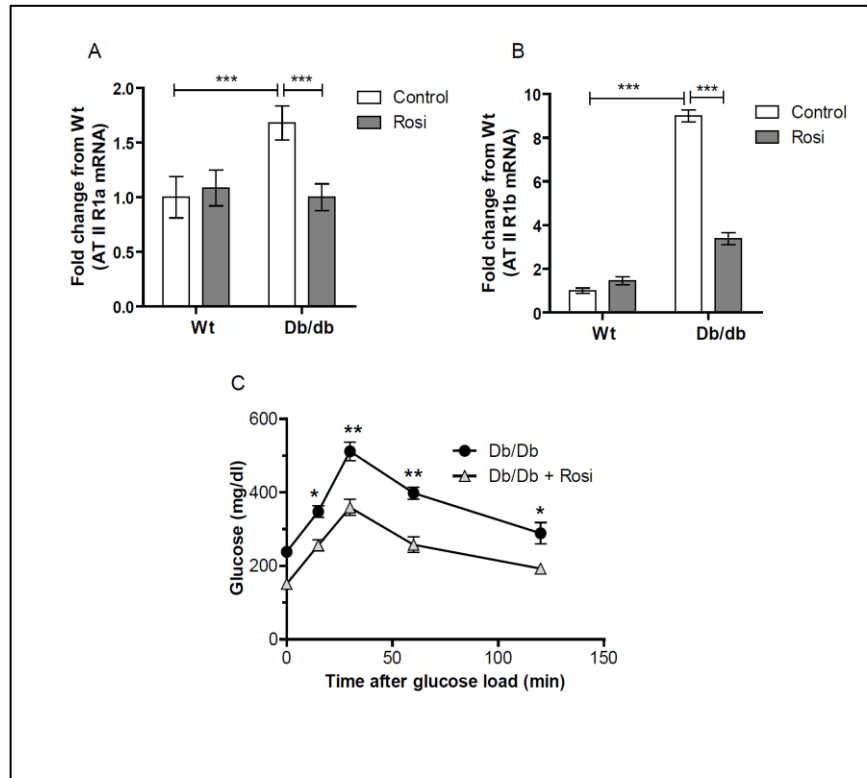


H2DCFDA (Invitrogen) (1  $\mu\text{mol/l}$ ) (A). Both Candesarten and Apocyanin prevented AT II induced ROS formation as measured by flow cytometry. (B) N-acetyl cystein (NAC) did not inhibit AT II induced ROS formation (C). Data are means  $\pm$  SEM from ventricular cardiomyocytes isolated from 4 animals per group for panel A through C. \*\*,  $P < 0.005$ , ns = not significant.



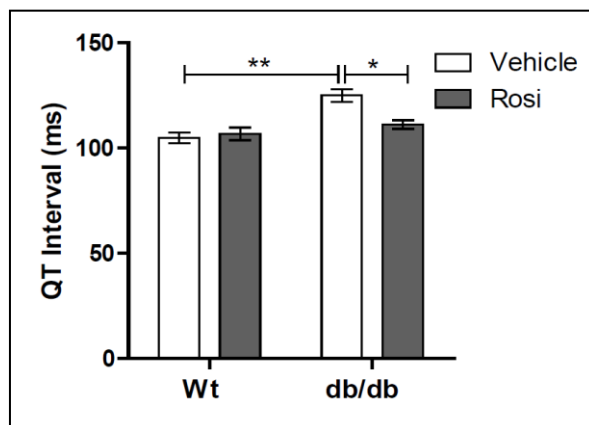
**Figure 3.4: PPAR  $\gamma$  activation attenuates AT II induced NADPH Oxidase activation.**

Western analysis reveals that AT II increases the association of p47 phox, a subunit of NADPH Oxidase, with the membrane fraction. Rosiglitazone (10 $\mu\text{M}$ ), prevents the increase in AT II mediated membrane association of p47 phox. Results indicate mean  $\pm$  SEM from 6 animals per group for the western analysis. \*,  $P < 0.01$ , \*\*,  $P < 0.005$ , \*\*\*,  $P < 0.0001$ .



**Figure 3.5: Activation of PPAR $\gamma$  improves the expression of AT II receptors in the diabetic heart.**

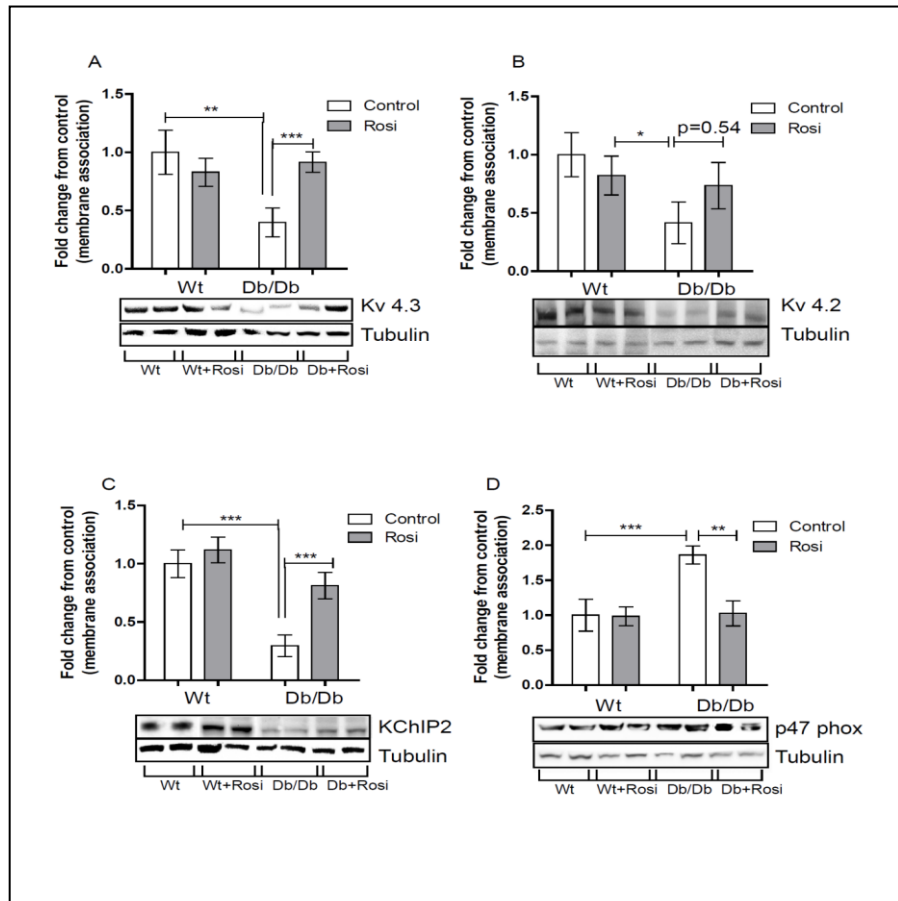
Two step qPCR analyses demonstrate that AT II R1a (A) At II R1b (B) increased in db/db mouse hearts when compared to hearts from wild type C57 Blk6J mice. Significant reduction is observed in diabetic mice (db/db) hearts after rosiglitazone treatment (10mg/kg). Results from the oral glucose tolerance test demonstrated significant improvement in circulating glucose levels after PPAR $\gamma$  agonist treatment, rosiglitazone (C).\*; P< 0.05, \*\*; P< 0.005, \*\*\*; P< 0.0001.



**Figure 3.6: Activation of PPAR  $\gamma$  improves the QT-Interval in the diabetic heart.**

Prolonged relaxation was observed in the diabetic (db/db) heart as assessed by prolonged QT intervals. Rosiglitazone treatment (10mgs/kg) improved the QT interval in the db/db mice hearts.

\*;  $P < 0.05$ , \*\*;  $P < 0.005$ .



**Figure 3.7: PPAR $\gamma$  activation improves membrane association of the potassium channel subunits associated with I<sub>to</sub> in diabetic hearts.** Hearts were isolated from -db/-db and wild type (Wt) mice after 14 days of rosiglitazone treatment by oral gavage (10mg/kg). Protein isolated from membrane fractions were resolved by western analysis for Kv 4.3 (A), Kv 4.2 (B), KChIP 2 (C) and P47phox. Bands representing ion channel expression levels were compared to  $\alpha$ -Tubulin. \*\*, P< 0.005. \*\*\*, P<0.0001

## Discussion

Angiotensin II (AT II) is highly up regulated during diabetes and results in the development of cardiac hypertrophy. During the evolution of this process the myocardium undergoes structural changes which, at the cellular level, are characterized by pathological remodeling of  $K^+$  ion channels. Clinical manifestation of these events includes prolongation in relaxation (QT Interval) times. The functional consequence of changes in expression of the pore forming components of transient outward current,  $I_{to}$  leads to marked increases in action potential durations in ventricular myocytes and to prolongation of the QT interval in surface ECG recordings (241). In the current study we found a profound protection by PPAR $\gamma$  activation against depressed  $I_{to}$  in AT II treated cardiomyocytes. Therefore the goal of the current study was to explore how AT II attenuates cardiac muscle  $I_{to}$  and understand the protective mechanism of PPAR $\gamma$  activation against this process.

Alterations in the angiotensin II type I receptor depress the transient outward current,  $I_{to}$  associated with cardiac hypertrophy, prolong relaxation (QT interval) and lead to development of severe cardiac arrhythmias and premature sudden death (242). Further in the diabetic heart,  $I_{to}$  is severely blunted while the delayed rectifying outward current  $I_k$  and the instantaneous non-inactivating steady state current  $I_{ss}$  remain unchanged (243-246). Further work by *Shimoni et al* indicate that  $I_{to}$  was attenuated in the db/db heart at 12 weeks of age and was augmented by quinapril; an angiotensin-converting enzyme inhibitor, which implicates the role of AT II in mediating diabetes induced blunting of  $I_{to}$  (245). However the mechanism as to how AT II mediates this process is not fully understood.

Our data from the isolated cardiomyocyte system suggest that the activation of PPAR $\gamma$  improves AT II mediated down regulation of  $I_{to}$  by preventing ROS formation. These data are supported by previous findings which report that activation of PPAR $\gamma$  by rosiglitazone prevented ROS formation (237, 239, 247). In our isolated cardiomyocyte system as well as in the db/db diabetic mouse hearts, we found that activation of PPAR $\gamma$  resulted in significant reduction of p47-phox protein membrane association. P47-phox is a major subunit involved in NADPH Oxidase activation resulting in ROS production. These findings suggest that activation of PPAR $\gamma$  exerts anti-oxidant properties, which improve the membrane association of the pore forming complexes of  $I_{to}$ . ROS formation and accumulation also prevents the palmitoylation and trafficking of caveolin-1 in endothelial cells (237). The core forming units (Kv components) are associated with the caveolin structures in the membrane. Secondly, palmitoylation occurs at the COOH-terminal site of Kv channel components, result in redirection of the channels to either lipid rafts or caveolin for the proper membrane tethering at the specific sites (248). Our results indicate that PPAR $\gamma$  activation protects against the AT II induced ROS formation and thus allows the proper membrane association of the core forming proteins for  $I_{to}$ .

PPAR $\gamma$  is a nuclear receptor that serves as a transcription regulator of many genes associated with fatty acid metabolism and energy regulation. However, transcriptional regulation of the  $I_{to}$  pore forming subunits by PPAR $\gamma$  has not been previously reported. Furthermore, we also did not find any significant changes in the mRNA expression of the pore forming subunits after PPAR $\gamma$  activation in isolated cardiomyocytes as well as in the hearts from db/db mice.

Obese-diabetic rodents experience cardiac dysfunction that may be ameliorated by treatment with PPAR $\gamma$  agonists (30, 249-251). However there is also great scrutiny for long term utilization of PPAR $\gamma$  agonists in diabetic patients because of the increased association of heart

failure with rosiglitazone (252, 253). Nevertheless, activation of PPAR $\gamma$  has cardiovascular benefits in many pathological models associated with cardiac dysfunction (250, 254-256). Furthermore findings from several studies implicate the anti-inflammatory effects of PPAR $\gamma$  activation in vascular muscle cells and macrophages (239). The mechanisms by which PPAR $\gamma$  activation endows these cardio-protective effects are not fully understood, but may be due to global metabolic changes. In regards to cardioprotection by the activation of heart specific PPAR $\gamma$  that heart specific knockout of PPAR $\gamma$  results in development of cardiac hypertrophy and in cultured cardiomyocytes, activation of PPAR $\gamma$  protects against adrenergic induced development of cardiac remodeling and hypertrophy (257).

Previous work by Singh et al indicates that the hyperglycemic condition in the diabetic rats induces cardiac specific increase in AT II (60). We also observed an increase in the mRNA levels of the AT II receptors in our db/db mice. However, we found that rosiglitazone dramatically down regulates AT II receptor expressions in the hearts from our db/db mice and may be a result from the improved insulin sensitivity associated with rosiglitazone treatment.

Certain limitations exist in our studies. Hyperglycemia induces ROS formation via several direct and indirect mechanisms. Improvements in circulating glucose levels may also have a synergistic protective effect against ROS formation in the diabetic heart. Therefore the profiles of improved circulating glucose levels by short term PPAR $\gamma$  activation cannot be neglected as demonstrated in figure 6. However AT II elevation induces cardiac hypertrophy in patients with hypertension and is an underlying mechanism in the development of diabetic cardiomyopathy via the development of ROS.

In summary, the process towards development of diabetic cardiomyopathy involves remodeling of the cardiac muscle architecture. Early phenotypic changes in this process are

characterized by altered expression of the potassium channels associated with  $I_{to}$ . These changes are central in the development of cardiac dysfunction, including prolonged relaxation and arrhythmias. Our data from the current manuscript suggest up regulation of AT II during diabetes impairs the membrane localization of the molecular components associated with  $I_{to}$  in cardiomyocytes. Further the antioxidant properties of PPAR $\gamma$  activation protects the cardiomyocyte from AT II mediated depressed  $I_{to}$  and prolonged QT interval. Previous work by several investigators including ours, shown that PPAR $\gamma$  activation protects against many forms of stress which induce the development of cardiac hypertrophy (258).



#### 4. Design and development of novel PPAR $\delta$ / $\gamma$ agonists

##### Abstract

Type 2 diabetes is at epidemic proportions and thus development of novel pharmaceutical therapies for improving insulin sensitivity has become of paramount importance. The objectives of the current study were to develop novel dual PPAR  $\gamma$ / $\delta$  agonists without the deleterious side effects associated with full PPAR  $\gamma$  agonists. Docking simulations of 23 novel compounds within the ligand binding domain of PPAR  $\gamma$ / $\delta$  were performed using AutoDock Vina which consistently reproduced experimental binding poses from known PPAR agonists. Comparisons were made and described with other docking programs AutoDock and Surflex-Dock (from SYBYL-X). Biological evaluation of compounds was accomplished by transcriptional promoter activity assays, quantitative PCR gene analysis for known PPAR  $\gamma$ / $\delta$  targets as well as *in vitro* assays for lipid accumulation and mitochondrial biogenesis verses known PPAR agonists. We found one (compound 9) out of the 23 compounds evaluated, to be the most potent and selective dual PPAR  $\gamma$ / $\delta$  agonist which did not display the deleterious side effects associated with full PPAR  $\gamma$  agonists.

## Introduction

As discussed in the previous chapter and in the literature review, we and others have found beneficial effects of PPAR  $\gamma$  activation in the heart in various cardiovascular pathologies. TZDs have potent insulin sensitizing action therefore, is beneficial for individuals with type 2 diabetes with insulin resistance. Unfortunately, the TZDs that are currently being clinically used (such as rosiglitazone and pioglitazone) are under strict regulation by FDA due to the adverse off target effects. Therefore, we focused on developing new selective PPAR  $\gamma$  modulators (SPPARM) which retains the agonist potential but devoid of these off target effects. This chapter will discuss in detail the *in silico* techniques we have utilized in designing these drugs and also the approaches we carried out to test the function of these drugs in biological systems. The work in this chapter had been already published (Bioorganic and Medicinal Chemistry Letters 2013; 23: 873-879).

Type 2 diabetes mellitus is at epidemic proportions characterized by excessive cardiovascular morbidity and mortality. Impaired insulin secretion and resistance contribute to the development of the disease. Thiazolidinediones (TZDs) represent a class of drugs that improve insulin sensitivity (259). TZDs were developed more than 20 years ago and are found to improve circulating hyperglycemia, hyperinsulinemia and increase insulin sensitivity in the liver, skeletal muscle and adipose tissue. In addition, circulating free fatty acids were lowered with a reduction in triglyceride levels. The beneficial effects are exerted by the activation of the nuclear receptor class of transcription factors: PPAR- $\gamma$ . However, TZDs use is associated with adverse effects such as increased cardiovascular events due mostly to peripheral edema and weight gain (260).

The PPARs family of nuclear receptors constitutes three members, PPARs- $\alpha$ ,  $\beta/\delta$  and  $\gamma$  that are highly conserved in mammals, each forming a functional heterodimeric complex with 9-*cis* retinoic acid receptor (RXR). PPAR  $\beta/\delta$  activation is found to be associated with improving overall circulating cholesterol levels (HDL, LDL and triglycerides) and is ubiquitously expressed throughout the body. Recently, it has been reported that overexpression of PPAR  $\beta/\delta$  in skeletal muscle improves the glycolytic muscle fiber type in animal models (261). However, agonist for this class of receptors does not have a significant impact upon improving circulating insulin levels. Consequently, as diabetes and metabolic syndrome are associated with defects in glucose oxidation and lipid metabolism, the concept of discovering dual agonists, which can activate both PPAR  $\beta/\delta$  and PPAR  $\gamma$  simultaneously, has emerged as a potential therapeutic target for improving outcomes for diabetes. Thus the goal of the current study is to develop a rational mechanism to find new compounds which would activate both PPAR  $\gamma$  and PPAR  $\delta$  targets without the deleterious side effects of increasing adiposity and weight gain.

To accomplish this task, a joint computational, synthesis, and gene regulation study of potential PPAR  $\gamma$  and PPAR  $\delta$  ligands was carried out. Twenty-three non-TZD compounds have been designed and synthesized as potential agonists. Docking simulations of the proposed compounds within the ligand binding domain of PPAR  $\gamma$  and  $\delta$  have been performed. Compounds were examined via *in vitro* cellular assays testing the transcriptional regulation of both PPAR  $\gamma$  and PPAR  $\delta$  targets.

## Materials and Methods

### Chemical Synthesis:

The general synthesis of compounds **9** and **3-121** are described below.

*Compound 9 (Scheme 1).* Treatment of 4-(Aminomethyl) benzoic acid **2** with Fmoc chloride, Na<sub>2</sub>CO<sub>3</sub>, dioxane, water, at 0° provided (3). [3] was treated with 2-chlorotrityl chloride resin, DIPEA, COMU, methanol, DCM, DMF, to create (4). [4] was reacted with 3,5-bis(trifluoromethyl) benzyl bromide, DIPEA, sodium hydride, DCM. This was treated with DCM, DMF, piperidine in a 1:1:2 ratio to remove Fmoc and generate (5). Treatment of 3-bromopropylamine hydrobromide with Fmoc chloride, sodium carbonate, dioxane, water, at 0° provided (6). [6] was reacted with (5), DIPEA, sodium hydride, DCM, DMF to make (7). [7] was treated with 4-methylbenzyl bromide, DIPEA, sodium hydride, DCM, DMF. This was reacted with DCM, DMF, piperidine in a 1:1:2 ratio to remove Fmoc to generate (8). [8] was treated with 90% TFA and DCM to remove resin and create our final compound **9**.

*Compound 3-121 (Scheme 2).* Treatment of Aminoacetaldehyde diethyl acetal (2) with 2-Mesitylenesulfonyl chloride, 10% NaOH, CH<sub>2</sub>Cl<sub>2</sub> provided (3). [3] was treated with 4-(Trifluoromethyl)benzyl bromide, NaH, DMF, under nitrogen to yield (4). [4] was treated with HBr, phenol, ethyl acetate to create **3-121**.

## Computational Methods:

AutoDock 4,(262) AutoDock Vina,(263) and Surflex-Dock from Sybyl-X(264) were used to dock known and proposed agonists of PPAR- $\gamma$  and PPAR- $\delta$ . Initial Cartesian coordinates for the protein-ligand structures were derived from a reported 1.95 Å crystal structure of PPAR  $\gamma$  (PDB ID: 3ET3)(265) and a 2.00 Å crystal structure of PPAR  $\delta$  (PDB ID: 3GZ9) (266). In the case of PPAR  $\gamma$  the present model included the active site and all residues within 15 Å of it. Clipped residues were capped with acetyl or N-methylamine groups. The reduced PPAR  $\gamma$  model consisted of ~2,800 atoms and 162 residues out of 275 residues. The PPAR  $\delta$  model used the protein in its entirety. The protein targets were prepared for molecular docking simulation by removing water molecules and bound ligands. AutoDockTools (ADT) (267) was used to prepare and analyze the docking simulations for the AutoDock and AutoDock Vina programs, whereas Sybyl-X was used to prepare, carry out runs, and analyze the Surflex-Dock simulations. All ligands were constructed using PyMOL (268) with subsequent geometry optimizations carried out using the semiempirical method PDDG/PM3(269-271) and the BOSS program.(272) Polar hydrogens were added, and in the case of AutoDock, Gasteiger charges were assigned. Nonpolar hydrogens were subsequently merged. The protonation state of the ligands was adjusted to the species assumed predominant at physiological pH, specifically, carboxylic acid moieties were deprotonated. Conjugate gradient minimizations of the systems were performed using MCPRO(272) and GROMACS.(273) A grid was centered on the catalytic active site region and included all amino acid residues within a box size set at  $x = y = z = 70$  grid points and 26 Å for AutoDock and AutoDock Vina, respectively. AutoGrid 4 was used to produce grid maps for AutoDock calculations where the search space size utilized grid points of 0.375 Å.

*AutoDock Vina details.* Standard flexible protocols of AutoDock Vina using the Iterated Local Search global optimizer<sup>1</sup> algorithm were employed to evaluate the binding affinities of the molecules and interactions with the receptors. All ligands and active site residues, as defined by the box size used for the receptors, were set to be rotatable. Calculations were carried out with the exhaustiveness of the global search set to 100, number of generated binding modes set to 20, and maximum energy difference between the best and the worst binding modes set to 5. Following completion of the docking search, the final compound pose was located by evaluation of AutoDock Vina's empirical scoring function where the conformation with the lowest docked energy value was chosen as the best.

*AutoDock details.* The Lamarckian Genetic Algorithm(274) was chosen to search for the best conformers. The rigid roots of each ligand were defined automatically, and the amide bonds were made nonrotatable. The docking process used for AutoDock mirrored that of a recent study by Perryman and McCammon.(275) For example, 100 conformers were considered for each compound. The population size was set to 150, and the individuals were initialized randomly. The maximum number of energy evaluation was set to  $9 \times 10^{10}$ . The docking parameters used were: maximum number of generations was  $9 \times 10^4$ , maximum number of top individuals that automatically survived set was 1, mutation rate of 0.02, crossover rate of 0.8, step sizes were 2 Å for translations,  $50^\circ$  for quaternions, and  $50^\circ$  for torsions, a cluster tolerance of 2 Å was employed, and the maximum number of iterations in the pseudo-Solis-and-Wets-minimization/local search was increased to 3000.

*Surflex-Dock details.* Following the receptor's preparation, all hydrogens were added and the side chain amides in all Asn and Gln were oriented to maximize hydrogen bonding. Minimization was performed for 100 iterations using the AMBER FF99 force field.(276) Prior to

docking runs, the Surflex docking algorithm(277) required the generation of a protomol(278) -- an idealized representation of the binding site that defines the search area. For this purpose, the prepared receptor files were loaded and the protomol generation constructed based on protein residues that constitute the active site, decreasing the threshold and increasing Bloat parameters values to 0.25 and 2 Å, respectively. The threshold has a default value of 0.50 and decreasing this number increases the volume while the Bloat on the other hand has a default of 0 and increasing this value inflates the protomol. During the docking runs, all other adjustable parameters were left at their default values.

### **Biological Methods:**

To validate the specificity of the compounds towards activation of PPAR  $\delta/\gamma$  targets, we tested the compounds capacity to activate AP2 PPRE (PPAR  $\gamma$  target) and PDK4 PPRE (PPAR  $\delta$  target) which was available inserted into PGL3 vectors containing CMV promoters from (ADDGENE). These vectors in association with RXR vectors and PPAR $\gamma$  or PPAR $\delta$  vectors were transfected into HEK-293 cells using Lipofectamine 2000 (Invitrogen). Relative lights units were ascertained using a Glomax Luminometer from Promega Corp. To further validate the physiological effects upon adipocytes of our compounds, 3T3L1 adipocytes were grown, differentiated and drugs were then added to cells for a period of 6 days and compared to cells treated with rosiglitazone (PPAR  $\gamma$  agonist). Recently, strong PPAR  $\delta$  agonist have been shown to induce mitochondrial biogenesis, thus compounds were applied to C2C12 muscle cell lines in culture for 4 days and mitochondrial genes were measured by quantitative real time PCR.

## Results

### **Compound 9 showed the highest binding affinities for PPAR $\gamma/\delta$ by Autodoc Vina**

Of the 23 compounds studied, the calculations predicted compound **9** to possess the highest binding affinity to both PPAR- $\gamma$  and PPAR- $\delta$  (Table 1). Also, according to the calculations compound **9** does not interact with Tyr473 at the PPAR $\gamma$  binding pocket. Another compound developed, **3-121**, ranked approximately in the lower end of the relative binding affinities of the 23 compounds calculated in both receptors (Table 4.1).

### **Compound 9 shows least affinity to AP2 promoter**

Our luciferase assays indicate that compound 9 has the least affinity for the AP2 promoter, while compound 3-121 induces the promoter activity by 4 fold (Figure 4.5 A). This observation was further confirmed by oil-red-o stain conducted on 3T3-L1 adipocytes (Figure 4.5 B).

### **Compound 9 has high affinity for PDK4 and promotes mitochondrial biogenesis**

Compound 9 activates the PDK4 promoter approximately more than 4 fold, as demonstrated by the luciferase assays (Figure 4.6 A). Further, we observed that the gene

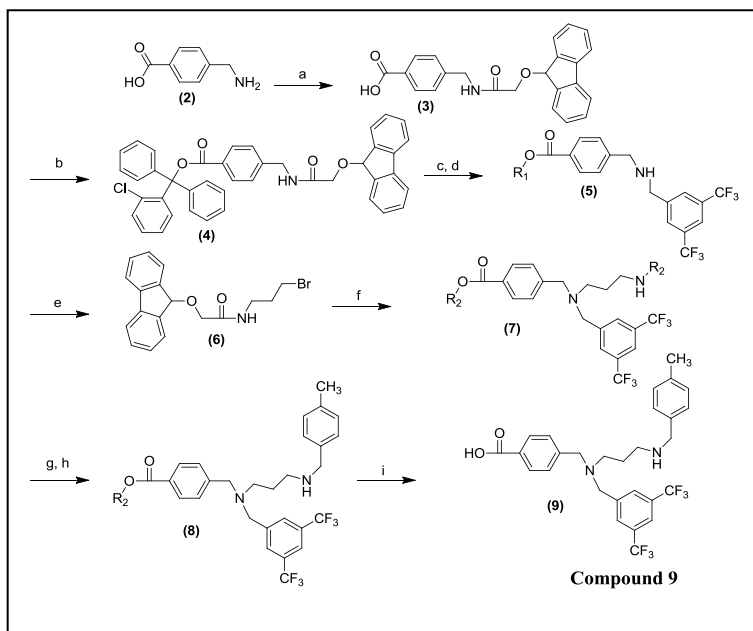


expression for mitochondrial markers was significantly increased with compound 9 treatment in C2C12 skeletal muscles (Figure 4.6 B).

### **Compound 9 promotes fatty acid oxidation but does not induce lipogenesis**

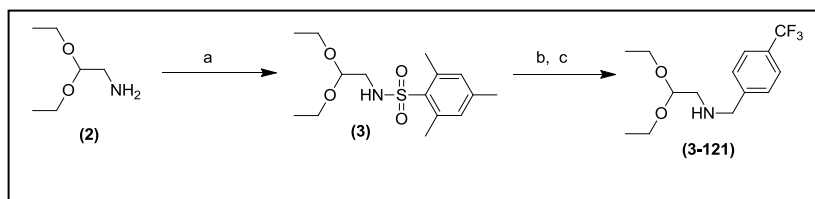
Compound 9 increases the genes that regulate fatty acid oxidation in 3T3-L1 adipocytes. Further, we observed that the genes that are involved in lipogenesis are attenuated with the compound 9 treatment (Figure 4.7).

## Figures and figure legends



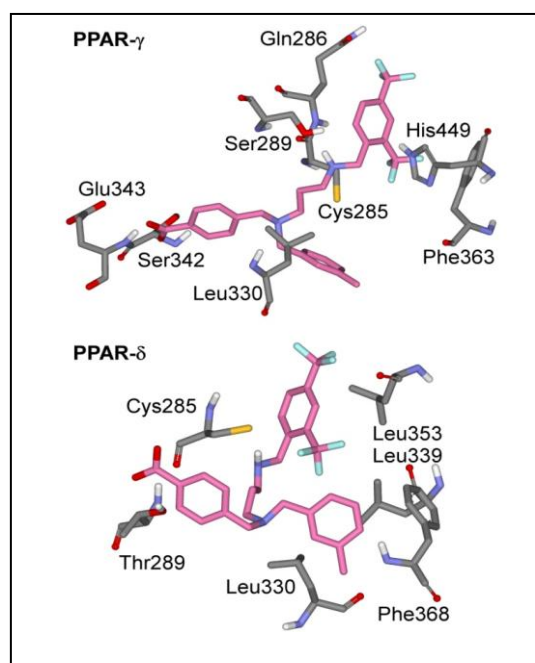
**Figure 4.1: Synthesize scheme of compound 9.**

Reagents and conditions: (a) Fmoc,  $\text{Na}_2\text{CO}_3$ , Dioxane, Water, at  $0^\circ\text{C}$ ; (b) 2-Chlorotrityl chloride resin, DIPEA, COMU, Methanol, DCM, DMF; (c) 3,5-Bis (trifluoromethyl) benzyl bromide, DIPEA, NaH, DCM; (d) DCM, DMF, Piperidine, 1:1:2; (e) 3-bromopropylamine hydrobromide, Fmoc Chloride,  $\text{Na}_2\text{CO}_3$ , Dioxane, Water at  $0^\circ\text{C}$ ; (f) (8), DIPEA, NaH, DCM, DMF; (g) 4-methylbenzyl bromide, DIPEA, NaH, DCM, DMF; (h) DCM, DMF, Piperidine, 1:1:2; (I) 90% TFA, DCM.

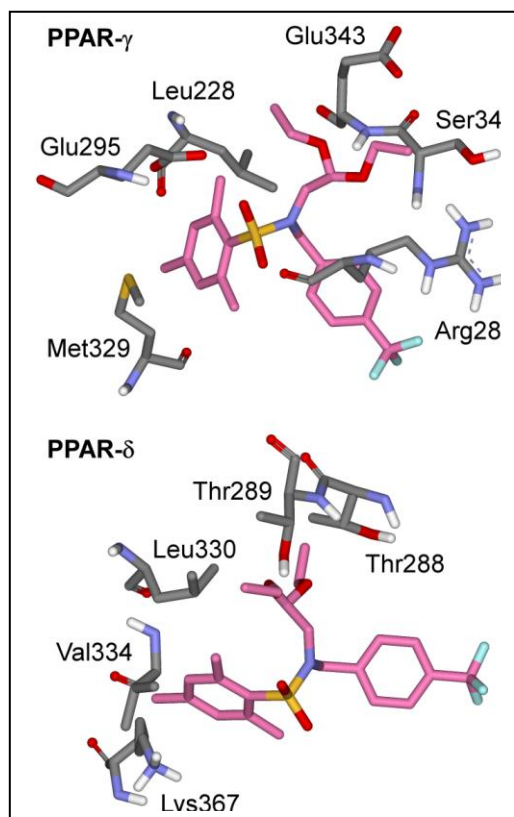


**Figure 4.2: Synthesize scheme of compound 3-121.**

Reagents and conditions: (a) 2 Mesitylenesulfonyl chloride, 10% NaOH, CH<sub>2</sub>Cl<sub>2</sub>; (b) 4-(Trifluoro-methyl) benzyl bromide, NaH, DMF; (c) HBr, Phenol, EtoAC.



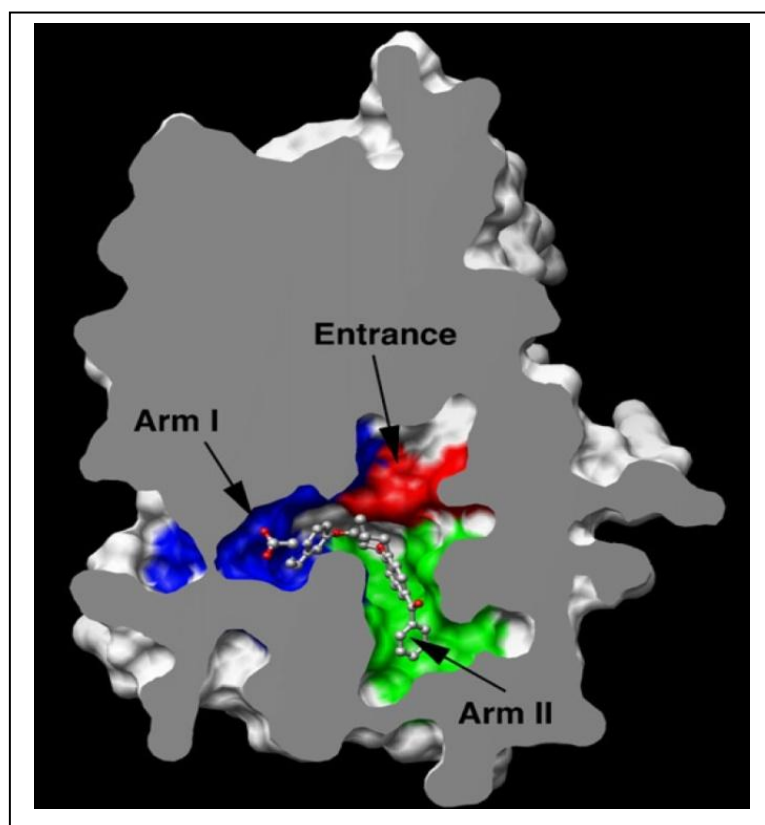
**Figure 4.3: Schematic representation of Compound 9 bound to the active site of PPAR-γ (top) and PPAR-δ (bottom) with key residues shown.**



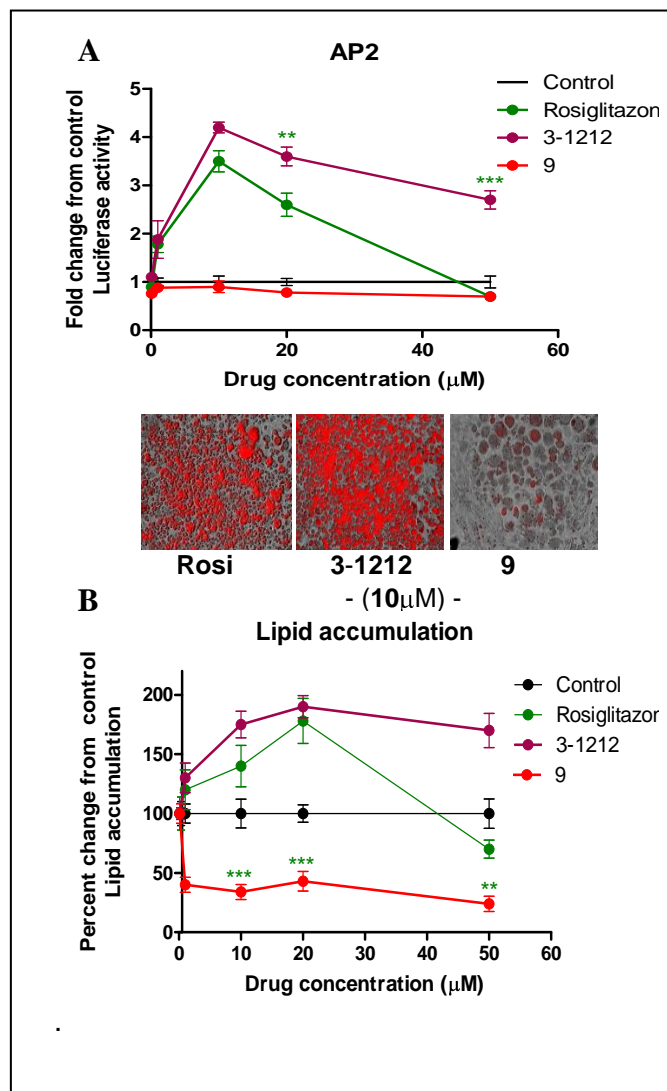
**Figure 4.4: Schematic representation of Compound 3-121 bound to the active site of PPAR- $\gamma$  (top) and PPAR- $\delta$  (bottom) with key residues shown**

PPAR $\gamma$		PPAR $\delta$	
Molecule	Affinity (kcal/mol)	Molecule	Affinity (kcal/mol)
<b>9</b>	<b>-12.0</b>	<b>9</b>	<b>-10.8</b>
8	-11.8	4-20	-10.6
7	-11.1	8	-10.6
3-91	-11.0	3-75	-10.4
4-20	-10.2	3-71	-10.2
4	-10.0	3-91	-10.2
3-95	-9.9	5	-10.1
5	-9.7	7	-9.8
6	-9.6	4	-9.6
3-71	-9.4	3-95	-9.5
3-65	-9.4	2	-9.3
3-75	-9.3	6	-9.2
4-21	-9.2	3	-9.2
4-23	-9.2	3-65	-9.0
3-137	-8.8	4-21	-8.9
2	-8.8	3-121	-8.6
3	-8.4	4-23	-8.6
3-115	-8.3	3-115	-8.3
1	-7.9	1	-8.2
3-121	-7.7	3-137	-7.7
3-133	-7.5	3-133	-7.4
4-19	-7.1	4-19	-7.1
3-81	-7.0	3-81	-6.9
<b>Rosi</b>	<b>-9.2</b>		

**Table 4.1: Predicted binding affinities for the proposed compounds for LBD of PPAR  $\gamma$  and PPAR  $\delta$**

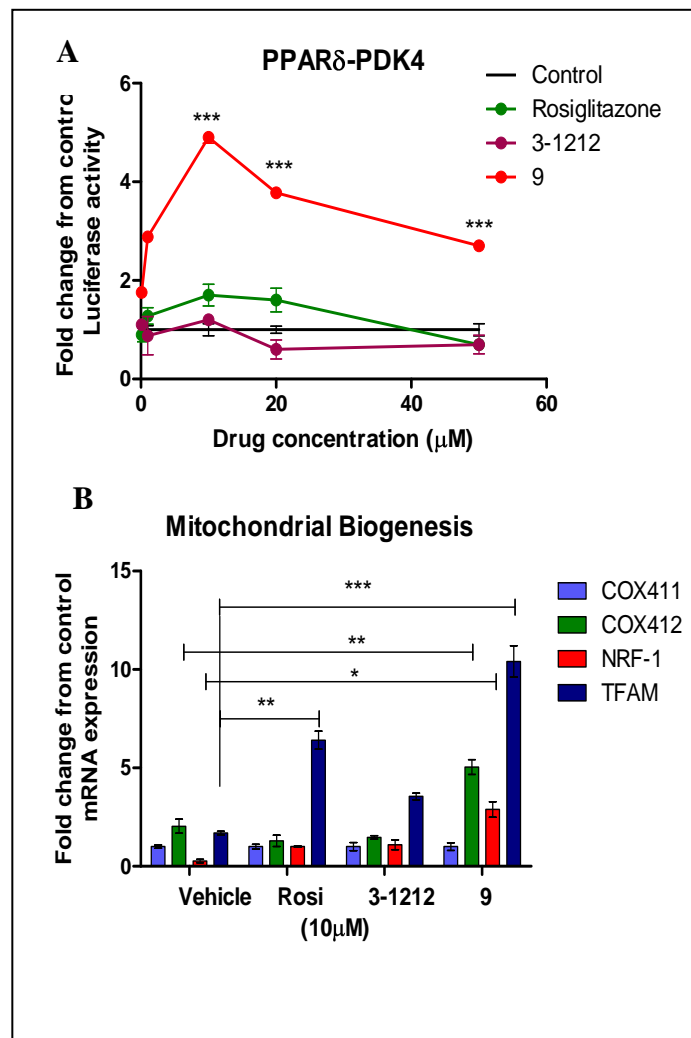


**Figure 4.5: The ligand binding domain** – Resembles a large Y-shaped cavity starting from the Entrance and extending in to Arm I and Arm II pockets.



**Figure 4.6: Compound 3-121 induces activation of AP2 promoter (A) and induces lipid accumulation in 3T3-L1 adipocytes (B)** Data are means  $\pm$  SEM from 3 independent experiments.

Values were set fold change from control. \*\*,  $P < 0.005$ , \*\*\*,  $P < 0.0001$

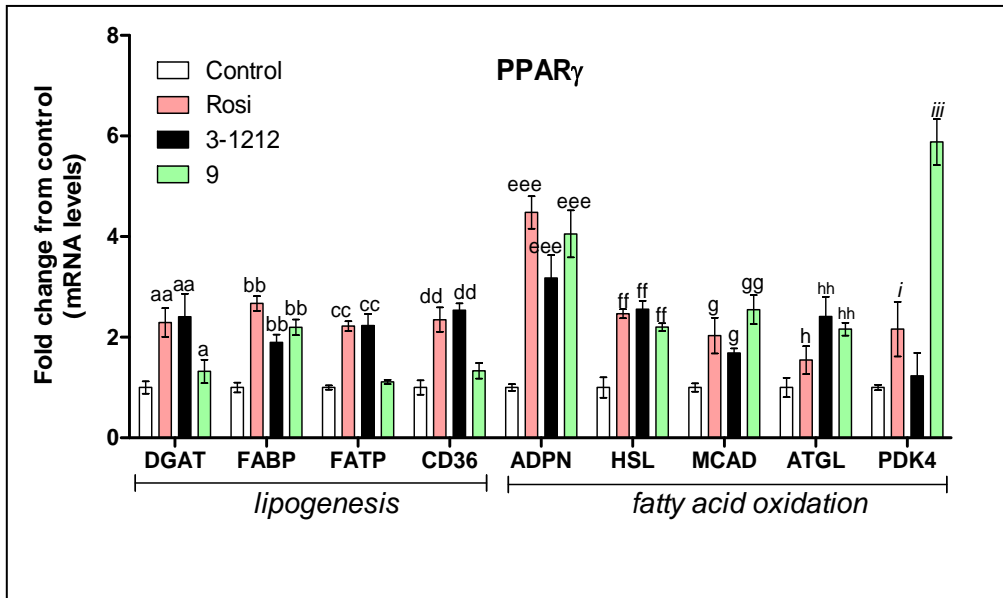


**Figure 4.7 - Compound 9 activates PDK4 promoter as measured by luciferase activity. (A).**

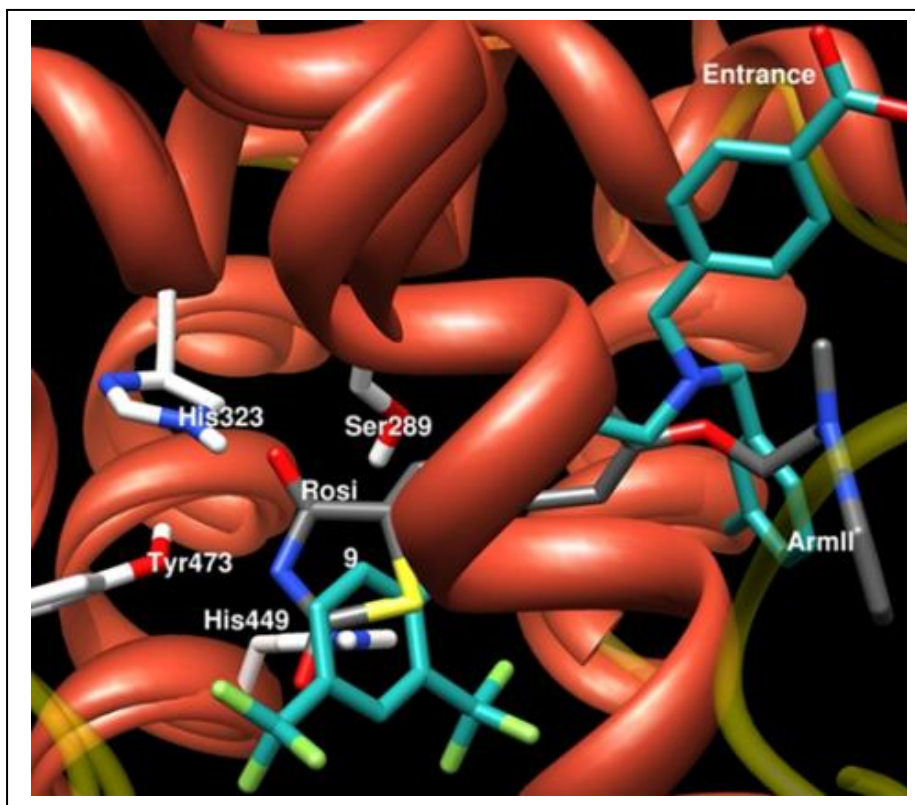
Further compound 9 also activates several genes associated with mitochondrial biogenesis (B).



Data are means  $\pm$  SEM from 3 independent experiments. Values were set fold change from control. \*,  $P < 0.05$ , \*\*,  $P < 0.005$ , \*\*\*,  $P < 0.0001$ .



**Figure 4.8: mRNA analysis of PPAR- $\gamma$  targets.** Rosiglitazone (10 $\mu$ M), GW0742 (10 $\mu$ M), **3-121** (10 $\mu$ M) and **9** (10 $\mu$ M), were applied to 3T3-L1 adipocytes to determine effects on PPAR- $\gamma$  target mRNA expression levels. a, b, d and f,  $P < 0.05$ , aa, cc, ee, ff, gg, hh;  $P < 0.005$ , hhhm *iii*;  $P < 0.0001$  when compared to control treated cell mRNA expression levels.



**Figure 4.9: Virtual representation of binding of rosiglitazone and compound 9 in the AF-2 domain.** Rosiglitazone (grey color) and compound 9 (aqua color) interacting with the amino acids in the AF-2 domain PPAR  $\gamma$ . Note absence of compound 9 binding with the Tyr 473 and increased binding with His 449.

## Discussion

The *in silico* design of small molecules that bind to a biological target has made great advancements in methodology in recent years (279). Of particular interest, virtual screening methods that dock ligands into a receptor allow for a large number of compounds to be vetted quickly and have been successfully applied to PPAR  $\gamma$  (280). However, virtual screening methods often neglect important statistical and chemical contributions in favor of computational efficiency (281). To examine the accuracy of the present docking methods, known agonists rosiglitazone (PDB ID: 2PRG),(282) indeglitazar (PDB ID: 3ET3),(265) and D321 (PDB ID: 3GZ9)(283) in PPAR  $\gamma$  or PPAR  $\delta$  have been calculated using AutoDock, AutoDock Vina, and Surflex-Dock and the resultant poses compared to the crystal structures. The flexible protocols of AutoDock Vina yielded computed RMSD values 0.88, 0.83, and 0.07 Å for rosiglitazone, indeglitazar, and D321, respectively, for all atoms between the controls and the docked conformations. AutoDock's computed RMSD values were 1.87, 1.40 and 1.07, while Surflex-Dock's were 1.16, 1.81 and 0.70 Å for the three agonists. Illustrations of the calculated versus crystal structure binding poses show that AutoDock Vina reproduced the controls significantly better than the other docking programs. Consequently, AutoDock Vina was used to dock and interpret data for the current compounds.

Twenty-three proposed synthetic agonists have been docked into both PPARs to elucidate any favorable interactions with active site residues (Table 4.1). The PPAR ligand binding domain resembles a large Y-shaped cavity starting from the Entrance and extending into Arm I and Arm II pockets (Figure 4.5) (284). Arm I is substantially polar while the Entrance and Arm II are

primarily hydrophobic. For most full PPAR agonists, hydrogen bonding typically occurs with Ser289, His323, Tyr473 and His449 (284).

Of the 23 compounds studied, the calculations predicted compound **9** to possess the highest binding affinity to both PPAR- $\gamma$  and PPAR- $\delta$  (Table 4.1). Inspection of the *in silico* PPAR  $\gamma$ /**9** complex suggests that the carboxylate group of compound **9** forms a hydrogen bond at a distance of 2.02 Å with the entrance residue Glu343 while the 2° amine donates a tighter hydrogen bond of 1.85 Å with the polar Ser289 residue of Arm I (Figure 4.5). The noncanonical X-H $\cdots\pi$  hydrogen bond is of great importance in proteins and the ligand's trifluoromethyl disubstituted phenyl ring is revealed to accept a N-H $\cdots\pi$  hydrogen bond of 2.69 Å from His449. It is interesting to note that **9** does not interact with Tyr473, a residue crucial to the stabilization of the AF2 helix H12 which allows the binding of co-activators that lead to the activation of the genes responsible for adipogenesis and insulin sensitivity (285). The results appear consistent with the present lipid accumulation assays, where the binding of **9** lowers the adipogenesis effect of rosiglitazone.

In PPAR  $\delta$ , however, the carboxylate group of the substrate forms a hydrogen bond of 3.03 Å with Thr289 in Arm I which is part of the His323, His449 and Tyr473 hydrogen bond network involving the carboxylate group of fatty and eicosanoic acids (the endogenous ligands) (284). The 2° amine also donates a H-bond of 2.87 Å to the sulfur atom of the polar residue Cys285 in Arm I. The phenyl ring substituted by a methyl group is located between the Entrance and Arm II and surrounded by the hydrophobic residues Leu330, Leu339 and Phe368 while the trifluoromethyl disubstituted phenyl ring is in close proximity to the hydrophobic residue Leu353 located in Arm II. These interactions could significantly stabilize the ligand binding domain translating to the observation from luciferase assays that determined that compound **9** activates a

direct PPAR- $\delta$  target. In general, the calculations suggests that **9** binds in Arm I and the Entrance of PPAR- $\gamma$ , whereas in PPAR- $\delta$ , it binds to Arms I and II of the binding pocket. Another compound developed, **3-121**, ranked approximately in the lower end of the relative binding affinities of the 23 compounds calculated in both receptors (Table 4.1). The ether oxygen atoms of **3-121** accept two hydrogen bonds of ca. 2.17 and 2.86 Å from the backbone NH group of the polar residue Glu343 in the Entrance region of PPAR  $\gamma$  (Figure 4.4). The calculations do not predict binding with any of the polar residues located in Arm I; this appears inconsistent with the experimental assays which point to it mediating lipid accumulation in the adipocytes. The docking studies nevertheless predicted **3-121** to bind weakly to PPAR- $\delta$  with the absence of the conserved hydrogen bonding interactions. The results correlate well with results from the luciferase assays indicating that **3-121** did not activate the direct PPAR  $\delta$  target PDK4.

Although experimental assays have yet to be carried out for compound **3-91**, its computed binding affinity to PPAR- $\gamma$  implies that it may be a potent activator (Table 4.1). The protein is predicted to form two hydrogen bonds with the isoindole-dione substituent group of **3-91** the hydrogen on His323 and a carbonyl oxygen, and the hydrogen on His449 and the nitrogen at distances of 2.38 and 2.66 Å, respectively. In addition, the OH group of Ser289 also forms a hydrogen bond of 2.57 Å with the N of the isoindole-dione substituent group. This binding mode is consistent with known full agonists, e.g., rosiglitazone.<sup>(286)</sup> **3-91** does not form substantial hydrogen bonds with PPAR- $\delta$  despite having a larger binding affinity relative to **3-121**. As a further point of reference, compound **4-23** ranks approximately in the mid-to-low range binding affinity compared to the other 23 compounds for both PPAR  $\gamma$  and PPAR  $\delta$  and lacks significant hydrogen bonding in the active sites of both PPARs.

Our transcriptional analysis (luciferase assay) of the compounds suggested that compound **3-121** was a potent PPAR- $\gamma$  activator as determined by the amount of activation of AP2, a transcriptional gene centrally involved in adipogenesis as well as lipid accumulation. In comparison to rosiglitazone, a known PPAR- $\gamma$  agonist, **3-121** appeared to be a stronger agonist for PPAR- $\gamma$  than rosiglitazone as determined by AP2 activation and lipid accumulation. In humans, rosiglitazone is found to mediate weight gain by increasing lipid accumulation and edema, which is described to be the deleterious effect of PPAR  $\gamma$  agonist therapy. Recent findings utilizing PPAR  $\delta$  agonist in diabetic mice have shown that these compounds increase fatty acid oxidation but do not have an effect upon improving insulin sensitivity. These data validate our compound **9** findings, which show that it does not induce ap2 activation nor an increase in lipid accumulation as demonstrated in Figure 4. However, it did increase genes associated with serving as a PPAR- $\delta$  agonist as demonstrated in Figure 5a. Over expression of PPAR- $\delta$  in skeletal muscle has been shown to increase mitochondrial biogenesis. Compound **9** is shown to induce the PDK4 promoter by PPAR  $\delta$  activation stronger than commercially available PPAR- $\delta$  agonist GW0742, by 2 fold (data not shown). Interestingly, **9** significantly increased COX-412, a transcription factor involved in inducing mitochondrial respiration and biogenesis in response to hypoxia in the heart and maybe a target of research for protection against ischemic injury in the diabetic heart. Improvement of insulin sensitivity in obese-diabetic animal models, have been found utilizing dual PPAR  $\gamma$ /PPAR  $\delta$  agonists. However the signaling mechanisms were not elucidated. Data from Figure 6 validates that compound **9** significantly activates effectors of PPAR  $\gamma$ , especially those involved in improving energy regulation such as adiponectin, MCAD, DGAT, and PDK4.

In summary, a joint computational and experimental study was carried out to develop a series of compounds that span from full to partial agonists of PPAR  $\gamma$  as well as PPAR  $\delta$ . Our docking simulations accurately elucidated which compounds would yield strong binding affinities for the PPARs. Of the 23 compounds studied, the calculations predicted compound **9** to possess the highest binding affinity in both PPAR  $\gamma$  and PPAR  $\delta$ . A detailed analysis of the binding pocket found **9** formed hydrogen bonding interactions with Ser289, Glu343, and His449 in PPAR  $\gamma$  but with Cys285 and Thr289 in PPAR  $\delta$ . Luciferase assays indicate that **9** partially activated PPAR  $\gamma$  while fully activating PPAR  $\delta$ . Interestingly, compound **9** is not predicted to interact with Tyr473 of the PPAR  $\gamma$  binding pocket: a residue crucial to the stabilization of the AF2 helix H12 which allows the binding of coactivators that lead to adipogenesis (285). These results appear consistent with the present lipid accumulation assays, where the binding of **9** lowers the adipogenesis yet as seen in Figure 6 activates several PPAR  $\gamma$  targets including adiponectin. Future work will involve further development of **9** derivatives and the effects upon insulin signaling in diabetic animal models.

## 5. Regulation of frataxin by HIF-1 $\alpha$ in the diabetic heart

### Abstract

Diabetes is at epidemic proportions, with the major form of fatality due to congestive heart failure triggered by myocardial infarction (MI). The impaired insulin signaling in the diabetic heart leads to myocardial energy dysregulation that compromises the cardioprotective mechanism against ischemic injury. We observed that db/db mice (leptin deficient, type 2 diabetic mice) have increased infarction size compared to wild type mice after ischemia/reperfusion (IR) injury by TTC stain. We also found that activity of Hypoxia inducible factor-1 (HIF-1 $\alpha$ ), a master transcription factor involved in the cardioprotective response to ischemia, is impaired in db/db hearts. HIF-1 $\alpha$  is known to transcriptionally regulate genes involved in myocardial energetics. We recently found that HIF-1 $\alpha$  transcriptionally regulates the mitochondrial protein frataxin in cardiomyocytes as determined by luciferase assays. In vitro studies indicate that hypoxic conditions increase frataxin protein expression in cardiomyocytes as determined by western analysis. Frataxin plays an important role in the Fe-S cluster biogenesis required for aconitase, succinate dehydrogenase and complexes in the mitochondria. Interestingly, we observed decreased expression of frataxin in the ischemic diabetic heart. Therefore, we postulate that attenuated HIF-1  $\alpha$ -frataxin signaling in ischemic db/db hearts leads to abnormally enlarged infarction size in response to IR. The decline in HIF-1 $\alpha$  activity in response to hypoxia was further validated in cardiomyocytes cultured in high glucose media. The significance for frataxin against hypoxic injury was confirmed by utilizing over-expressed



frataxin cardiomyocytes via MTT, ATP and aconitase activity assays. Currently we are attempting to identify the hypoxia response element (HRE) in frataxin promoter to further validate the transcriptional activity of HIF-1 $\alpha$ . In addition, we are completing the IR surgeries on HIF-1 $\alpha$  KO mice to address the cardioprotective nature of HIF-1 $\alpha$ - frataxin signaling against MI.

## Introduction

We have observed that treatment of PPAR  $\gamma$  exert an increased expression of the HIF-1 $\alpha$  protein in H9C2 cardiomyocytes. Interestingly, we observed that in the db/db hearts and in H9C2 cardiomyocytes grown in high glucose medium, the expression and activation of HIF-1  $\alpha$  is significantly suppressed compared to the controls. In addition we observed that the db/db hearts sustain a larger infarction when subjected to surgical I/R injury compared to c57bl6 control mice. This chapter will focus on our attempt to elucidate our observation of diabetic animals incurring a larger infarction during an I/R injury. Further, our data suggests that PPAR  $\gamma$  agonists can be a potential therapy to mitigate the damage to the diabetic heart when subjected to I/R injury.

Diabetes is at epidemic proportions in the United States, with the major form of fatality is due to congestive heart failure triggered by myocardial infarction (MI). Diabetics sustain extensive cardiac damage when subjected to I/R injury compared to a non diabetic individual. (155). The impaired insulin signalling in the diabetic heart leads to contractile dysfunction and myocardial energy dysregulation, which compromises the cardioprotection mechanism against ischemia/reperfusion injury (IR). Attenuation of this cardioprotection mechanism is associated with increased morbidity and mortality rates observed in diabetic patients.

We have observed that db/db mice (leptin deficient, type 2 diabetic mice) sustain a larger infarction when subjected to IR (>30%) compared to wild type mice. Further, we observed a significant attenuation of Hypoxia inducible factor-1 alpha (HIF-1  $\alpha$ ) activity in the db/db hearts

in response to ischemia. HIF-1 $\alpha$  is the master transcription factor that upregulates myriad of genes to promote survival in response to hypoxia. The transcriptional activity of HIF-1  $\alpha$  on genes such as VEGF, GLUT-1, HK-I & II is well established. The activation of these genes offer profound protection to cells and tissues under hypoxic/ ischemic stress. HIF-1 $\alpha$  mediated transcription requires the recruitment of co-activators P300/CBP and binding of this complex to Hypoxia Response Elements (HRE) located in the promoter regions of the target genes.

Attenuated activity of HIF-1  $\alpha$  in response to hypoxia in diabetic tissues and in cells cultured in high glucose medium has been previously reported (178). Interestingly, the elevated formation of MG in diabetic dermal fibroblasts binds covalently bind with p300 and prevent HIF-1 transactivation. MG is a highly reactive by product which is produced by many metabolic pathways. The main adverse effect of MG is facilitating AGE production (181). Interestingly, we observed an increase in MG levels in db/db cardiac tissues and in H9C2 cells treated with high glucose media.

During an attempt to identify the downstream targets that are affected by HIF-1 $\alpha$  attenuation, we found a significant reduction in frataxin (fxn) protein in the diabetic cardiac tissues compared to the control mice. The nuclear encoded mitochondrial protein Fxn plays an important role in the Fe-S cluster biogenesis required for aconitase, succinate dehydrogenase and complexes in the mitochondria. Defects in Fxn expression is associated with genetically inherited diseases such as Friedreich's ataxia (FRDA), which is known to produce severe cardio-neurological anomalies (224).

In the current study we observed that HIF-1  $\alpha$  can transcriptionally regulate fxn expression by luciferase analysis. Further, we observed that knock down of HIF-1  $\alpha$  by utilizing RNAi significantly reduced the fxn expression in response to hypoxia in H9C2 cardiomyocytes.

Therefore, we postulate that attenuated HIF-1  $\alpha$  –fxn signaling in ischemic db/db hearts lead to abnormally enlarged infarction size in response to I/R.

In regards, to validate this hypothesis, we have constructed a cardiac specific HIF-1  $\alpha$  knockout mice. Also, we have established a fxn overexpressed H9C2 cardiomyocyte cell line. The details of the animal construction of the animal model and cell line will be provided below. Further, our data suggests that treatment of PPAR  $\gamma$  in the diabetic mice will rescue the attenuated HIF-1  $\alpha$  – fxn signaling in the heart, and will mitigate the diabetes associated extensive cardiac damage when subjected to I/R injury.

## **Materials and methods**

### **I/ R injury in mice**

The experimental protocol and the breeding protocol were approved by the Auburn University Institutional Animal Care and Use Committee. Animals were housed in Auburn University's Biological Research Facility.

Prior subjecting the mouse to I/R surgery, the animal was weighed and anesthetized with sodium pentobarbital given through intraperitoneally. The animals were ventilated via a tracheotomy tube and blood pressure and ECG were constantly monitored throughout the procedure. Anesthesia was maintained with supplemental doses of pentobarbital. The hearts were exposed by left thoracotomy. Briefly the procedure involves reversible ligation of the proximal left anterior descending (LAD) coronary artery with a 8.0 silk suture mounted on a tapered needle. After 30 minutes of LAD occlusion, the ligature was loosened and reperfusion was completed after 2 hours. Following reperfusion, Evans Blue dye was perfused by an intra cardiac injection to visualize the area at risk. Hearts were then immediately removed and weighed and the tissues were snap frozen for further analysis.

The infarction size of the hearts was determined by the TTC (triphenyltetrazolium) stain. The hearts were cut in to 1.5mm sections. These tissue slices were incubated in 1% TTC solution at 37 C for 15 minutes. Afterwards the heart sections were imaged and analyzed by Kodak Gel Logic 2200 to determine the area at risk and infarct area.

## Construction of cardiac specific HIF knock out mouse

To construct the above mentioned animal model, we utilized a Cre – lox P system. The transgenic animals with HIF-1  $\alpha$  gene flanked between 2 lox P sites and mice with Cre recombinase expression driven by  $\alpha$  – MHC promoter were purchased from the Jackson laboratories. As the Cre –recombinase expression is driven by the  $\alpha$  – MHC promoter which is specific for the heart, the enzyme will be only expressed in the cardiac tissue. Herein, the mice with HIF-1  $\alpha$  gene flanked by lox P sites and mice with cardiac specific Cre recombinase will be referred to as lox P and Cre mice respectively. The information of the strains of the animals purchased is given below.

- Lox P mice – B6.129 – Hif1a<sup>tm3Rsjo</sup>/J – (stock number: 007561)
- Cre mice - B6 FVB – Tg (Myh 6 – Cre) 218 2 MDS/ J – (stock number: 011038)

The lox P mice are homozygous for lox P gene and the Cre mice are heterozygous for the gene. From here onwards, the dominant lox P allele will be depicted as P and the wild type allele will be depicted as p. Similarly, Cre dominant allele will be referred to as C and the recessive allele will be depicted as c.

Briefly the breeding protocol is as follows. The lox P mice (ccPP) and Cre mice (Ccpp) will be mated to obtain heterozygotes in the first progeny. We assumed the genes will behave according to the Mendelian genetics and the genotypes for the animals are given below.

ccPP \* Ccpp



CcPp – 50%

ccPp - 50%

50% heterozygotes for both genes are expected in the first progeny.

These heterozygotes obtained from the first progeny will be backcrossed again with a lox P animal to obtain cardiac specific HIF-1 knockout mice.

CcPp \* ccPP



CcPP – 25% - cardiac specific HIF – 1 a knockout mice

CcPp – 25% - Heterozygotes for both genes

ccPP - 25% - lox P mice

ccPp – 25% - null mice

The mice will be subjected to I/R surgery as explained in detail above.

## **H9C2 cardiomyocyte cell culture**

H9C2 ventricular rat embryonic cardiomyocytes were purchased from ATCC. Cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% pen strep. Cells were passaged when the confluency reached to 70%. Cells treated with high and low glucose media contained glucose at a concentration of 33mM and 5mM respectively. Cells were maintained at 37 C, 5% CO<sub>2</sub> levels with humidity. The cells were treated with rosiglitazone (10μM).

## **Induction of hypoxia in cell culture**

Cells were exposed to hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> with N<sub>2</sub> for the balance) in an air tight Billups Rothernberg hypoxia chamber with adequate humidity at 37 C for 18-24 hours. The chemical hypoxia was induced by treating the cells with CoCl<sub>2</sub> (400μM) and DMOG (1mM).

## **Construction of frataxin over-expressed H9C2 cardiomyocytes**

Frataxin over-expressed cardiomyocyte cell line was constructed using a lentivirus constructed based on gateway system. First cDNA of frataxin was cloned in to a gateway donor vector with antibiotic resistance for Spectinomycin. Then the donor vector was cloned to a pLX 302 destination vector with ampicillin resistance using LR clonase. pLX 302 is a mammalian expression, lentiviral gateway destination vector. This vector has a puromycin selectable marker.



Then the cells were transformed in to DH5alpha and streaked on agar plates containing ampicillin and incubated at 37 C overnight. The single colonies were isolated and inoculated in liquid broth containing ampicillin. After overnight incubation the plasmid was isolated using Promega pure yield plasmid midi prep system (A2495). The purified gateway vector was co-transfected with lentiviral packaging vector (psPAX2) and envelope vector (pMD2.G) in to HEK 293T cell line. The media with the virus was extracted after the third day of transfection. The media containing the virus was filtered using 0.45µm syringe filters. The 1ml of purified media was mixed with 2 ml of complete media and the H9C2 cells were treated with this mixture for 24 hours. Then the media was changed and the cells were fed with fresh complete media. On the second day the cells were incubated with media containing 1µg/ ml puromycin to select the cells with successful incorporation of the fxn gene.

### **Promoter activity assays**

2.5 kb upstream of the fxn promoter was cloned in to a PGL3 vector using Kpn1 and Xho1 restriction enzymes. Then the vector was transformed in the bacteria and was isolated with the Promega pure yield midi prep system. The H9C2 cardiomyocytes were transfected with this PGL3 vector and pSV –  $\beta$  – galactosidase vector to enable accurate control for transfection efficiency and indexing of luciferase activity. Then the cells were exposed to hypoxia as explained above and the luciferase activity was measured by luciferase reporter assays system (Promega).  $\beta$  – galactosidase activity was measured by spectrophotometrically using ONPG as a substrate. All values and results were expressed as an index of relative light units (RLU).

### **Protein extraction and western immunoblots**

Proteins were extracted from the dissected heart tissues and the H9C2 cells using chilled lysis buffer (Cell signaling) containing protease and phosphatase inhibitors. Proteins were resolved in 12% polyacrylamide gels and probed with fxn (Santa Cruz), MG (3mM), HIF-1 (Novus, Santa Cruz) and tubulin (Iowa Hybridoma center). Signals were quantified by densitometric analyses using Quantity – One analysis software (Bio – Rad).

### **Immunocytochemistry**

The H9C2 cells were exposed to  $\text{CoCl}_2$  to induce chemical hypoxia. Another group of H9C2 cells were transfected with constitutively active HIF-1  $\alpha$  and were cultured in normoxia. After 18 hours, the mitochondria was stained with Mitotracker - red (150nM – Invitrogen) and incubated with fxn. Then the cells were probed with secondary antibody conjugated with FITC. Fluorescence microscopy was performed using a Nikon Ti fluorescence microscope.

## Results

### **HIF-1 $\alpha$ attenuate the infarction size in the diabetic heart**

To better understand the impact of diabetes on cardiac injury during ischemia, we conducted I/R surgeries in db/db mice and c57bl6 wild type mice. We observed a significant increase in the infarction size in db/db hearts. Interestingly, we observed a significant protection offered by DMOG treatment (40mg/kg) prior the animals are subjected to IR injury in both wild type and db/db mice (>20%). We have subjected HIF-1 $\alpha$  heterozygote animals to I/R surgery and these mice sustained extensive cardiac damage which is comparable to db/db mice (Figure 5.1).

### **Diabetes can impact adversely on HIF-1 $\alpha$ localization in the nucleus**

We observed a reduction of HIF-1 $\alpha$  localization in the nucleus of the diabetic animals and also in H9C2 cells cultured in high glucose media (33mM). The nuclear proteins isolated from the cardiac tissues of db/db animals treated with DMOG significantly had attenuated levels of HIF-1  $\alpha$  localized in the nucleus compared to c57bl6 control mice (Figure 5.2 A). Previously, it has been shown that insulin can stabilize the HIF-1 $\alpha$  expression via Akt/ PI3K pathway. We also observed an increased expression of HIF-1  $\alpha$  protein in H9C2 cells treated with insulin (100nM) cultured under normoxic conditions (Figure 2B). We also observed an increase in HIF-

HIF-1  $\alpha$  localization in the nucleus in H9C2 cells after exposure to hypoxia (Figures 5.2 C1 and C2). Further, we observed that HIF-1  $\alpha$  localization is significantly impaired in H9C2 cardiomyocytes cultured in high glucose medium (33mM) (Figure 5.2 C3). DMOG treatment improves the HIF-1 expression (Figure C4) and use of constitutively active HIF-1 a vector also promote HIF-1 localization in the nucleus in normoxic conditions (Figure 5.2 C5).

### **High glucose and hypoxia induce the formation of MG in cardiomyocytes**

MG is known to inhibit the transactivation of HIF-1  $\alpha$  in dermal fibroblasts of diabetic animals (181). Therefore, we assessed the MG levels in the cardiac tissues and the H9C2 cardiomyocytes to explain the low transactivation of HIF-1  $\alpha$  we observed. We observed approximately four fold increase of MG in db/db cardiac tissues (Figures 5.3 A & B). Interestingly we observed that the MG formation is increased in H9C2 cardiomyocytes exposed to hypoxia. The cells cultured in 33mM glucose media exposed to hypoxia had the highest levels of MG (Figure 5.3C). By immunocytochemistry, we observed that the H9C2 cultured in high glucose medium contained elevated levels of MG (Figure 3D1) and most of this MG is associated with the CBP in the nucleus (Figures 5.3 D2, D3 & D4).

## **Diabetes down regulates the active frataxin protein levels in the heart**

We observed a significant reduction of the active version of frataxin (18kDa) in the db/db hearts (Figure 5.4 A). Our gene expression studies confirm that the diabetic cardiac tissues have a significant reduction in frataxin gene expression (Figure 5.4 B1). Densitometry analysis confirms that the active/ inactive ratio of frataxin is significantly lower in the db/ db hearts.

## **HIF-1 $\alpha$ increases the expression of frataxin**

We transfected the cells with a PGL3 luciferase vector containing 2.5 kB region of the frataxin promoter. This vector was co transfected with another vector which transcribe HIF-1  $\alpha$ . Then the cells were treated with CoCl<sub>2</sub> for 18 hours and the luciferase activity was measured after 18 hours. We observed a 2 fold increase in luciferase activity in cells transfected with HIF-1  $\alpha$  after normalization to  $\beta$  – gal values (Figure 5.5 A).

Our immunocytochemistry analysis shows that frataxin is localized in mitochondria and the cytosol under normoxic conditions (Figure 5.5 B1). However, when the cells are exposed to 18 hours of hypoxic conditions, we observed that frataxin is localized predominantly in to the mitochondria in H9C2 cells. Further, we observed the same effect when the cells were transfected with constitutively active HIF-1  $\alpha$  under normoxic conditions (Figure 5.5 B3). The increase in frataxin expression by HIF-1  $\alpha$  was further validated by immunoblots. Our western analysis indicates that treatment of H9C2 cells with CoCl<sub>2</sub> and DMOG for 18 – 24 hours significantly increased the total frataxin expression in the cells. The direct effects of HIF-1  $\alpha$  on

frataxin was further utilized using a RNAi system to knock down frataxin. Figure D shows that the HIF-1  $\alpha$  expression is significantly attenuated with RNAi. Further, there was a significant increase in frataxin expression in the cells treated with  $\text{CoCl}_2$ . This effect was ameliorated in the group that is treated with  $\text{CoCl}_2$  and RNAi for HIF-1  $\alpha$ .

### **Over expression of frataxin increases the cell viability compared to the control after 24 hours of exposure to hypoxia**

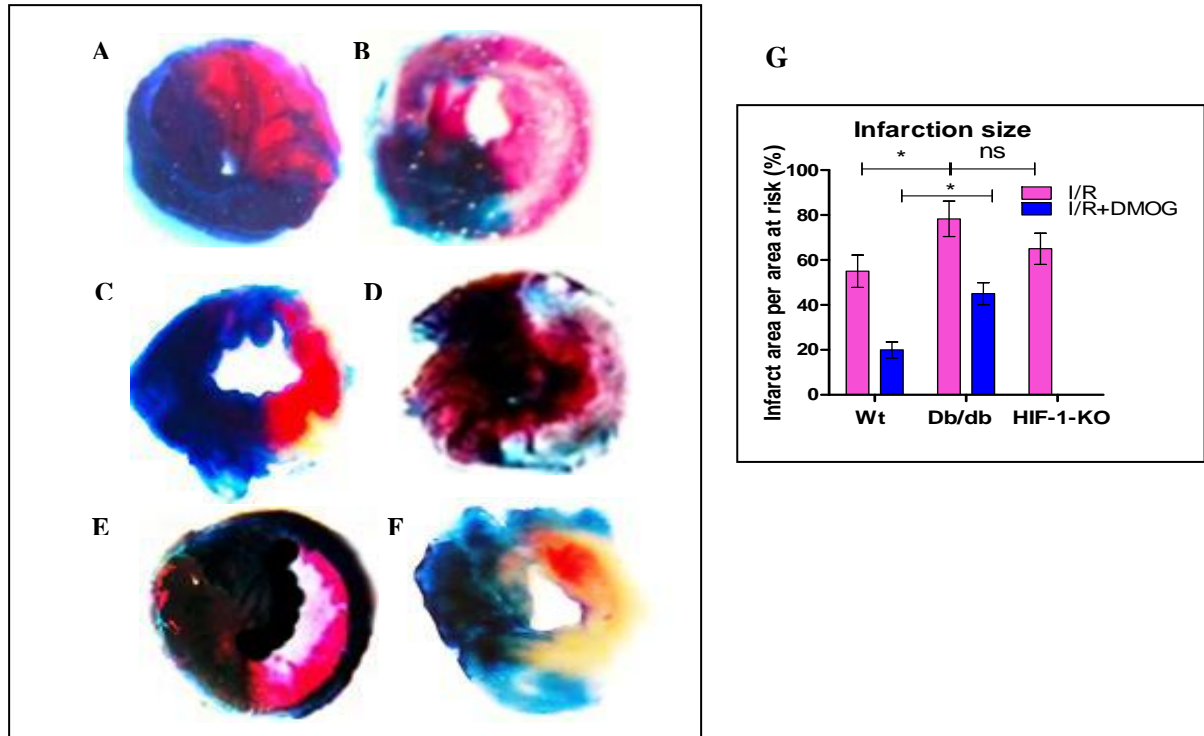
To determine the effects of increased frataxin levels under hypoxic conditions we constructed a frataxin overexpressed H9C2 cardiomyocytes by utilizing a lentiviral system. The figure 5.6 A demonstrates that the frataxin expression is significantly elevated in our frataxin over expressed cardiomyocytes. We exposed the normal H9C2 cardiomyocytes and frataxin over expressed cells to 24 hours of hypoxia and the cell viability was assessed with MTT assays. We observed that the frataxin over expressed myocytes can retain the cell viability comparable to the control cells (Figure 5.6 B).

### **PPAR $\gamma$ activation significantly rescue the infarction in db/db hearts**

As mentioned previously in this chapter, we observed that the db/db hearts sustain extensive cardiac damage when subjected to I/R injury compared to the control mice. However, we observed that 14 days of PPAR  $\gamma$  treatment (10mg/ kg) significantly reduced the infarction

size in both wildtype and the db/db mice (Figure 5.7). In order to find out the mechanism behind this protection offered by the PPAR  $\gamma$  treatment, we conducted promoter activity assays. We co-transfected the cells with PGL3 vector containing HRE region and constitutively active PPAR  $\gamma$  (CA – PPAR  $\gamma$ ) vector. Our luciferase data implicate that CA – PPAR  $\gamma$  significantly increase the luciferase values which implicate that PPAR  $\gamma$  can indirectly/ directly activate the transcription of HIF-1  $\alpha$  (Figure 5.7 E).

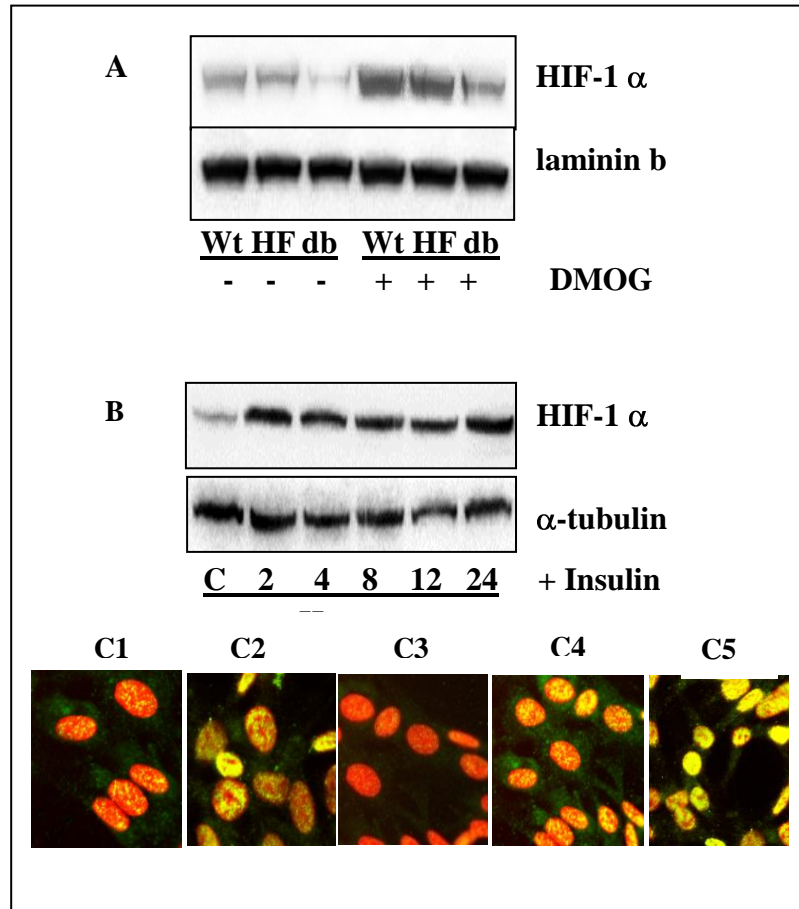
## Figure and figure legends



**Figure 5.1: HIF-1  $\alpha$  mitigates ischemia reperfusion injury in c57bl6 and diabetic hearts**

(A) Sham surgery. (B) Ischemia/reperfusion injury in wild type C57bl6 mice. (C) DMOG reduces the infarct size in C57bl6 hearts. (D) Infarction in db/db mouse is larger in comparison to C57bl6. (E) DMOG attenuates enlargement of infarction size in the db/db mouse heart. (F) HIF-1 $\alpha$  heart specific knockout mice display large infarction in the hearts. (G) Graphical representations for measured infarction sizes compared to areas at risk. Where n=4 per group.\*; P<0.05. Where n=4 per group.\*\*\*; P<0.0001

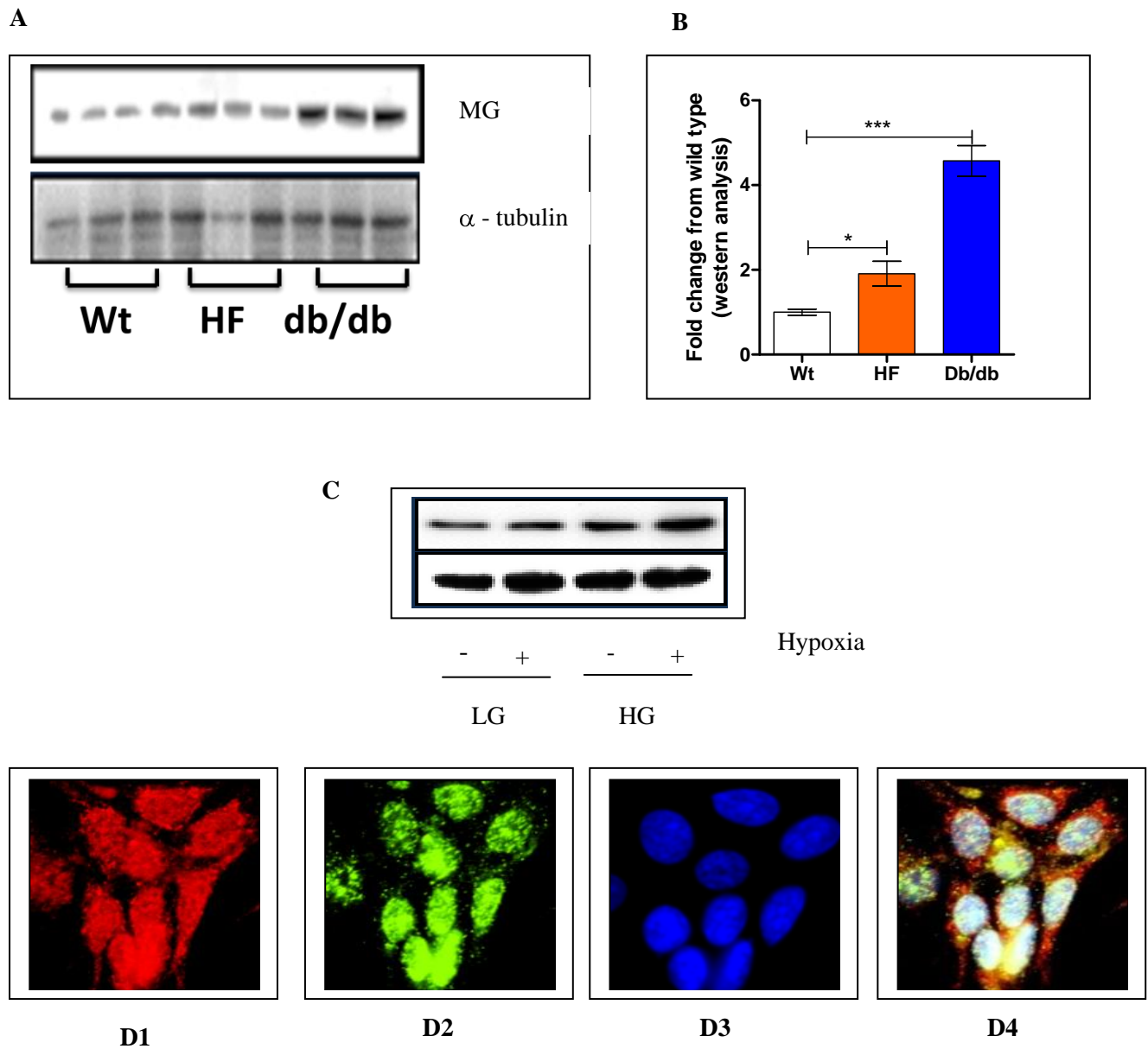




**Figure 5.2: Alteration of HIF-1 localization in the diabetic heart**

HIF-1 $\alpha$  localizes to the nuclei after DMOG (40 mg/kg) prior to heart enucleation. **(A)** Western analysis of left ventricular nuclear preps from Wt: wild type mice, HF: High fat diet, db/db: leptin deficient mice hearts, n=3 mice per group. HIF-1 $\alpha$  is standardized to nuclear laminin B. **(B)** Insulin stabilizes HIF-1 $\alpha$  levels in H9C2 cells. **(C1)** HIF-1 $\alpha$  is localized in H9C2 cells under normoxia. **(C2)** HIF-1 $\alpha$  is localized in the nucleus after hypoxia. **(C3)** High glucose prevents the localization of HIF-1  $\alpha$  in the nucleus in response to hypoxia. **(C4)** DMOG improves HIF-1 $\alpha$  expression after hypoxia. **(C5)** Mutant HIF-1 $\alpha$  increased nuclear localization in normoxic

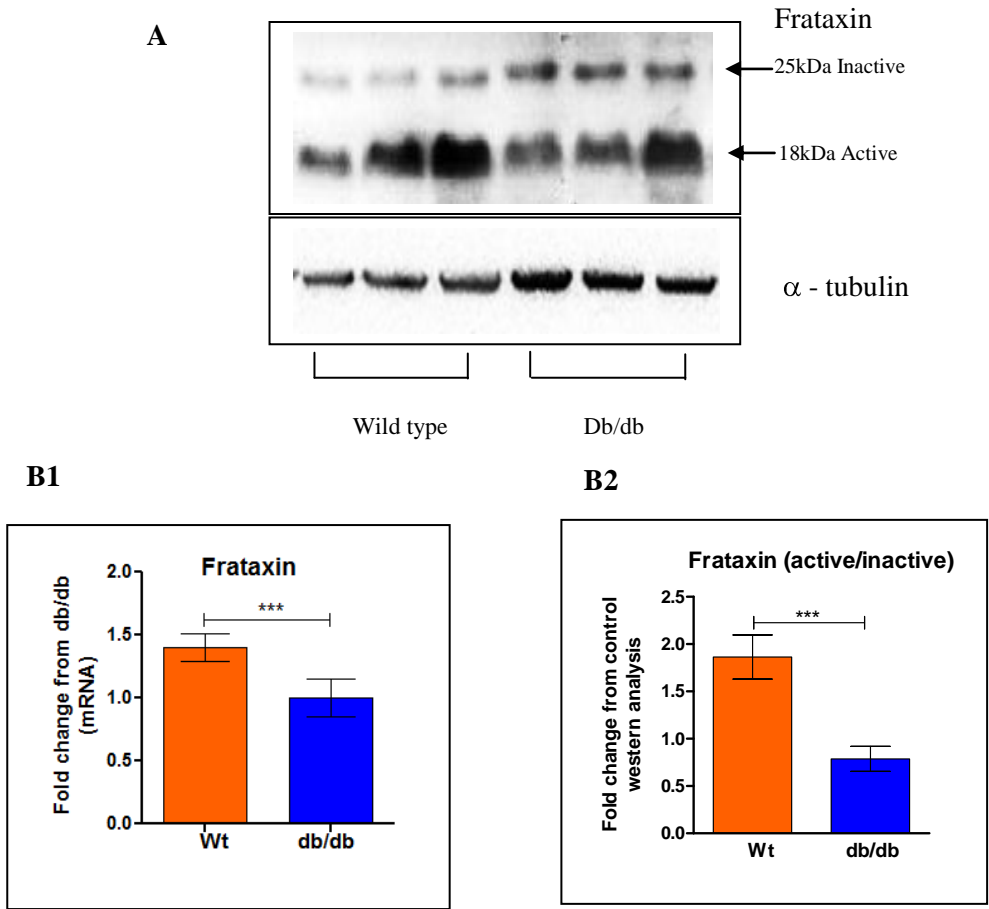
conditions. Note levels in all panels of faint cytosolic HIF-1 $\alpha$  (green) and nuclear HIF-1 $\alpha$  (Yellow). Red is nuclear staining.



**Figure 5.3: High glucose and hypoxia induce formation of methylglyoxal**

(A) & (B) Db/db and high fat diet display more methyl glyoxal (MG) levels than wild type hearts as determined by western analysis. N=3 mice per group. (C) H9C2 cells cultured in low glucose

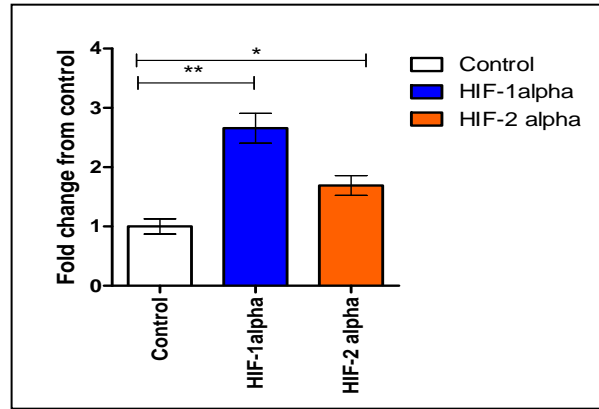
medium (LG) (5mM) and high glucose (HG) (33mM) show higher levels of MG than non-hypoxia high glucose or low glucose (LG) with or without hypoxia. **(D1)** H9C2 cells in high glucose (33 mM) show high levels of MG (Red) after hypoxia. **(D2)** CBP **(D3)** Dapi. **(D4)** High glucose (33mM) after hypoxia induces high levels of methylglyoxal (red).



**Figure 5.4: Diabetes down regulates the active version of frataxin**

(A) Reduced expression of the active version (18kDa) of frataxin is found in diabetic (db/db) hearts as demonstrated by western analysis. (B1) Db/db hearts have significantly reduced frataxin gene expression in cardiac tissues. (B2) The protein expression of active frataxin/inactive frataxin ratio is significantly attenuated in the db/db hearts.

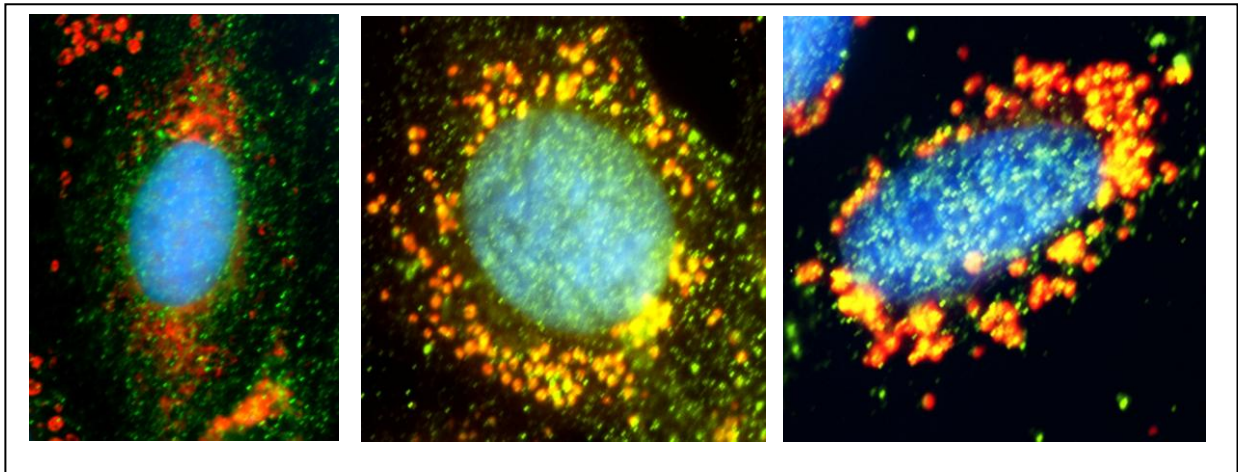
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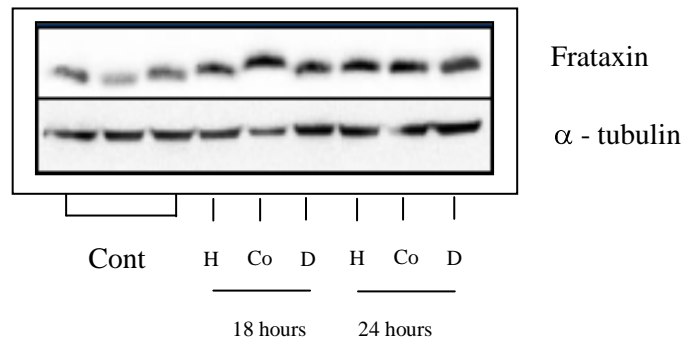
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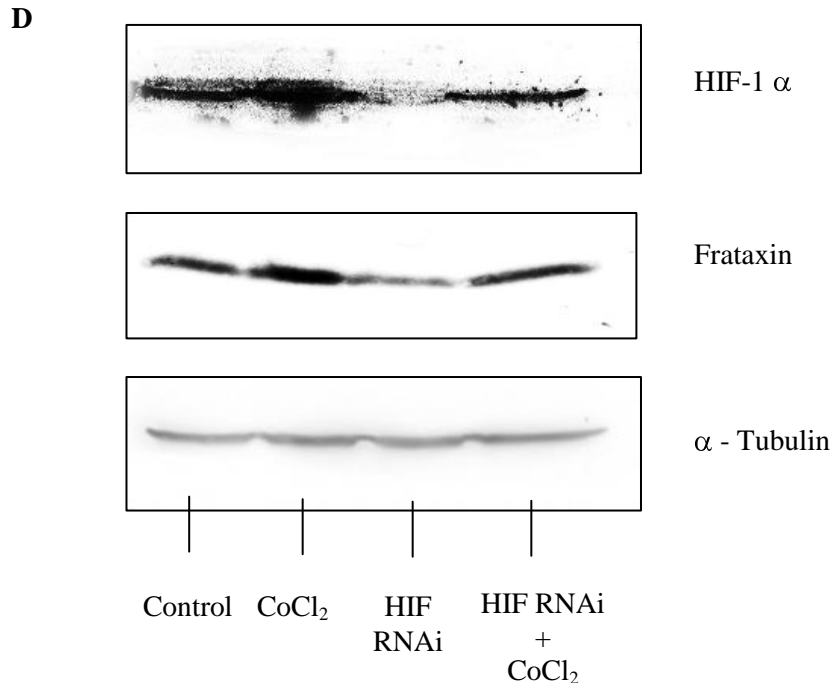
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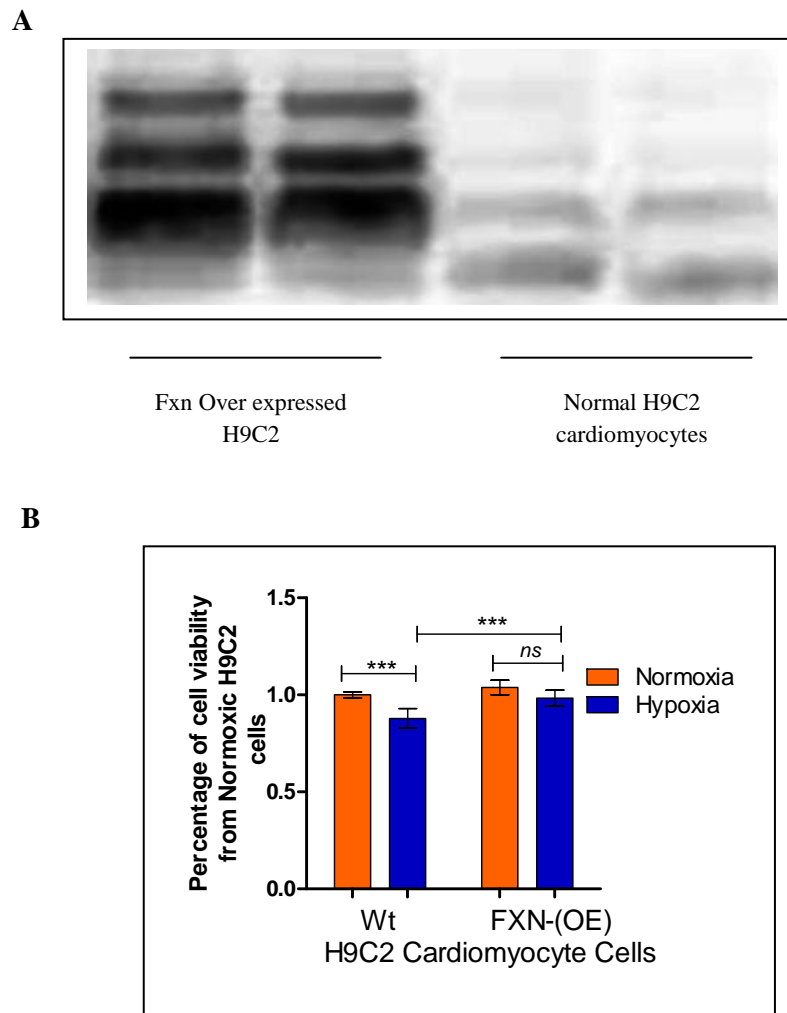
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**Figure 5.5: HIF-1 $\alpha$  increases the expression of Frataxin**

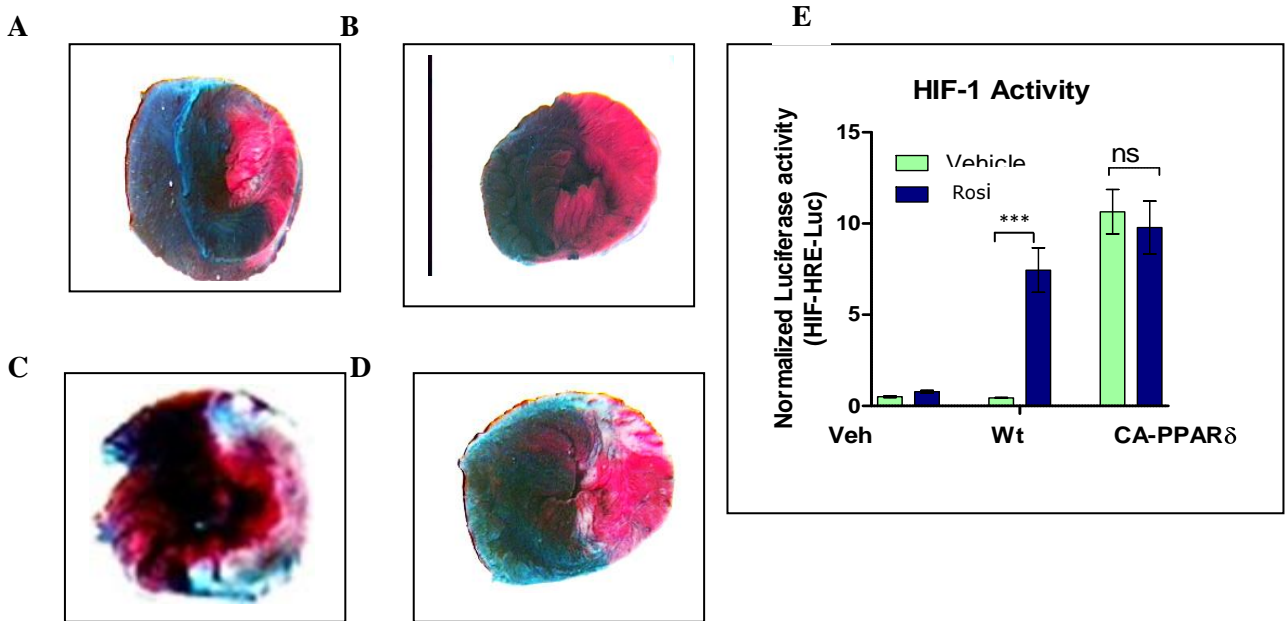
(A) Frataxin expression is significantly increased in the presence of HIF-1 $\alpha$ . Transcriptional activity verifies that HIF-1 $\alpha$  regulates frataxin expression in the 2.5 kbp region of the frataxin promoter, which indicates the transcriptional regulation role of HIF-1 $\alpha$ . (B1), Frataxin (green) is localized in cytosol and mitochondria (red) under normoxia. (B2) Hypoxia induces increased frataxin localization in mitochondria. (B3) Over expression of constitutively active HIF-1 $\alpha$  induces mitochondrial localization of frataxin. (C) 18 and 24 hours of hypoxia, either by hypoxia (H), Cobalt chloride (Co) or DMOG (D) induce an increase in frataxin expression as determined by western analysis. (D) RNAi for HIF-1 a significantly reduce the HIF-1  $\alpha$  expression with CoCl<sub>2</sub> treatment. Further, CoCl<sub>2</sub> treatment significantly increased the frataxin expression while HIF RNAi ameliorate this effect.



**Figure 5.6: Over-expression of frataxin increases the cell viability**

(A) Frataxin expression is significantly increased with the lentiviral infection. (B) Hypoxia (24 hours) reduced the mitochondrial activity (cell viability) as determined by MTT assay in control H9C2 cells. Frataxin over expressing cells have significant improvement in mitochondrial activity under hypoxia vs control H9C2 cells under hypoxia.





**Figure 5.7: PPAR  $\gamma$  activation significantly rescue the infarction in the db/db hearts**

The wild type mice had less infarction size compared to the db/db animals when subjected I/R surgery. Rosiglitazone treatment significantly reduced the infarction in both wild type and db/db animals. (A) Wild type (B) Wild type treated with rosiglitazone (c) db/db (d) db/db treated with rosiglitazone. Our luciferase assays demonstrate that rosiglitazone treatment and transfection of CA – PPAR  $\gamma$  significantly increase the HRE luciferase activity.

## Discussion

Many clinical and epidemiological studies show that the hearts of diabetic individuals are more sensitive to I/R injury. This leads to higher mortality rates amongst diabetics than that of non diabetics having a heart attack. Experimental studies on transgenic diabetic animal models also implicate the susceptibility of attaining a larger infarction by I/R injury compared to the non diabetic controls. The increase in infarction size and impaired recovery observed diabetic individuals and animal models are attributed to multiple factors. One such factor is altered metabolic substrate utilization in the diabetic myocardium. Additionally, increased oxidative stress is also identified as a risk factor for incurring extensive cardiac damage due to I/R in the diabetic hearts (287).

The altered metabolism and increased oxidative stress in the diabetic heart alters many biological signaling pathways. It has been found that the transcriptional activity of HIF-1  $\alpha$  is attenuated in response to hypoxia in many diabetic tissues. HIF-1  $\alpha$  activation protects the cells/ tissues from hypoxic damage by increasing the transcription of protective genes that regulate angiogenesis and metabolism. The increased MG formation in the diabetic dermal fibroblasts found to interact with CBP/ p300 and prevent it from binding it to HIF-1  $\alpha$ . This significantly attenuate the transactivation of HIF-1  $\alpha$  in response to ischemia/ hypoxia and suppresses the protection offered by HIF-1  $\alpha$ .

We also observed an increase in the infarction when the db/db animals were subjected to I/R injury. Interestingly we observed that the prior treatment with DMOG (40mg/kg) in db/db mice offered a profound protection against I/R injury in the db/db hearts (Figure 5.1). In order to explain these observations, we analyzed the HIF-1  $\alpha$  localization in to the nucleus in these

hearts. Compared to the controls mice, the db/db hearts had significantly attenuated levels of HIF-1  $\alpha$  localized with the DMOG treatment. This data suggested that in the db/db heart may have impaired HIF-1  $\alpha$  stabilization levels or impaired transactivation. Our cell culture data further consolidated the findings from the animals (Figure 5.2). We observed that the H9C2 cells cultured in high glucose media (33mM) also had less HIF-1  $\alpha$  localized in the nucleus after hypoxia treatment (Figure 5.2).

To better explain the impaired transactivation of HIF-1  $\alpha$  in the diabetic tissues, we measure the MG levels in our db/db cardiac tissues. Interestingly we observed a significant increase in MG levels in diabetic hearts. Further, we observed that the H9C2 cardiomyocytes grown in high glucose media produce increased levels of MG in response to hypoxia compared to the cells grown with low glucose medium (5mM). It is well established that the MG formation is increased in the tissues and cells under hyperglycemia. This is because increased availability of glucose and the attenuation of removal mechanisms of MG in the cells. However, how MG production increases in the presence of hypoxia remains to be further elucidated. The immunocytochemistry studies also confirmed that increased MG production in H9C2 cardiomyocytes exposed to high glucose. Further, we observed that most of this MG is co-localized to CBP which is a necessary cofactor for HIF-1  $\alpha$  function. Therefore, this data may explain the impaired transactivation of HIF-1  $\alpha$  under hyperglycemic conditions in cardiomyocytes.

In order to identify the novel downstream targets which are modulated by HIF-1  $\alpha$ , we found that the expression of frataxin is significantly induced when the H9C2 cells exposed to chemical hypoxia (Figure 5.4). Frataxin is a nuclear encoded mitochondrial protein, which plays a vital role in iron homeostasis in the mitochondria. This protein acts as a chaperone protein in

biosynthesis of heme. Also, it is required for formation of Fe-S clusters which are prosthetic groups much needed for the proper functioning of aconitase, succinate dehydrogenase and also the complex I and III of the electron transport system. Therefore, frataxin plays a pivotal role in regulating cellular metabolism and oxidative stress. The work by *Guccini et al* demonstrates that frataxin expression is increased in response to hypoxia in tumor cell lines and tissues. This work suggests that frataxin expression promotes cell survival and progression in cancer. However, the role of frataxin in hypoxia/ ischemia has not been studied extensively.

Our immunocytochemistry data also shows that frataxin predominantly gets localized in the mitochondria due to a hypoxic insult. Furthermore, we observed less expression of frataxin in H9C2 cardiomyocytes transfected with RNAi system for HIF-1  $\alpha$ . This observation led us to formulate a hypothesis that HIF-1 may have the potential to transcriptionally regulate frataxin expression in hypoxia.

We utilized luciferase assays to test whether HIF-1  $\alpha$  can transcriptionally regulate frataxin in response to hypoxia in cardiomyocytes. A PGL3 vector containing 2.5kB upstream of frataxin promoter was cloned and transfected to the cells. We observed that the luciferase activity is increased more than 2 fold in the cells co transfected with HIF-1  $\alpha$  vector and chemical hypoxia. These data suggest that HIF-1  $\alpha$  can directly/ indirectly regulate frataxin expression in hypoxia. Future studies will include conducting ChIP assays to further validate whether HIF-1  $\alpha$  directly regulate transcriptional activity in response to hypoxia in cardiomyocytes.

To further explore the protective effects of frataxin in cardiomyocytes, we constructed a frataxin over-expressed H9C2 cardiomyocyte cell line using a lentiviral system. We assessed the cell viability of these cells and normal H9C2 cells after exposure to hypoxia for 24 hours. Our

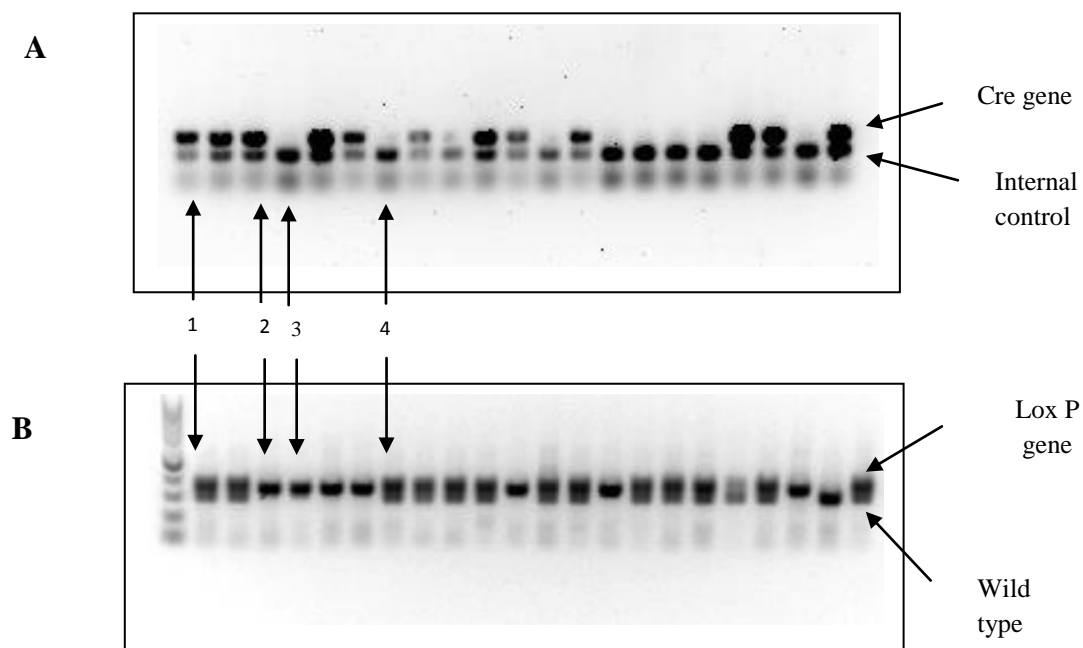
MTT assay demonstrates that over-expression of frataxin protects the cell viability comparable to control cells. This suggests that frataxin expression exerts beneficial effects and promote cell survival under hypoxic insult (Figure 5.6).

Our data confirms attenuation of HIF-1  $\alpha$  in response to hypoxia/ ischemia in hyperglycemic conditions in cells as well as in tissues. Identification of potential therapeutic targets to increase the HIF-1  $\alpha$  expression in the diabetic heart may offer a profound protection against I/R injury and improve the recovery in diabetic individuals. Previously we have found that PPAR  $\gamma$  activation can increase the HIF-1  $\alpha$  activity in cardiomyocytes. The promoter activity assays we conducted by utilizing a PGL3 vector which has HRE, indicated a significant increase in activity with PPAR  $\gamma$  treatment. We observed the same results with cells transfected with a constitutively active PPAR  $\gamma$  vector (Figure 5.7). These data confirms that the PPAR  $\gamma$  agonism may have a direct/ indirect transcriptional activity on HIF-1  $\alpha$ . The *in silico* studies we have conducted validate the presence of PPRE regions in the upstream of 2.5kB of HIF-1  $\alpha$  promoter. Further, studies are required to validate the functionality of the PPRE sites we found in the HIF-1  $\alpha$  gene promoter. We also observed a significant protection against I/R injury in db/db animals treated with rosiglitazone (10mg/kg) for 14 days.

### **Future directions:**

We have constructed a transgenic cardiac specific HIF-1 $\alpha$  knockout mouse in our lab. We will utilize these mice to validate the HIF-1  $\alpha$ – frataxin signaling in response to I/R injury in the heart. Further, we will induce diabetes in these mice by feeding a high fat diet to explore the effects of diabetes on HIF-1  $\alpha$ – frataxin signaling. We will conduct further analysis on our

frataxin over-expressed H9C2 cardiomyocytes to identify the protective mechanisms offered by frataxin in response to hypoxia. Also, we are at the final stage of constructing a frataxin knock down H9C2 cell line as well. If we are able to produce this cell line successfully, it will be utilized to further validate the role of frataxin during hypoxic stress. ChiP assays, and point directed mutagenesis will be carried out to validate the direct transcriptional regulation of HIF-1  $\alpha$  on frataxin gene.



**Figure 2.8 Confirmation of the cardiac specific HIF-1  $\alpha$  knockout animal model**

(A) Cre blot: Cre band indicates the presence of the Cre gene driven by the  $\alpha$  - MHC promoter. (B) The gel image for the presence of lox P gene. (1) Heterozygous HIF-1  $\alpha$  knockout (CcPp) (2) Homozygous cardiac specific knockout (CcPP) (3) Lox P animal (ccPP) (4) null (ccPp)

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