EFFECT OF HYPERGLYCEMIA AND THIAZOLIDENEDIONES ON CARDIAC ANGIOTENSIN CONVERTING ENZYME (ACE2) AND NEPRILYSIN (NEP)
in db/db DIABETIC MICE

A thesis submitted in partial fulfillment
Of the requirements for the degree of
Master of Science

By

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2017
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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Rucha Fadnavis ENTITLED “Effect of hyperglycemia and thiazolidinediones on cardiac angiotensin converting enzyme 2 (ACE2) and neprilysin (NEP) in db/db diabetic mice.” BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Fadnavis, Rucha M.S., Department of Pharmacology and Toxicology, Wright State University, 2017. Effect of hyperglycemia and thiazolidinediones on cardiac angiotensin converting enzyme 2 (ACE2) and neprilysin (NEP) in db/db diabetic mice.

Type 2 diabetes mellitus is strongly associated with increased risk of cardiovascular disease. Despite availability of several antidiabetic medications, the cardiovascular outcomes remain unchanged. Activation of renin angiotensin system is one of the critical factors in development of diabetic complications. Hyperglycemia causes an increase in tissue and circulating Angiotensin II. Angiotensin converting enzyme (ACE2) and neprilysin (NEP) are angiotensin (1-7) forming enzymes. ACE2 was first cloned from heart failure patients and has cardioprotective and renoprotective properties. Combination of angiotensin receptor antagonism and NEP inhibition is a new therapeutic strategy for treatment of heart failure. The aim of this study was to investigate whether there is an alteration of cardiac NEP and ACE2 in db/db mice and to study the effect of glycemic control with rosiglitazone and pioglitazone on these enzymes. Lean control and db/db mice were fed 20 mg/Kg/day rosiglitazone and pioglitazone. Diabetic mice demonstrated hyperglycemia which was attenuated by rosiglitazone and pioglitazone treatment. Western blot revealed two bands for NEP and ACE2 in both lean and db/db mice in both the studies. Mature NEP was downregulated while fragment was upregulated in db/db mice. Expression of mature ACE2 was upregulated in db/db mice while the fragment was not affected. Both rosiglitazone and pioglitazone had no effect on cardiac NEP and ACE2 expression. There was a significant increase in cardiac ADAM 17 expression in db/db mice. Another aim was to optimize the fluorescence based enzyme activity assay for renal and cardiac ACE2 and NEP. Protease inhibitors for prolyl endopeptidase, prolyl carboxypeptidase, aminopeptidase A-M were introduced in the assay buffer. Addition of protease inhibitors improved the specificity of the renal ACE2 activity assay. Protease inhibitors however, did not affect renal NEP activity assay. This is the first study to report the presence of two immunoreactive bands for cardiac ACE2 and NEP expression. We also report the possibility of fully mature form of cardiac NEP being degraded to form fragmented form. To the best of our knowledge, this study is also the first to report an upregulation in cardiac ACE2 in db/db mice.
# TABLE OF CONTENTS

**INTRODUCTION** ....................................................................................................................... 1

Diabetes Mellitus .......................................................................................................................... 1

Epidemiology ............................................................................................................................... 1

Risk Factors for Type 2 Diabetes ............................................................................................... 2

Diagnosis of Diabetes .................................................................................................................. 3

Pathophysiology of Diabetes Mellitus .......................................................................................... 4

- Hyperglycemia ......................................................................................................................... 5
- Hyperinsulinemia ...................................................................................................................... 5
- Reactive oxygen species (ROS) and oxidative stress ............................................................... 5
- Lipotoxicity ............................................................................................................................... 6
- Impaired mitochondrial function ............................................................................................. 6
- Activation of Renin Angiotensin System (RAS) ...................................................................... 7

Complications of Diabetes .......................................................................................................... 7

- Cardiovascular Complications ............................................................................................... 7
- Diabetic Kidney Disease (DKD) ............................................................................................. 9

Management of Diabetes ............................................................................................................ 9

- Lifestyle Modification ............................................................................................................. 9
- Pharmacological Intervention ................................................................................................. 10

**Peroxisome proliferator activating receptors $\gamma$** ............................................................... 11

- Thiazolidinediones: PPAR$\gamma$ agonists in treatment of diabetes ........................................... 12
- Rosiglitazone & Pioglitazone .................................................................................................. 12
- Safety Concerns of Thiazolidinediones ................................................................................... 14

**Evidence of RAS activation in Diabetes related cardiovascular complications** ...................... 14

**Components of Renin Angiotensin System (RAS)** ................................................................. 15
<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin converting enzyme 2 (ACE2)</td>
<td>16</td>
</tr>
<tr>
<td>Neprilysin (NEP)</td>
<td>17</td>
</tr>
<tr>
<td>Dual AT1 Receptor Blockade and Neprilysin Inhibition in Heart Failure</td>
<td>18</td>
</tr>
<tr>
<td>A Disintegrin and Metalloprotease-17 (ADAM 17)</td>
<td>19</td>
</tr>
<tr>
<td>Animal Model of Diabetes</td>
<td>20</td>
</tr>
<tr>
<td>Hypothesis and Specific Aims</td>
<td>22</td>
</tr>
<tr>
<td>Hypothesis</td>
<td>22</td>
</tr>
<tr>
<td>Specific Aims</td>
<td>22</td>
</tr>
<tr>
<td>Significance</td>
<td>23</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>24</td>
</tr>
<tr>
<td>Experimental design</td>
<td>24</td>
</tr>
<tr>
<td>Treatment with Rosiglitazone</td>
<td>24</td>
</tr>
<tr>
<td>Treatment with Pioglitazone</td>
<td>24</td>
</tr>
<tr>
<td>Body Composition Measurement</td>
<td>24</td>
</tr>
<tr>
<td>Blood Glucose Measurement</td>
<td>25</td>
</tr>
<tr>
<td>Organs Perfusion</td>
<td>25</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>25</td>
</tr>
<tr>
<td>Western Blot</td>
<td>26</td>
</tr>
<tr>
<td>Optimization of ACE2 Activity</td>
<td>27</td>
</tr>
<tr>
<td>Optimization of NEP Activity</td>
<td>27</td>
</tr>
<tr>
<td>Results</td>
<td>69</td>
</tr>
<tr>
<td>Specific Aim 1</td>
<td>69</td>
</tr>
<tr>
<td>Physiological and metabolic parameters</td>
<td>69</td>
</tr>
<tr>
<td>Plasma hormone and lipid parameters</td>
<td>71</td>
</tr>
<tr>
<td>Cardiac Protein expression of NEP, ACE2, ACE and ADAM 17</td>
<td>72</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1: Interaction between various risk factors and condition of prediabetes and type 2 diabetes...... 2
Figure 2: Pathophysiology of type 2 diabetes mellitus................................................................. 4
Figure 3: Effect of rosiglitazone on blood glucose and body weight ........................................... 31
Figure 4: Effect of rosiglitazone on food intake and water intake ............................................. 32
Figure 5: Effect of rosiglitazone on absolute body fat and absolute lean mass ............................. 33
Figure 6: Effect of rosiglitazone on total body water and heart to body weight ratio .................... 34
Figure 7: Immunoblot demonstrating effect of rosiglitazone on cardiac ACE2 expression ........... 35
Figure 8: Effect of rosiglitazone on cardiac ACE2 (95 kDa) .......................................................... 36
Figure 9: Effect of rosiglitazone on cardiac ACE2 (30 kDa) .......................................................... 37
Figure 10: Immunoblot demonstrating effect of rosiglitazone on cardiac NEP expression .......... 38
Figure 11: Effect of rosiglitazone on cardiac NEP (100 kDa)......................................................... 39
Figure 12: Effect of rosiglitazone on cardiac NEP (65 kDa) ........................................................... 40
Figure 13: Effect of rosiglitazone on cardiac ACE expression ....................................................... 41
Figure 14: Effect of rosiglitazone on cardiac ADAM 17 expression .............................................. 42
Figure 15: Immunohistchemistry for cardiac ACE2 expression expression in 14 week old mice ...... 43
Figure 16: Immunohistchemistry for cardiac NEP expression expression in 14 week old mice ....... 44
Figure 17: Immunohistchemistry for cardiac ADAM 17 expression expression in 14 week old mice .... 45
Figure 18: Effect of pioglitazone on blood glucose and body weight ........................................... 46
Figure 19: Effect of pioglitazone on fat intake and water intake ................................................... 47
Figure 20: Effect of pioglitazone on absolute body fat and absolute lean mass ............................. 48
Figure 21: Effect of pioglitazone on total body water and heart to body weight ratio ..................... 49
Figure 22: Immunoblot demonstrating effect of pioglitazone on cardiac ACE2 expression .......... 50
Figure 23: Effect of pioglitazone on cardiac ACE2 (95kDa) expression ......................................... 51
Figure 24: Effect of pioglitazone on cardiac ACE2 (30kDa) expression ......................................... 52
Figure 25: Immunoblot demonstrating effect of pioglitazone on cardiac NEP expression ............ 53
Figure 26: Effect of pioglitazone on cardiac NEP (100kDa) expression ......................................... 54
Figure 27: Effect of pioglitazone on cardiac NEP (65kDa) expression ........................................ 55
Figure 28: Effect of pioglitazone on cardiac ACE expression ..................................................... 56
Figure 29: Immunohistochemistry for cardiac ACE2 expression in 18 week old mice ...................... 57
Figure 30: Immunohistochemistry for cardiac NEP expression in 18 week old mice ....................... 58
Figure 31: Optimization and effect of MLN-4760 on renal ACE2 expression ................................. 59
Figure 32: Optimization and effect of MLN-4760 on cardiac ACE2 expression .............................. 60
Figure 33: Effect of MLN-4760 and rosiglitazone on cardiac ACE2 expression in buffer A .............. 61
Figure 34: Effect of MLN-4760 and rosiglitazone on cardiac ACE2 expression in buffer B ............... 62
Figure 35: Optimization and effect of thiorphan on renal NEP expression .................................... 63
Figure 36: Optimization and effect of thiorphan on cardiac NEP expression ................................. 64
Figure 37: Effect of thiorphan and rosiglitazone on cardiac NEP expression in buffer A .................. 65
Figure 38: Effect of thiorphan and rosiglitazone on cardiac NEP expression in buffer B ................. 66
Figure 39: Effect of pioglitazone on cardiac ACE2 activity in 18 week old mice ............................. 67
Figure 40: Effect of thiorphan and pioglitazone on cardiac NEP activity in 18 week old mice ............ 68

LIST OF TABLES

Table 1: Age dependent changes in metabolic parameters of lean and db/db mice .......................... 29
Table 2: Effect of rosiglitazone on plasma hormone and lipids in lean and db/db diabetic mice ........ 30
I. INTRODUCTION

Diabetes Mellitus

Scientific research throughout the 19th and 20th century has led to rapid advancement in drug discovery and better healthcare practices. The average lifespan has risen due to the decline in mortality caused by infectious diseases. On the flip side, lifestyle diseases have been an emerging trend since late 20th century and are rapidly rising. Among these lifestyle diseases, diabetes mellitus is one of the biggest health challenges. Diabetes mellitus is a chronic disease associated with increased morbidity and mortality syndrome of metabolic disorders that is most commonly associated with impaired glucose processing. Diabetes mellitus is manifested in many forms namely prediabetes, type 1 diabetes, type 2 diabetes, gestational diabetes, neonatal diabetes, monogenic diabetes syndrome, cystic fibrosis induced diabetes and post transplantation diabetes (1). Type 2 diabetes mellitus (T2DM) is the prevalent form of diabetes in patients, about 98% of diabetic patients suffer from T2DM. T2DM is characterized by impaired glucose tolerance, insulin resistance by target organs and relative reduction in insulin secretion by pancreatic β-cells leading to hyperglycemia (1). Diabetes control and complications trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) showed that risks and complications in diabetic patients are directly related to glycemic control as measured by glycated hemoglobin (HbA1C) levels (2). Effective treatment of hyperglycemia of type 2 diabetes is of critical importance in management of macrovascular and microvascular complications.

Epidemiology

Worldwide projections by the International Diabetes Federation number of patients suffering from diabetes 51% by 2030 (3;4). Diabetes is prevalent in 10% of total adult population of North America. According to the National Diabetes Statistics Report, 7.6% of non-Hispanic white, 9.0% of Asian American, 12.8% of
Hispanic, 13.2% of non-Hispanic black and 15.9% of American Indians/Alaskan Native populations suffer from some form of diabetes (5). The number of patients suffering from diabetes is projected to grow by 98% (4). Growing number of patients concurrently leads to increase in healthcare expenditure which is currently projected to around 612 billion USD and 1010 billion USD (3). The enormous costs associated with treatment is an enormous burden on the economy. It is important to study the pathophysiological mechanisms of type 2 diabetes to devise effective strategies for diagnosis, treatment and management of diabetes complications.

**Risk Factors for Type 2 Diabetes**

![Diagram showing the interaction between various risk factors and condition of prediabetes and type 2 diabetes.](image)

*Figure 1: Interaction between various risk factors and condition of prediabetes and type 2 diabetes. Modified from Chatterjee et. al 2016 (6)*
Multiple genetic and lifestyle factors contribute towards the development of type 2 diabetes (7). It has thus been characterized as a polygenic disease (7). According to the guidelines provided by the American Diabetes Association, family history, lack of physical activity, hypertension, obesity (BMI ≥ 25Kg/m2), dyslipidemia, polycystic ovary disease, low testosterone are the most common risk factors that lead to prediabetic state (8). Prediabetic state is usually asymptomatic and thus patients with any of the aforementioned conditions have been advised to undergo regular screening tests for type 2 diabetes after age of 45 years (8).

**Diagnosis of Diabetes**

Diagnostic criteria for the diagnosis of diabetes are elucidated in the guidelines provided by the American Diabetes Association (9). Since patients do not always exhibit classic symptoms of hyperglycemia, a variety of criteria have been set by the advisory committee of the American Diabetes Association. One of the following criteria need to be fulfilled for appropriate diagnosis:

1. A fasting plasma glucose ≥ 126mg/dL, where fasting plasma glucose is defined as having no food intake at least 8 hours prior
2. Plasma glucose levels ≥ 200 mg/dL in an oral glucose tolerance tests, performed according to the World Health Organization guidelines with a glucose load of 75g anhydrous glucose solution in water
3. Glycosylate hemoglobin (HbA1C) ≥ 6.5% in an National Glycohemoglobin Standardization Program (NGSP) certified standardized laboratory test. Random plasma glucose ≥ 200mg/dL in a patient with classic symptoms of hyperglycemia.

However, progressive organ damage leads to severe complications until proper diagnosis of type 2 diabetes in most patients. This organ damage takes place through several pathophysiological mechanisms.
Pathophysiology of Diabetes Mellitus

Figure 2: Pathophysiology of type 2 diabetes mellitus.

Patients with type 2 diabetes require excessive levels of plasma insulin. Rise in blood glucose levels stimulate production of insulin from the pancreas. The insulin normally produced fails to stimulate the muscle and liver. This results in reduced glucose uptake from the blood. The liver contributes to high glucose levels by breaking down and releasing stored glucose (glycolysis) and, to a lesser extent, making new glucose (gluconeogenesis). Some of the excess glucose is taken up by the fat cells or taken up by the liver and converted to triglycerides. Accumulation of excess triglycerides lead to lipotoxicity. Hyperglycemia in turn leads to impaired mitochondrial function, reactive oxygen species (ROS) production and renin angiotensin system (RAS) activation.
To reduce the impact of diabetes on lives and the economy, it is important to understand the underlying pathophysiological mechanisms leading to complications of diabetes. Although insulin resistance and beta-cell dysfunctions are the main drivers for type 2 diabetes, other factors also contribute to the pathogenesis of the disease.

(i) **Hyperglycemia**

Circulating levels of blood glucose are significantly elevated in patients with type-2 diabetes. Higher levels of glucose in the blood are associated with generation of reactive oxygen species (ROS), cytokine activation, a decreased expression of glucose transporter type (GLUT) receptors and increased production of advanced glycated end-products (AGEs)(10). Role of hyperglycemia in the development and progression of renal dysfunction in type 1 and type 2 diabetes was demonstrated in experimental animals previously in our laboratory(11;11-13). In this study, the focus will be on the effect of hyperglycemia on cardiovascular system.

(ii) **Hyperinsulinemia**

Type 2 diabetes mellitus is characterized by insulin resistance in the target organs mainly muscle and liver (14). Insulin has a significant influence on glucose transport, glycolysis, glycogen synthesis, cardiac hypertrophy, protein synthesis and lipid metabolism (15;16). Additionally, insulin participates in the regulation of myocardial contractility (17) and offers protection against ischemic myocardial death (18), autophagy (19) and cell survival directly or via IGF-1 action. Hyperinsulinemia starts a chain of pathophysiological events that lead to obesity (20).

(iii) **Reactive oxygen species (ROS) and oxidative stress**

Nitric oxide is endothelium derived relaxing factor (EDRF), which acts an endogenous vasodilator (21). Loss of endothelial nitric oxide biosynthesis has been observed in diabetic patients (22). ROS are produced
in the vasculature of diabetic patients by sources like endothelial nitric oxide oxidase, cytochrome 450, mitochondrial electron transport chain and many others (23). Hyperglycemia increases production of superoxide ion through the mitochondrial electron transport chain (24;25). Formation of AGEs are also implicated in formation of ROS in hyperglycemia induced cellular injury to cardiomyocytes (26). Increased production of AGE products is a result of oxidation of protein and lipids along with their enzyme-independent glycation (24;25).

(iv) Lipotoxicity

Metabolism of glucose and free fatty acids (FFAs) contribute to energy production in most tissues of the body (23). Hyperglycemia and increased levels of plasma triglycerides are characteristic of T2DM (27). Glucose transport is suppressed in the myocardium in diabetic state as a downstream effect of insulin resistance (27). Therefore, FFAs act as the primary substrates for energy production and result in lipotoxicity (27). This lipotoxicity is a result of accumulation byproducts of excessive FFA metabolism (27). Lipotoxicity has been found to cause an increase in calcium in the cytoplasm which might led to ventricular stiffness in diabetic cardiovascular disease (27;28).

(v) Impaired mitochondrial function

Mitochondrial production of energy in any cell of the body is a chief source for ATP generation (29). The number of mitochondria is higher in cardiomyocytes compared to other tissues in the body (29). Due to the high oxygen demand in the cardiomyocytes, the heart muscle is rendered vulnerable with small amount of mitochondria damage (29). Substantial research demonstrates that the mitochondrial function is impaired in the cardiomyocytes in type 2 diabetes (29;30) in the myocardium of patients with type 2 diabetes. There is severe mitochondrial dysfunction in rodent models of type 1 and type 2 diabetes (31;32). Human studies have also confirmed the impaired ability of cardiomyocytes (27) and the resulting decreased ATP generation.
(vi) Activation of Renin Angiotensin System (RAS)

Activation of RAS has been implicated in development of impaired endothelial dysfunction, vascular inflammation and prothrombotic state (33). Enhanced expression of Angiotensin converting enzyme (ACE) and subsequent Angiotensin II (Ang II) is associated with atherosclerotic disease in patients (34). Increased ACE was observed in patients with acute coronary syndromes (35). Resulting oxidative stress due to upregulation of RAS in the cardiomyocyte and endothelial cells in diabetic patients leads to various conditions like ventricular dysfunction and fibrosis (36). RAS inhibition by with ACE inhibitors like enalapril in several clinical trials has shown significant improvement in cardiovascular symptoms (37-39). Thus modulation of RAS is a cornerstone in the management of cardiovascular symptoms in patients with type 2 diabetes.

Complications of Diabetes

Deleterious effects of impaired glucose handling due to type 2 diabetes are seen in various organs (8). Pre-existing diabetes has been found to increase risk of certain types of cancers, cardiovascular disease, kidney disease, neuropathic disorders, cognitive decline, fatty liver disease, bone disease, reproductive dysregulation and psychological disorders (8). Among all these complications, cardiovascular disease and kidney disease are one of the major complications that are associated with burdensome management and a huge cost to the economy (8).

(i) Cardiovascular Complications

Damage to the cardiovascular system by glucose dysregulation is a major complication of diabetes (40). Cardiovascular complications are the top cause of morbidity in type 2 diabetes mellitus patients (41)(42). Diabetic cardiomyopathy was first described by Rubler et al in 1972 as a distinct condition after studying etiological factors in heart failure patients (43). Cerebrovascular disease, cardiovascular abnormalities,
nephropathy and peripheral neuropathy are the most commonly associated complications with type 2 diabetes (44).

Human and rodent studies establish diabetic cardiomyopathy being chiefly associated with left ventricular dysfunction (45;46). Associations between hyperglycemia and microvascular and macrovascular complications of diabetes were found in diabetes complications and complications trial (DCCT) and other trials (8). The Framingham study with 5,209 men and women with pre-existing diabetic condition were found to be associated with increased risk for congestive heart failure over 18 years of follow-up (47). There is a marked increase in incidence of cardiovascular conditions like heart failure, coronary heart disease in patients with pre-existing diabetic condition (29;47). Diastolic dysfunction in diabetic and hypertensive patients was associated with aortic stiffness (48).

The cardiovascular complication in patients with type 2 diabetes have been characterized by structural and functional changes (27). Structural changes are characterized by left ventricular hypertrophy (LVH) (49;50) in male and female patients with type 2 diabetes as seen in various studies (47;51). Chronic hyperglycemia and insulin resistance was found to be associated with increased left ventricular mass (52) (53). Myocardial fibrosis and collagen deposition was found in patients with diabetes without pre-existing condition of hypertension and coronary artery disease (54). Thickening in blood vessel walls like capillaries and hypertrophic increase in size of cardiomyocytes is observed in diabetic patients with no apparent symptoms of cardiovascular disease (55). Collagen type I and III deposition was observed in biopsy samples from both right and left ventricles of type 2 diabetic patients (55). Patients with type 2 diabetes without pre-existing conditions like hypertension and coronary artery disease seem to demonstrate the interstitial fibrosis and collagen deposition (55-57). Increased levels of myocardial triglycerides are also found to be present in type 2 diabetes patients with cardiovascular symptoms (58). Cardiovascular disease symptoms are manifested as early diastolic dysfunction followed by subsequent systolic dysfunction (59). Low contractile reserve due to reduced ability in raising ejection fraction has also been found to be associated with diabetes-induced cardiovascular disease (27;60).
(ii) Diabetic Kidney Disease (DKD)

Diabetes is one of the most common causes of chronic kidney diseases, and accounts for about 50% of cases for end-stage renal disease in developed countries (61). However, damage to the kidney structure and function is too severe till the time it gets detected with the help of existing diagnostic criteria (62). The cardiovascular risks associated with kidney diseases are compounded by conditions like diabetes, hypertension atherosclerosis, arteriosclerosis, abnormal sympathetic stimulation to the heart and structural heart defects (61;63). Alteration of RAS components in diabetic patients and various mouse models has been found to be a critical factor in the development of renal dysfunction and eventual ESRD (64). The current study will investigate the effect of hyperglycemia and pharmacological therapy on alteration of cardiac RAS system.

Management of Diabetes

Guidelines from the American Diabetes Association elucidate the recommended therapies and pharmacological interventions for the treatment of patients with T2DM. The recommendations encompass normalizing blood glucose levels, management of cardiovascular risks through lifestyle and pharmacological interventions (65).

(i) Lifestyle Modification

For effective management of type 2 diabetes modifications in diet to include healthier, fiber rich nutritious food is the most important recommendation by the American Diabetes Association (8). Diabetes-specific medical nutritional therapy is catered to each patient for inclusion of proteins, fats, carbohydrates, fiber in a balanced fashion to make up a food plan (8). Along with modifications in diet, physical exercise has also been an effective tool for management of diabetes based on several clinical trials (66). Aerobic exercise has been shown to increase energy demand, thus showing improvement in glycemic control and lipid parameters (66). Improvement in insulin sensitivity and significant weight loss has been observed with
intense exercise (67). Release of irisin has been found to convert unhealthy white adipose tissue to healthier brown adipose tissue after exercise (68). Resistance exercise increases muscle strength, increase bone mineral density, increase lean muscle mass leading to improved glycemic control and improvement in insulin sensitivity (69;70). Previous work in our laboratory has demonstrated the benefits of physical exercise in normalizing hyperglycemia and attenuation of albuminuria in *db/db* diabetic mice (11).

(ii) Pharmacological Intervention

Monotherapy with metformin is the front-line treatment for type 2 diabetes (8). Metformin is safe, cost effective and has been found to decrease risk of cardiovascular events (8;71). However, long term treatment with metformin has been found to cause vitamin B12 deficiency and anemia (8). Dual therapy with metformin has been recommended with a diverse class of drugs for patients who need better glycemic management. Those class of drugs are sulfonylureas, thiazolidinedione, dipeptidyl peptidase 4 (DPP-4) inhibitors, SGLT2 inhibitors, glucagon like peptide 1 (GLP-1) receptor agonists and insulin in high risk patients (8). Triple therapy of metformin with a combination of two classes of drugs mentioned before is recommended in patients whose diabetic symptoms do not get controlled with mono or dual therapy (8).

Despite the availability of numerous medications for treatment of diabetes, none to few are free of side-effects. There is a surprising lack comparative analysis among the antidiabetic medications about their side-effects. Thiazolidinedione are peroxisome proliferator activating receptors γ (PPARγ) agonists have been extremely successful in management of hyperglycemia. These have recently have come under scrutiny for associated cardiovascular risks and increased mortality. We studied the effect of rosiglitazone and pioglitazone in *db/db* diabetic mice.
Peroxisome proliferator activating receptors γ

Peroxisome proliferator activating receptors (PPARs) belong to a family of nuclear transcription factors consisting of isoforms like PPAR α, PPARβ/δ and PPARγ (72;73). After entering the nucleus, heterodimer formation takes place with retinoid X receptor as a result of binding with ligands specific to PPARs and thus they regulate gene expression (72). The different isoforms are expressed in various organs and activate three different type of genes and thus perform different functions in the biological system (74). Several studies have established PPAR γ as one of the crucial modulator in the pathogenesis of diabetes (29).

Fat-specific adipocyte protein 2 (FABP), FA-binding protein, acyl-CoA synthase, glucokinase, and glucose transporter type 4 (GLUT4) are gene targets of PPAR γ. Moreover, PPAR-γ also regulates genes involved in insulin signalling and the expression of pro-inflammatory cytokines, such as tumor necrosis factor- (TNF-) α [6, 41]. PPARγ is a cellular target for the antidiabetic thiazolidinediones (TZDs), which sensitize cells to insulin and improve insulin activity to improve glucose uptake in hepatocytes and skeletal muscles (74-76). Expression of PPAR γ is upregulated in diabetic rats (77;78).

PPARγ has a relatively lower expression in the heart when compared to adipocytes or kidneys (79). Alteration in the expression of PPARγ from in-vitro studies and in-vivo studies has been implicated in cardiovascular conditions like atherosclerosis, hypertension and ischemic injury to the heart (80;81). Rosiglitazone, a PPARγ agonist demonstrated cardioprotective in OLEF rat model of diabetes mellitus via suppression of cardiac fibrosis with a concurrent improvement in diastolic heart function (82). There was a decrease in the receptors of advanced glycated end products (AGEs) heart tissues along with reduction with connective tissue growth factor (82). Pre-treatment with pioglitazone in patients of type 2 diabetes with acute myocardial infarction demonstrated improvement due to better rate of perfusion and a reduction in cardiac reperfusion injury (83).
(i) Thiazolidinediones: PPARγ agonists in treatment of diabetes

Thiazolidinediones are called ‘insulin sensitizers’ as they reduce circulating plasma levels of insulin as a result of improved uptake in the myocytes and adipocytes so that glucose regulation is improved. Thiazolidinedione demonstrated beneficial effects in decreasing insulin resistance and overall improvement in steady state glucose levels, plasma triglycerides and insulin levels in 1983 (84). Ligand binding assays performed in adipocytes cells derived from mouse embryo line established selective agonism for the PPAR γ (85). These class of anti-diabetic agents are called anti-hyperglycaemic agents as they normalize the elevated glucose levels with low risk of hypoglycemia. Use of thiazolidinediones demonstrated significant lowering in glycosylated hemoglobin (HbA1C) ~1% (86). The randomized ADOPT trial comparing rosiglitazone, metformin or glyburide in about 4,500 patients concluded rosiglitazone showed better outcomes in stabilizing steady state glucose levels than metformin, a first line drug in antidiabetic therapy (87).

(ii) Rosiglitazone & Pioglitazone

Rosiglitazone has been a prominent member of the thiazolidinedione class of drugs in management of diabetes since its entry in the market in 1999 (88). Comparative analysis in ADOPT trial with glyburide and metformin demonstrated better maintenance in glycemic control with rosiglitazone (89). Treatment with rosiglitazone showed increased sensitivity of insulin in skeletal muscles, adipocytes and liver (90), plasma blood glucose is subsequently reduced due to improved glucose uptake by skeletal cells, adipocytes and liver, decreased endogenous gluconeogenesis (90)(91). Diastolic and systolic blood pressure was also lowered in patients with rosiglitazone treatment (91). However, myocardial triglyceride levels were largely unchanged with therapy with a possible increase in low density lipoproteins (LDLs) (92).

ADOPT trial also showed rosiglitazone to be beneficial in treatment of renal diseases associated with type 2 diabetes(89). Rosiglitazone treatment demonstrated inhibition of collagen IV deposition in primary mouse
podocyte cultures (93). The AT1-ROS pathway is inhibited with the use of rosiglitazone (93). Rosiglitazone attenuates renal injury in hepatic pancreatitis model (94). Previous studies in our laboratory have found attenuation of hyperglycemia, albuminuria and renal fibrosis with rosiglitazone treatment in db/db diabetic mice (95).

A meta-analysis study in 42 trials with 24 weeks of rosiglitazone treatment found association between increased risk of myocardial infarction and other cardiovascular complications (96). This led to safety concerns being associated with rosiglitazone treatment and led to its ultimate withdrawal from the market (97). A large cohort study called the RECORD trial was conducted on FDA advisory committee recommendation was inconclusive about any increase in cardiovascular risk with the use of rosiglitazone (98;99). However, rosiglitazone has been banned in Europe and it sold in the US market with a caution advisory on the label (100). The FDA since then has imposed stricter regulation for antidiabetic medications where in addition to control of hyperglycemia, a low cardiovascular risk should be demonstrated (101).

Pioglitazone is another PPAR-α agonist in the class of thiazolidinedione approved for oral monotherapy for diabetic patients in 2002 (102). The PROactive trial evaluated the role of pioglitazone in reducing CVD risk in diabetic patients (103). There was no significant decrease in CVD risks like overall mortality, myocardial infarction and stroke but there was a significant decrease in patients with previous incidences of myocardial infarction (101). A significant decrease in recurrent stroke incidence was also observed in patients with previous stroke (101). A comparison between rosiglitazone and pioglitazone demonstrated that while there is no significant increase in cardiovascular risks with rosiglitazone (101). Pioglitazone has demonstrated mild cardioprotective properties along with normalizing hyperglycemia.

Pioglitazone has been suggested as a therapy for non-alcoholic steatohepatitis (NASH) (104). It has also been useful in treatment of Parkinson’s disease (105;106). Pioglitazone is also useful in control of atherosclerosis by its lipid lowering action (107). Besides normalizing hyperglycemia, pioglitazone also has antibacterial properties and has shown improvement in cognitive function (108;109). There are still
concerns of increased risk of congestive heart failure (CHF) and bladder cancer with pioglitazone use (100). There needs to be a similar meta-analysis study for pioglitazone like that of rosiglitazone to make better comparisons. In the present study we have used both rosiglitazone and pioglitazone to normalize glycemia to investigate whether thiazolidinediones alter the balance of cardiac RAS.

(iii) Safety Concerns of Thiazolidinediones

Concerns have been raised about Thiazolidinediones causing water retention leading to peripheral edema in the extremities (110). Patients with symptomatic heart failure are not able to tolerate Thiazolidinediones due to concerns about exacerbation of heart failure and thus are contraindicated in these patients (110). Fluid retention was observed with monotherapy and also with combination therapy (110). However, fluid retention was managed with the use of diuretic therapy and thus no increase in mortality was observed (111). Cardiotoxicity of high doses of rosiglitazone has been found to be independent of PPAR-γ actions and associated with mitochondrial damage and dysfunctions (112). In-vitro studies show that thiazolidinediones associated with lipid deposition in cardiomyocytes (113). Pioglitazone however, is useful in lowering lipid levels in patients but has no demonstrated beneficial effect on echocardiographic parameters of heart function (114). One of the frontline approaches for treatment of cardiovascular complications in patients with or without diabetes is RAS inhibition. We thus aim to compare and contrast the effects of rosiglitazone and pioglitazone on the cardiac RAS system in db/db diabetic mouse model.

Evidence of RAS activation in Diabetes related cardiovascular complications

Activation of RAS through the sympathetic nervous system has been strongly linked to cardiovascular risks associated in patients with diabetes (115). Cardiac fibrosis (116;117), cardiomyocyte atrophy (117), atherosclerosis (118;119) lead by diabetic hyperlipidemia are the pathological manifestations of this RAS activation.
Guidelines provided by American Diabetes Association (120) reference ACE inhibitors and Angiotensin type 1 (AT1) receptor blockers as the front-line treatment for patients with existing diabetic condition at high risk of developing cardiovascular complications. In diabetic patients with atherosclerotic cardiovascular disease (ASCVD), RAS modulators like Angiotensin receptor blockers and ACE inhibitors attenuate albuminuria, lower blood pressure, normalize albumin-to-creatinine ratio to indicate slowing progression of kidney disease. RAS modulation is thus a very important component of management of diabetes and its renal and cardiovascular outcomes.

In this study we aim to study the effect of diabetes on the RAS components in the heart of db/db diabetic mice. As there are concerns of increased cardiovascular risk with the use of thiazolidinediones, we also investigated the effect of rosiglitazone and pioglitazone on cardiac RAS components in these mice.

Components of Renin Angiotensin System (RAS)

Many successful therapeutic strategies for control of diabetes related cardiovascular disease employ modulators of the RAS. Improper activation of the RAS is linked to diabetes, hypertension and organ damage. All of these are identified as a major cause of chronic kidney diseases (121;122). RAS is a major component in the physiological control of blood pressure and fluid equilibrium in the human body. In response to lowered blood perfusion of blood, a peptide renin is released from the Macula Densa cells located at the juxta-glomerular cells in the kidney. Renin cleaves angiotensinogen to form Angiotensin I. This Angiotensin I is cleaved by Angiotensin converting enzyme (ACE) to form Angiotensin II (Ang II). Ang II is the major active component of the RAS having a powerful effect on the arterial pressure regulation, natriuresis and cell proliferation. The acute effect of Angiotensin II on arterial pressure is primarily by renal vasoconstriction which then leads to enhanced tubular sodium reabsorption (123;124). Abnormally increased activity of Angiotensin II leads to elevated arterial pressure, increased sodium reabsorption from proximal and distal tubules, proliferation, fibrosis and renal injury.
Studies have shown that a low sodium diet causes a downregulation in ACE activity and subsequent lower blood pressure. Upregulation ACE2, an enzyme that degrades Ang II to form vasodilatory Ang (1-7) suggests that RAS system has its own homeostasis to maintain (125).

(i) Angiotensin converting enzyme 2 (ACE2)

The classical view of how the renin-angiotensin system works was revolutionized with the discovery of ACE2 in 2000 by Donoghue et al and Tipnis et al (126;127). Structurally, ACE2 is roughly 42% homologous to Angiotensin Converting enzyme (ACE). This enzyme is a carboxypeptidase that causes cleavage of the Leucine amino acid residue at the C-terminal end of Ang I to form Ang (1-9). Further studies show that is also involved in the formation of a biologically active renoprotective peptide Ang (1-7) by the degradation of Ang II (128;129). Various studies have demonstrated that ACE2 is catabolically more active towards degrading Ang II rather than Ang I (128). Considering that Ang (1-7) plays a renoprotective role due to its vasodilatory properties, ACE2 is a renoprotective enzyme. Studies have shown an ADAM 17 mediated increased shedding of ACE2 in the urine of diabetic mice (130-132). This shedding is ameliorated by exercise, insulin treatment and anti-hyperglyaemic agent rosiglitazone (130;133;134). Urinary ACE2 activity in late stages of diabetes in diabetic mice is increased which was ameliorated with the administration of insulin (135). It however, remains to be seen whether this decrease in ACE2 shedding is due to control of glycemia or improvement in the kidney function. A recent study with Sprague Dawley rats in a renovascular hypertension model has shown downregulation of ACE2 in the peripheral membrane of both injured and contralateral kidney(136).

Studies with genetically deficient ACE2 mice demonstrate that release of ACE2 is critical for nitric oxide (NO) and oxidative stress in the vascular endothelium (137). Deletion of ACE2 in streptozotocin-induced diabetic rats exacerbates progressive glomerular and interstitial cell damage (138). Downregulation of ACE2 is implicated in diabetic nephropathy and the injury to kidney tubules resulting from it (139). Cardiac hypertrophy and cardiac remodeling was a consequence of increased Ang II levels and pressure overload...
in ACE2 knockout mice (140;141). Plasma soluble form of ACE2 is elevated in patients after an episode of myocardial infarction (142). ACE2 plays a major role in the heart. ACE2 knockout mice have increased Ang II levels intracardially along with reduction in cardiac contractility (143). In mice with myocardial infarction an upregulation in matrix metalloproteinase-2 and 9 associated with loss of ACE2 was demonstrated (144). This loss of ACE2 could also lead to neutrophil infiltration and subsequent upregulation of inflammatory cytokines like IL-6, chemokine, interferon γ (144).

(ii) Neprilysin (NEP)

NEP, also known as neutral endopeptidase, enkephalinase or E.C. 3.4.24.11 is involved in the production of Ang (1-7) from Ang I and from Ang (1-9) (145). It is a membrane bound enzyme and is a metalloproteinase with the presence of zinc at its active site (146;147). It was originally isolated by Kerr and Kenny from rabbit kidney brush border in 1974 where they found its ability to cause a cleavage in the β-chain of Insulin peptide (148). NEP is inhibited by the thermolysin inhibitor phosphoramidon (147). Studies performed by Schwartz and his colleagues have shown NEP to be present in the brain and categorized it as an enzyme causing cleavage of encephalin, it is thus called an enkephalinase (149). NEP also helps in the degradation of Ang II and helps in the formation of Ang (1-7) (150) which has major nephroprotective properties. Research conducted thereafter have demonstrated expression of NEP in a variety of organs like brain, heart, kidney, lungs. NEP causes cleavage of its substrate at the amino side of its peptide linkage (151). It is critical in the processing and breakdown of the vasoactive peptides that are essential for salt and water regulation of the body, namely atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP)(152). ANP enhances natriuresis and thus helps in regulating blood pressure along with playing a role in diminishing hypovolemia by increasing water retention (153).
(iii) Dual AT1 Receptor Blockade and Neprilysin Inhibition in Heart Failure

Inhibition of NEP/ACE combined produced marked decrease in peripheral vascular resistance than with ACE inhibition alone (154). This encouraged research to investigate dual inhibition of NEP/ACE as a therapeutic intervention. However, this dual inhibition degrades bradykinin which increased the risk of angioedema (155;156). Numerous trials CHARM-Preserved, PEP-CHF and I-PRESERVE failed to provide better outcomes for heart failure patients (157-159). Dual inhibition of NEP enzyme with AT1 receptor blockers has thus gained a lot of attention in the recent years. Sacubitril/ Valsartan (LCZ696) is the new candidate for improving clinical outcomes in patients with heart failure. The 2012 PARAMOUNT trial comparing overall outcomes for patients with heart failure using LCZ676 compared to valsartan showed improved outcomes with decreased blood pressure (160). However, there was a rise in urinary albumin to creatinine ratio, showing signs of kidney damage (161). LCZ696 was then tested for comparison with Enalapril and was successful in providing better efficacy and heart outcomes in the PARDIGM-HF trial in 2014. The trial was stopped before completion because the drug candidate showed overwhelming data in support if its efficacy and outcomes and fewer side effects when compared to the enalapril (162). As patients with diabetes are one of the biggest demographics for cardiovascular episodes and heart failure, it is important to understand whether there is an alteration in the expression and activity of cardiac NEP levels in diabetes. One of the aims of this thesis is to investigate whether there is an alteration in cardiac NEP levels in diabetes. Recent study of diabetic nephropathy induced by streptozotocin in mREN2 rats showed a decrease in proteinuria and albuminuria using AT1 receptor blocker (irbesartan) and NEP inhibitor (thiorphan) therapy compared to AT1 receptor blockade alone (163).

There is evidence of NEP downregulation in Alzheimer’s animal model studies. Combined NEP/ACE inhibition improved femoral blood flow and femoral vascular conductance in male STZ induced diabetic rats (164). Inhibition of NEP has been linked to an increase in insulin sensitivity (165;166). Plasma concentrations of NEP are increased in obese and insulin resistant mice (167).
As NEP is a multifunctional enzyme, it has different effects upon different organs of the body. One of the most important effects is its role in the degradation of amyloid deposits in the brain. Neurofibrosis and plaques in the brain autopsy of a patient were observed in 1905 by Alois Alzhemier. However, Alzheimer was not able to characterize what the plaques were made from (168;168). These plaques are made of amyloid- beta peptide which accumulate in the brain and cause neuronal cell loss which leads to Alzheimer’s (169). Lentivirus linked overexpression of NEP in hippocampal cells showed that NEP inhibits the neurotoxicity of amyloid beta peptides (170). The same authors also show that NEP might be involved in slowing down the progression of Alzheimer’s disease (171). Adenovirus-mediated expression of soluble form of NEP in the plasma inhibits deposition and aids in clearing up amyloid proteins in mouse transgenic model (172). The same authors demonstrate that NEP expressed inside the skeletal muscles also causes a reduction in Amyloid deposits in transgenic Alzheimer’s mouse model (173). Urinary and renal studies in db/db diabetic mice showed active fragments of ACE2 and NEP in the urine. There is evidence of ADAM 17, a metalloprotease to be responsible for cleavage of ACE2 and NEP. This study aimed to study the cardiac modulation of ADAM 17 in db/db diabetic mice.

(iv) A Disintegrin and Metalloprotease-17 (ADAM 17)

ADAM 17 or tumor necrosis factor- α (TNF-α) converting enzyme (TACE) belongs to a family of proteins composed of about 750 amino acids called as the disintegrins or metalloproteinases (174). These enzymes play important roles in various organs by promoting cell adhesion and cell maturation. These enzymes prevent formation of amyloid β-protein from its precursor protein and thus protective in Alzheimer’s disease (174). However, the activation of epidermal growth factor receptor (EGFR) by these enzyme promotes tumor formation in cancer studies (174;175;175). ADAM 17 plays an important role in the ectodomain shedding of various enzymes by regulating the expression of TNF-α (174). ADAM 17 is responsible for release of membrane bound TNF-α from the cell surface in a soluble form. (176;177). ADAM 17 is expressed in heart, kidney, brain and skeletal muscles (178).
It has been shown before that AngII treatment induces ADAM 17 and TNF-α in mice models (179). There is a reduction in symptoms of renal diseases like glomerular damage and renal fibrosis after Ang II infusion in mice lacking TGF-α and those given a specific TACE inhibitor (180). Inhibition of ADAM 17 in murine model of polycystic kidney disease ameliorated symptoms of kidney dysfunction (181). Previous studies in our lab have shown increased renal ADAM 17 expression in db/db mice (182). ADAM 17 expression is increased in the peripheral mononuclear cells (PMNC) in patients with congestive heart failure (183). Upregulation of ADAM 17 was also observed in patients with myocarditis. In animal studies with short hairpin RNA knockdown of ADAM 17 in rat model there was an attenuation in cardiac hypertrophy (184). ADAM 17 is an upstream factor to several other metalloproteases in the development of cardiac hypertrophy (185). Ageing and loss in elasticity of coronary arteries is associated with upregulation of TNF-α (186). One of the aims of the present study is to investigate whether there is an alteration in ADAM 17 in diabetic hearts.

**Animal Model of Diabetes**

Type 2 diabetes is characterized by progressive loss in pancreatic efficiency which leads to development of insulin resistance. In these conditions, normal or elevated levels of insulin fail to produce an effective response against hyperglycemia (187). Research on diabetes in mouse models is focused on complications of diabetes in addition to hyperglycemia. There are several genetic models like polygenic and monogenic models. There are also diet induced mouse models for type 2 diabetes. The polygenic mouse models are KK mice (188), new Zealand obese mice (189) among many others. Monogenic mouse models include the Lep ob/ob and Lep db/db mice. The ob/ob mice are deficient in the satiety factor leptin while the db/db mice are deficient in the leptin receptor (190).

In db/db mice, the presence of a point mutation in the gene coding for leptin receptor renders this mouse insulin resistant (191). This is accompanied by high circulating levels of both insulin and glucose (191;192) (193). None of the mouse models above, are able to develop all of the complications of type 2 diabetes.
However, the db/db mice demonstrated cardiovascular dysfunction (194,195), nephropathy (196), retinopathy (197), hypertension (193) and cognitive decline (198). This mouse model is thus useful in studying organ specific complications of type 2 diabetes like cardiovascular disease and diabetic nephropathy. Previous studies in the lab have employed db/db and Akita mice model to study the RAS system alteration in the kidney. In this project, db/db diabetic mice were used to investigate whether there is an alteration of cardiac ACE2, NEP and ADAM 17. In addition, we will also investigate of the effect of normalizing hyperglycemia with rosiglitazone and pioglitazone on these RAS candidates.
II. HYPOTHESIS AND SPECIFIC AIMS

1. Hypothesis

In diabetic db/db mice, there is an alteration in the balance of cardiac Ang (1-7) forming enzymes NEP and ACE2. This alteration could be a result of upregulation in cardiac ADAM17. Treatment of hyperglycemia with pioglitazone and rosiglitazone will normalize cardiac NEP, ACE2 and ADAM 17.

2. Specific Aims

1. To investigate the effect of rosiglitazone on hyperglycemia and cardiac ACE2, NEP and ADAM 17 protein expression.
2. To investigate the effect of pioglitazone on hyperglycemia and cardiac ACE2, NEP and ADAM 17 protein expression.
3. To optimize the fluorescence based enzyme activity assay for cardiac and renal ACE2 and NEP. The effect of rosiglitazone and pioglitazone will also be investigated on cardiac ACE2 and NEP activity.
III. SIGNIFICANCE

Most of the research investigating the physiological role of NEP has been focused on the brain and lungs. Recently, with the introduction of new therapy combining NEP inhibition with RAS blockade there is an emerging interest in studying role of cardiac NEP. Therefore, this study aims to elucidate the expression and activity of cardiac NEP.

Despite its discovery from cDNA library of heart failure patients, there are limited studies examining the effect of ACE2 in the heart. Patients suffering from type 2 diabetes are the largest demographic for heart failure. Yet, there is very limited data on cardiac ACE2 in diabetic conditions. This study thus aims to investigate if there is alteration of cardiac ACE2 in db/db diabetic mice.

Following a meta-analysis, there are reports of increased myocardial infarction and heart failure risk in patients with rosiglitazone treatment. Safety concerns have been raised regarding contraindication for the use of thiazolidinedione class in diabetic patients with symptomatic heart failure. This is due to reports of fluid retention leading to increased cardiac pre-load. Therefore, this study aims to compare and contrast rosiglitazone and pioglitazone in db/db diabetic mice.
IV. MATERIALS AND METHODS

Experimental design

Male 6 week old BKS.Cg-Dock^+/+ Lepr^db/db: db/db diabetic mice and their age-matched controls were purchased (Jackson Laboratories, Bar Harbor, ME, USA). Individual housing was provided to these mice in plastic cages at room temperature with regular 12:12 hour light: dark cycle for 12 weeks. Free access to water and standard 18% protein rodent chow was provided to these animals (Harlan Teklad, Madison, WI, USA).

Treatment with Rosiglitazone

Mice were randomly assigned into 4 groups. They were: Lean control fed standard chow (n= 9), Lean control fed rosiglitazone diet (n=7, 20mg/Kg/day, Envigo Teklad, Madison, WI, USA), db/db fed normal chow (n=10) and db/db fed rosiglitazone diet (n=10, 20mg/Kg/day, Envigo Teklad, Madison, WI, USA) for 7 weeks.

Treatment with Pioglitazone

Mice were randomly assigned into 4 groups. They were: Lean control fed standard chow (n= 10), Lean control fed pioglitazone diet (n=7, 20mg/Kg/day, Envigo Teklad, Madison, WI, USA), db/db fed normal chow (n=10) and db/db fed pioglitazone diet (n=10, 20mg/Kg/day, Envigo Teklad, Madison, WI, USA) for 10 weeks.

Body Composition Measurement

ECHO MRI absolute body composition analyzer (Houston, TX, USA) was used to study the body composition of animals. The instrument was calibrated and body weight of each mouse was recorded.
Mouse was then placed in a clear plastic cylinder and a plastic plunger was placed on top to keep the mouse in place. This was done to ensure a stable and reliable reading. This whole setup was then placed inside the before mentioned instrument and the measurements for the animal were recorded. The mouse was then returned to its cage after recording the measurement. Readings were taken one week before treatment with drugs and after 6 weeks after treatment.

**Blood Glucose Measurement**

Test Strips from FreeStyle Neo® Blood Glucose Monitoring System (Abbott, Santa Clara, CA, USA) was used to measure blood glucose levels. Blood sample was collected by tail puncture method. The tail was pricked with surgical scissors to make a minute cut and the drop of blood was placed on glucose test strips. Blood glucose measurement was performed weekly between 10 am and midday, or at intervals thereafter as indicated. Blood glucose concentration values were expressed in mg/dL.

**Organs Perfusion**

Mice were anesthetized with euthasol (Preparation: 50mg/ml, Dose: 1µL/g mouse) and the chest cavity was exposed. A needle was inserted in the left ventricle wall and cold 1X PBS was perfused through the heart using perfusion pump (Bio-Rad, Hercules, CA). A slit was made in the right ventricle of the heart to let the blood flow out of the heart. 1X PBS was perfused till the organs become pale and all the blood runs out of the body. Using the same perfusion pump, a solution of 4% Paraformaldehyde was perfused in the organs for fixing them. The mice were then dissected and organs were collected and stored in 4% paraformaldehyde at 4 °C.

**Immunohistochemistry**

The previously perfused paraffin-embedded heart sections were de-paraffinised with 98.5% Xylene. The sections were then rehydrated sequentially for 5 minutes each in 100%, 95%, 50% and 30% ethyl alcohol
solutions. These sections were then rinsed with phosphate buffer saline (PBS) and distilled water. For that purpose, the sections were boiled for 30 minutes within a solution of 10 mM sodium citrate buffer (pH 8.5). Incubation with methanol was then performed for about 20 minutes at -20°C temperature. In order to avoid any non-specific antibody binding, the sections were blocked with 3% normal donkey serum at 4°C for an hour. Primary antibodies were diluted with 3% normal donkey serum. The sections were then incubated with primary antibodies NEP (Goat anti-mouse, R&D, AF1123, 1:500), ACE2 (Rabbit anti-mouse, Sigma, HPA 000288, 1:200) and ADAM 17 (Rabbit anti-mouse, Abcam, ab2051, 1:1000) overnight at 4°C. The excess antibodies were removed by washing the sections thrice with PBS. Secondary antibody incubation was then performed with respective Alexa fluor-488 conjugated (Fisher Scientific, NH) and CY3 conjugated (Jackson Immunoresearch, West Grove, PA, USA) antibodies for signal amplification for 2 hours at 4°C. The slides were mounted using a DAPI containing mounting medium (Vector Laboratories, Burlingame, CA, USA).

**Western Blot**

Excised heart samples stored in dry ice were homogenized using bead homogenization method in Precellys 24 organ homogenizer (Bertin Corp., Rockville, MD, USA). EDTA-free lysis buffer was used to homogenize the heart samples (Roche, NYC, NY, USA). Heart lysate samples were prepared for electrophoresis by mixing 10μg protein with a sample buffer solution containing β-mercaptoethanol (volume ratio 1:1). These samples were then separated using electrophoresis in a 8% sodium dodecyl sulphate gel. The separated proteins were then transferred and blotted upon a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The membrane was then blocked with 5% milk solution in 1XPBS+0.1% Tween 20 to avoid non-specific protein binding with the antibodies. The membranes were then probed for specific protein detection using monoclonal and polyclonal antibodies such as: ACE2 (goat, R&D, 1:1000), NEP (goat, R&D, 1:1000), ACE (goat, Santa Cruz,1:500) and ADAM 17 (Abcam, 1:1000). The incubation with each of the primary antibodies was performed overnight. After washing
excess primary antibody with 1XPBS+0.1% Tween 20 solution, these membranes were then incubated with HRP-conjugated secondary antibodies like donkey anti-goat (R&D, 1: 2000) and anti-rabbit (Santa Cruz, 1: 20,000). Membranes were incubated for 2 minutes with Immobilon chemiluminescent substrate (Millipore Sigma, Bedford, MA, USA) and imaging was performed by exposing the membranes for specific times to autoradiography films (Denville Scientific, Holliston, MA, USA) and developing the films in a dark room.

**Optimization of ACE2 Activity**

A quenched fluorogenic substrate called 7-Mca-APK (Dnp) (Biomol International, NY, U.S.A) was used to measure the activity of ACE2. Cleavage by ACE2 enzyme of this substrate at the Dnp (2, 4 dinitrophenyl) site develops fluorescence. This fluorescence had been quenched due to the presence of Dnp and cleavage by ACE2 leads to development of fluorescence. ACE and ACE2 are very similar in their structure thus specificity of the activity was ensured by addition of 10μM Lisinopril, ACE inhibitor was used in the reaction mixture. 10μM of dual prolyl carboxypeptidase (PCP) and prolyl endopeptidase (PEP) inhibitor ZPP, aminopeptidase A& M (AP-A & AP-M) inhibitors 5 μM Amastatin and Bestatin respectively were also added in the buffer. Whole kidney lysate (35 μg protein) and heart lysates (35μg) were incubated with MLN-4760 (10mM, a gift from the former Millennium Pharmaceuticals, Cambridge, MA, USA) for 15 minutes at room temperature. Enzyme activity assay buffer with 0.01 M fluorogenic substrate was added to the samples. The fluorescence thus developed was then detected at 328 nm excitation with the help of a Biotek Synergy H1 plate reader. The results were recorded at 0, 0.5, 1, 2, 4, 8 and 24 hours. The results obtained were then expressed in terms of Flu.Units/μg protein/hour

**Optimization of NEP Activity**

Enzyme activity for Neprilysin was measured using an indirect coupled enzyme assay method. 15 μg of sample lysate and 4mM of Succinyl-Ala-Ala-Phe-7-amido-4- methylcoumarine substrate (Sigma Aldrich, St.Louis, MO, USA) are allowed to incubate for an hour. This results in cleavage of fluorogenic substrate
by NEP forming a hydrophobic residue. Subsequent incubation with 400mU of Leucine aminopeptidase results in removal of phenylalanine residue to get 4-amido 7 methylcoumarnine (AMC) which produces fluorescence. NEP dependent activity was determined by decrease in rate of digestion due to addition of 1 μM of specific NEP inhibitor Thiorphan. Assay buffer was prepared using Tris buffer (pH 7.6) containing 0.05M tris, 0.15M NaCl, 10μM Lisinopril and 5mM of Zinc Chloride. Additional inhibitors like 10μM of specific PCP/ PEP inhibitor, 5 μM specific AP-A & AP-M inhibitors Amastatin and Bestatin were added in the buffer respectively. This buffer was mixed with NEP substrate for 4mM final concentration. In a dark 96 well plate, substrate buffer mixture was added to each well containing 15 μg of heart tissue lysate. After one hour incubation with samples, 4mU of Leucine aminopeptidase and 0.2mM Phosphoramidon was added to each well. Fluorescence produced was measured at excitation/emission of 390nm/460nm after 15 minutes, 30 minutes and 1 hour after addition of Leucine aminopeptidase and Phosphoramidon using Biotek Synergy H1 microplate reader. The results obtained were then expressed in terms of Flu.Units/µg protein/hour.

**Statistical analysis**

Statistical analysis was performed using Graph pad prism 5.01 and Statistica software (v.10). All of the data was expressed as mean ± SEM. The differences in blood glucose, body weight, food intake, water intake, absolute body fat, lean mass and total body water were assessed by repeated measures two-way ANOVA followed by Bonferroni’s multiple comparison test. For more than two groups one-way and two-way ANOVAs were used. Unpaired student’s t-test was used to evaluate the differences between two groups. A value of *p<0.05 was considered statistically significant.
Table 1: Age dependent changes in metabolic parameters of lean and db/db mice

<table>
<thead>
<tr>
<th>Animal Strain</th>
<th>Lean</th>
<th>db/db</th>
<th>Lean</th>
<th>db/db</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>6</td>
<td>6</td>
<td>14</td>
<td>14</td>
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<tr>
<td>Group Size</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Body Weight</td>
<td>20.80±0.5</td>
<td>34.92±1.8*</td>
<td>26.6±0.5</td>
<td>40.01±0.48*</td>
</tr>
<tr>
<td>Blood Glucose</td>
<td>105.42±17.7</td>
<td>382.6±21.9*</td>
<td>116.0±19.6</td>
<td>600.0±0.00*</td>
</tr>
<tr>
<td>Food Intake</td>
<td>3.42±0.2</td>
<td>8.31±0.3*</td>
<td>3.38±0.1</td>
<td>6.84±0.8*</td>
</tr>
<tr>
<td>Water Intake</td>
<td>8.06±0.2</td>
<td>26.98±2.9*</td>
<td>6.11±0.3</td>
<td>31.43±3.7*</td>
</tr>
<tr>
<td>Absolute body fat</td>
<td>2.43±0.4</td>
<td>20.1±1.1*</td>
<td>6.20±0.8</td>
<td>20.20±1.7*</td>
</tr>
<tr>
<td>Absolute lean mass</td>
<td>12.5±0.7</td>
<td>14.59±0.6</td>
<td>16.61±0.9</td>
<td>14.59±0.6</td>
</tr>
<tr>
<td>Total body water</td>
<td>12.53±0.7</td>
<td>12.65±0.5</td>
<td>12.70±0.7</td>
<td>12.9±0.5</td>
</tr>
</tbody>
</table>

Values represent mean± SEM *p<0.05 vs. age-matched lean control mice is considered statistically significant.
Table 2: Effect of rosiglitazone on plasma hormone and lipid parameters in lean and *db/db* diabetic mice

<table>
<thead>
<tr>
<th>Animal Strain</th>
<th>Lean</th>
<th>Lean+Rosi</th>
<th><em>db/db</em></th>
<th><em>db/db</em>+Rosi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>9</td>
<td>6</td>
<td>6</td>
<td>6</td>
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<tr>
<td>Group Size</td>
<td>9</td>
<td>6</td>
<td>6</td>
<td>6</td>
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<tr>
<td>Duration</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Plasma Insulin (ng/ml)</td>
<td>1.1±0.4</td>
<td>1.5±0.1</td>
<td>7.0*±0.3</td>
<td>2.2*±0.3</td>
</tr>
<tr>
<td>Plasma adiponectin (µg/ml)</td>
<td>2.1±0.08</td>
<td>1.2±0.1</td>
<td>4.9*±0.08</td>
<td>4.1*±0.4</td>
</tr>
<tr>
<td>Plasma leptin (ng/ml)</td>
<td>2.8±0.2</td>
<td>1.5±0.3</td>
<td>26.8*±4.4</td>
<td>56.18*±3.7</td>
</tr>
<tr>
<td>Plasma glucagon (ng/ml)</td>
<td>0.07±0.01</td>
<td>0.05±0.01</td>
<td>1.7*±0.03</td>
<td>0.4*±0.01</td>
</tr>
<tr>
<td>Absolute triglyceride (mg/dL)</td>
<td>75.56±5.52</td>
<td>101.8±4.46</td>
<td>281.7*±15.77</td>
<td>89.65*±7.94</td>
</tr>
<tr>
<td>Plasma cholesterol (mg/dL)</td>
<td>94.14±4.61</td>
<td>82.35±11.27</td>
<td>157.3±13.32</td>
<td>168.5±6.15</td>
</tr>
</tbody>
</table>

Values represent mean± SEM *p<0.05 vs. age-matched lean control mice and #p<0.05 vs. age-matched *db/db* mice considered statistically significant.
Figure 3: (A) Blood glucose levels in lean and db/db mice fed chow with and without rosiglitazone. Repeated measured two way ANOVA analysis with Bonferroni’s posthoc test demonstrated significantly higher blood glucose in db/db mice (*p<0.001 vs. lean mice). Treatment with rosiglitazone significantly attenuated hyperglycemia in db/db mice (#p<0.001 vs untreated db/db mice). (B) Body weight measurement (g in lean and db/db mice chow with and without rosiglitazone. Repeated measured two way ANOVA analysis with Bonferroni’s Posthoc test demonstrated higher body weight in db/db mice (*p<0.001 vs. lean mice). Treatment with rosiglitazone significantly increased body weight in db/db mice (#p<0.001 vs. untreated db/db mice). Data is represented as mean± SEM of group size (n= 6-7).
Figure 4: (A) Food intake in (g) in lean and db/db mice fed chow with and without rosiglitazone. Repeated measures two way ANOVA analysis with Bonferroni’s posthoc test demonstrated higher food intake in db/db mice (*p < 0.001 vs. lean control). Treatment with rosiglitazone has no effect on food intake of db/db and lean mice. (B) Water intake in lean and db/db mice chow with and without rosiglitazone. Repeated measured two way ANOVA analysis with Bonferroni’s posthoc test demonstrated increased water intake in db/db mice (*p < 0.001 vs. lean mice). Treatment with rosiglitazone showed a significant decrease in water intake of db/db mice (#p < 0.001 vs untreated db/db mice). Data is represented as mean± SEM of group size (n= 7-8).
Figure 5: (A) Absolute body fat in lean and db/db mice fed chow with and without rosiglitazone. Repeated measured two way ANOVA analysis with Bonferroni’s posthoc test demonstrated higher body fat in db/db mice (*p<0.01 vs. lean mice). There was a significant increase in absolute body fat of db/db after treatment with rosiglitazone (#p<0.001 vs. untreated db/db mice). (B) Absolute lean mass in lean and db/db mice chow with and without rosiglitazone. Repeated measured two way ANOVA analysis with Bonferroni’s posthoc did not show any difference in lean mass of db/db and lean mice. Treatment with rosiglitazone did not affect lean mass in both lean and db/db mice. Data is represented as mean± SEM of group size (n=7-8).
Figure 6: (A) Total body water in lean and db/db mice fed chow with and without rosiglitazone. Repeated measured two way ANOVA analysis with Bonferroni’s posthoc test did not demonstrate any difference in total body water of db/db and lean mice. Treatment with rosiglitazone did not affect total body water in both lean and db/db mice. (B) Heart weight to body weight ratio in lean and db/db mice chow with and without rosiglitazone. One way ANOVA demonstrated no significant difference in the ratio of weight of heart to body weight from db/db mice compared to lean mice. Treatment with Rosiglitazone did not demonstrate any difference in the ratio of heart weight to body weight ratio of lean and db/db mice. Each bar represents mean±SEM of group size (n=6-8).
Figure 7: Immunoblot demonstrating cardiac ACE2 expression in 14 week old lean, lean+ rosiglitazone (L+R), db/db and db/db +rosiglitazone (db+R) mice after treatment with rosiglitazone. Two immunoreactive bands were observed at 95 kDa and 30 kDa (n=3).
Figure 8: Western blot analysis of cardiac ACE2 (95 kDa) expression in lean and db/db mice fed chow with and without rosiglitazone. One way ANOVA demonstrated significant increase in ACE2 expression in db/db mice compared to lean mice (*p<0.001). Treatment with rosiglitazone did not affect ACE2 expression in both lean and db/db mice. Each bar represents mean± SEM of group size (n=6-8).
Figure 9: Western blot analysis of cardiac ACE2 (30 kDa) expression in lean and \( db/db \) mice fed chow with and without rosiglitazone. One way ANOVA did not demonstrate any difference in ACE2 expression in \( db/db \) mice compared to lean mice. Treatment with rosiglitazone did not affect ACE2 expression in both lean and \( db/db \) mice. Each bar represents mean± SEM of group size (n=6-8).
Figure 10: Immunoblot demonstrating cardiac NEP expression in 14 week old lean (L), db/db and db/db +rosiglitazone (db+Rosi) mice after 7 weeks of treatment with rosiglitazone. Two immunoreactive bands were observed at 100 kDa and 65 kDa (n=3).
Figure 11: Western blot analysis of cardiac NEP (100kDa) expression in lean and \textit{db/db} mice chow with and without rosiglitazone. One way ANOVA demonstrated significant decrease in NEP (100kDa) expression in \textit{db/db} mice compared to lean mice (*p<0.05). Treatment with rosiglitazone did not show significant difference in NEP expression of lean control and \textit{db/db} mice. Each bar represents mean± SEM of group size (n=6-8).
Figure 12: Western blot analysis of cardiac NEP (65kDa) expression in lean and db/db mice chow with and without rosiglitazone. One way ANOVA demonstrated significant increase in cardiac NEP (65 kDa) expression in db/db mice compared to lean mice (*p<0.01). Treatment with rosiglitazone did not show significant difference in cardiac NEP expression in lean and db/db mice. Each bar represents mean± SEM of group size (n=6-8).
**Figure 13:** Western blot analysis of cardiac ACE expression in lean and *db/db* mice chow with and without rosiglitazone. One way ANOVA demonstrated no significant difference in ACE expression in *db/db* mice compared to lean mice. Treatment with rosiglitazone did not show significant difference in ACE expression of lean and *db/db* mice. Each bar represents mean± SEM of group size (n=6-8).
Figure 14: Western blot analysis of cardiac ADAM 17 expression in lean and db/db mice chow with and without rosiglitazone. One way ANOVA demonstrated a significant increase in ADAM 17 expression in db/db compared to lean mice (*p<0.001). Treatment with rosiglitazone did not show significant difference in ADAM 17 expression in both lean and db/db mice. Each bar represents mean± SEM of group size (n=6-8).
Figure 15: Representative photomicrographs from Lean, *db/db* and *db/db*+ rosiglitazone mice are shown displaying staining for ACE2 protein (green) in heart sections. The cardiomyocyte nuclei are stained by DAPI (blue). Original magnification: x400. ACE2 staining was obtained in the epicardium.
Figure 16: Representative photomicrographs from Lean, *db/db* and *db/db*+ rosiglitazone mice are shown displaying staining for NEP protein (red) in heart sections. The cardiomyocyte nuclei are stained by DAPI (blue). Original magnification: x400. NEP staining was obtained in the epicardium.
Figure 17: Representative photomicrographs from Lean, db/db and db/db+ rosiglitazone mice are shown displaying staining for ADAM 17 protein (green) in heart sections. The cardiomyocyte nuclei are stained by DAPI (blue). Original magnification: x400. ADAM 17 staining was obtained in the epicardium.
Figure 18: (A) Blood glucose levels in lean and db/db mice chow with and without pioglitazone. Repeated measured two way ANOVA analysis with Bonferroni’s posthoc test demonstrated significantly higher blood glucose in db/db mice (*p<0.001 vs. lean mice). Treatment with pioglitazone significantly attenuated hyperglycemia in db/db mice (#p<0.001 vs untreated db/db mice). (B) Body weight measurement (g) in lean and db/db mice chow with and without pioglitazone. Repeated measured two way ANOVA analysis with Bonferroni’s Posthoc test demonstrated higher body weight in db/db mice (*p<0.001 vs. lean mice). Treatment with pioglitazone significantly increased body weight in db/db mice (#p<0.001 vs. untreated db/db mice). Data is represented as mean± SEM of group size (n= 6-7).
Figure 19: (A) Food intake in (g) in lean and \(db/db\) mice chow with and without pioglitazone. Repeated measures two way ANOVA analysis with Bonferroni’s posthoc test demonstrated higher food intake in \(db/db\) mice (*\(p<0.001\) vs. lean control). Treatment with pioglitazone has no effect on food intake of \(db/db\) and lean mice. (B) Water intake in lean and \(db/db\) mice chow with and without pioglitazone. Repeated measured two way ANOVA analysis with Bonferroni’s posthoc test demonstrated increased water intake in \(db/db\) mice (*\(p<0.001\) vs. lean mice). Treatment with pioglitazone showed a significant decrease in water intake of \(db/db\) mice (#\(p<0.001\) vs untreated \(db/db\) mice). Data is represented as mean± SEM of group size (n= 7-8).
Figure 20: (A) Absolute body fat in lean and *db/db* mice chow with and without pioglitazone. Repeated measured two way ANOVA analysis with Bonferroni’s posthoc test demonstrated higher body fat in *db/db* mice (*p<0.01* vs. lean mice). There was a significant increase in absolute body fat of *db/db* after treatment with pioglitazone (*p<0.001* vs. untreated *db/db* mice). (B) Absolute lean mass in lean and *db/db* mice chow with and without pioglitazone. Repeated measured two way ANOVA analysis with Bonferroni’s posthoc did not show any difference in lean mass of *db/db* and lean mice. Treatment with pioglitazone did not affect lean mass in both lean and *db/db* mice. Data is represented as mean±SEM of group size (n=7-8).
Figure 21: (A) Total body water in lean and $db/db$ mice chow with and without pioglitazone. Repeated measured two way ANOVA analysis with Bonferroni’s posthoc test did not demonstrate any difference in total body water of $db/db$ and lean mice. Treatment with pioglitazone did not affect total body water in both lean and $db/db$ mice. (B) Heart weight to body weight ratio in lean and $db/db$ mice chow with and without pioglitazone. One way ANOVA demonstrated no significant difference in the ratio of weight of heart to body weight from $db/db$ mice compared to lean mice. Treatment with pioglitazone did not demonstrate any difference in the ratio of heart weight to body weight ratio of lean and $db/db$ mice. Each bar represents mean±SEM of group size (n=6-8).
**Figure 22:** Immunoblot demonstrating cardiac ACE2 expression in 18 week old lean (L, n=3), db/db (D, n=3) and db/db +pioglitazone (Dp, n=3) mice after treatment with pioglitazone. Two immunoreactive bands were observed at 95 kDa and 30 kDa.
Figure 23: Western blot analysis of cardiac ACE2 (95 kDa) expression in lean and db/db mice chow with and without pioglitazone. One way ANOVA did not demonstrate any difference in ACE2 expression in db/db mice compared to lean. Treatment with pioglitazone did not affect expression of ACE2 in db/db mice. Each bar represents mean± SEM of group size (n=6-8).
Figure 24: Western blot analysis of cardiac ACE2 (30 kDa) expression in lean and db/db mice chow with and without pioglitazone. One way ANOVA did not demonstrate any difference in ACE2 expression in db/db mice compared to lean. Treatment with pioglitazone did not affect ACE2 expression in both lean and db/db mice. Each bar represents mean± SEM of group size (n=6-8).
**Figure 25:** Immunoblot demonstrating cardiac NEP expression in 18 week old lean (L, n=3), *db/db* (D, n=3) and *db/db* +pioglitazone (Dp, n=3) mice after treatment with pioglitazone. Two immunoreactive bands were observed at 100 kDa and 65 kDa.
Figure 26: Western blot analysis of cardiac NEP (100 kDa) expression in lean and db/db mice chow with and without pioglitazone. One way ANOVA did not demonstrate any difference in NEP expression in db/db mice compared to lean. Treatment with pioglitazone did not affect NEP expression in both lean and db/db mice. Each bar represents mean± SEM of group size (n=6-8).
Figure 27: Western blot analysis of cardiac NEP (65 kDa) expression in lean and db/db mice chow with and without pioglitazone. One way ANOVA demonstrated significant increase in cardiac NEP (65kDa) expression in db/db mice compared to age-matched lean control mice (*p<0.05). Treatment with pioglitazone did not show significant difference in cardiac NEP expression in lean control and db/db mice. Each bar represents mean± SEM of group size (n=6-8).
Figure 28: Western blot analysis of cardiac ACE expression in lean and \( db/db \) mice chow with and without pioglitazone. One way ANOVA demonstrated no significant difference in cardiac ACE expression in \( db/db \) mice compared to age- matched lean control mice. Treatment with pioglitazone did not show significant difference in cardiac ACE expression in lean control and \( db/db \) mice. Each bar represents mean± SEM of group size (n=3).
Figure 29: Representative photomicrographs from Lean, *db/db* and *db/db*+ pioglitazone mice are shown displaying staining for ACE2 protein (green) in heart sections. The cardiomyocyte nuclei are stained by DAPI (blue). Original magnification: x400. ACE2 staining was obtained in the epicardium.
Figure 30: Representative photomicrographs from Lean, \( db/db \) and \( db/db+ \) pioglitazone mice are shown displaying staining for NEP protein (red) in heart sections. The cardiomyocyte nuclei are stained by DAPI (blue). Original magnification: x400. NEP staining was obtained in the epicardium.
Figure 31: (A) Renal ACE2 activity in 35µg of kidney homogenate lean and db/db mice. Assay buffer contains Tris hydrochloride (0.05M), Zinc Chloride (5mM) and Lisinopril (0.01mM). (Buffer A, pH 7.5) and Tris, Zinc Chloride, Lisinopril, ZPP(10 µM), Amastatin (5 µM) and Bestatin (5 µM) (Buffer B). MLN-4760, a specific inhibitor for ACE2 confirming presence of ACE2. Fluorescence is significantly higher in db/db mice than lean control mice (*p<0.01). Significant inhibition with MLN-4760 in both lean and db/db (#p<0.001) confirmed the presence of ACE2. In buffer A, residual fluorescence is observed in lean and db/db mice after incubation with MLN-4760. In buffer B, fluorescence is inhibited almost completely in both lean and db/db mice. (B) Renal ACE2 activity in lean and db/db mice based on inhibition in hydrolysis of substrate after incubation with MLN-4760. Two buffer conditions were used Buffer A and Buffer B. A significant increase in ACE2 activity is observed in db/db mice compared to lean control in both buffer A and B (*p<0.001).
Figure 32: (A) Cardiac ACE2 activity in 35 μg of heart homogenate in lean and db/db mice. Two buffer conditions, Buffer A and Buffer B were used. Heart homogenate from age-matched lean and db/db mice was used. MLN-4760, a specific inhibitor for ACE2 confirmed presence of ACE2. Significant inhibition with MLN-4760 in both lean and db/db (#p<0.001) confirmed the presence of ACE2 in both buffer A and B. In buffer A, residual fluorescence is observed in lean and db/db mice after incubation with MLN-4760 (#p<0.001). In buffer B, fluorescence is inhibited almost completely in both lean and db/db (#p<0.001). (B) Cardiac ACE2 activity in lean and db/db mice based on inhibition in hydrolysis of substrate after incubation with MLN-4760. Two buffer conditions were used Buffer A and Buffer B. A significant increase in ACE2 activity is observed in db/db mice compared to lean control in both buffer A (*p<0.001). In, Buffer B there was no difference between lean and db/db mice.
Figure 33: (A) Effect of rosiglitazone on cardiac ACE2 activity in lean and db/db mice in buffer A. Specific inhibitor MLN-4760 produced significant inhibition in total fluorescence (*p<0.05) in all four groups. (B) Effect of rosiglitazone on cardiac ACE2 activity in lean and db/db mice based on inhibition in hydrolysis of substrate after incubation with MLN-4760. A significant increase in ACE2 activity is observed in db/db mice compared to lean control (*p<0.01). Treatment with rosiglitazone did not have any effect on cardiac ACE2 activity.
Figure 34: (A) Effect of rosiglitazone on cardiac ACE2 activity in lean and db/db mice in buffer B. Specific inhibitor MLN-4760 produced significant inhibition in total fluorescence (*p<0.05) in all four groups. (B) Effect of rosiglitazone on cardiac ACE2 activity in lean and db/db mice based on inhibition in hydrolysis of substrate after incubation with MLN-4760. There was no significant difference in ACE2 activity in db/db mice compared to lean control. Treatment with rosiglitazone did not have any effect on cardiac ACE2 activity.
Figure 35: (A) Renal NEP activity in 35 μg of kidney homogenate from lean and db/db mice. Two buffer conditions were used, Buffer A and Buffer B. Thiorphan, a specific inhibitor for NEP was used to measure NEP activity in lean and db/db mice based on inhibition in hydrolysis of substrate after incubation with Thiorphan. Significant inhibition with Thiorphan in both lean and db/db (#p<0.001) confirmed the presence of NEP. In buffer A, residual fluorescence is observed in lean and db/db mice after incubation with Thiorphan. In buffer B, fluorescence is inhibited almost completely in both lean and db/db (#p<0.001). The fluorescence is brought to almost the baseline after incubation with Thiorphan when buffer B is used. (B) Renal NEP activity in in lean and db/db mice based on inhibition in hydrolysis of substrate after incubation with Thiorphan. Two buffer conditions were used, Buffer A and Buffer B. A significant decrease activity is observed in db/db mice compared to lean control in both buffer A and buffer B (*p<0.001).
Figure 36: (A) Cardiac NEP activity in 35 μg of heart homogenate in lean and db/db mice. Two buffer conditions were used, Buffer A and Buffer B. Thiorphan, a specific inhibitor for NEP was used to measure NEP activity in lean and db/db mice based on inhibition in hydrolysis of substrate after incubation with Thiorphan. A significant decrease in NEP activity is observed in db/db mice compared to lean control in buffer A and buffer B (#p<0.001). Residual fluorescence is observed in lean and db/db mice after incubation with Thiorphan in buffer A. In buffer B, fluorescence is inhibited almost completely. (B) Cardiac NEP activity in lean and db/db mice based on inhibition in hydrolysis of substrate after incubation with Thiorphan. A significant decrease in NEP activity is observed in db/db mice compared to lean control in both buffer A and buffer B (*p<0.001).
Figure 37: (A) Effect of rosiglitazone on cardiac NEP activity in lean and db/db mice in buffer A. Specific inhibitor thiorphan produced significant inhibition (*p<0.05) in all the four groups. (B) Effect of rosiglitazone on cardiac NEP activity in 35µg of heart homogenate in lean and db/db mice based on inhibiton produced after incubation with MLN-4760. A significant decrease in NEP activity is observed in db/db mice compared to lean control (*p<0.01). Treatment with rosiglitazone did not have any effect on cardiac NEP activity.
Figure 38: (A) Effect of rosiglitazone on cardiac NEP activity in lean and db/db mice in buffer A. Specific inhibitor Thiorphan produced significant inhibition in total fluorescence (*p<0.05) in all four groups. (B) Effect of rosiglitazone on cardiac NEP activity in lean and db/db mice based on inhibition in hydrolysis of substrate after incubation with thiorphan. There is a significant decrease in NEP activity in db/db mice compared to lean control (*p<0.05). Treatment with rosiglitazone did not have any effect on cardiac NEP activity.
Figure 39: Effect of pioglitazone on cardiac ACE2 activity in 18wk old lean and db/db mice. Specific inhibitor thiorphan produced significant inhibition (*p<0.05) in all the four groups. (B) Effect of pioglitazone on cardiac ACE2 activity in 35µg of heart homogenate in lean and db/db mice based on inhibition produced after incubation with thiorphan. There was no difference in ACE2 activity is observed in db/db mice compared to lean control (*p<0.01). Treatment with pioglitazone did not have any effect on cardiac ACE2 activity.
**Figure 40:** (A) Effect of pioglitazone on cardiac NEP activity in 18wk old lean and *db/db* mice. Specific inhibitor thiorphan produced significant inhibition in NEP activity (*p<0.05*) in all the four groups. (B) Effect of pioglitazone on cardiac NEP activity in 35μg of heart homogenate in lean and *db/db* mice based on inhibition produced after incubation with MLN-4760. There was no difference in NEP activity is observed in *db/db* mice compared to lean control (*p<0.01*). Treatment with pioglitazone did not have any effect on cardiac NEP activity.
V. RESULTS

Specific Aim

To investigate the effect of rosiglitazone on hyperglycemia and cardiac ACE2 and NEP expression.

Physiological and metabolic parameters

To determine the age dependent changes of diabetes and effect of rosiglitazone in db/db diabetic mice, the following parameters were measured weekly.

(A) Effect of rosiglitazone on blood glucose levels

There was a significant increase in blood glucose levels in 6 week old db/db mice compared to lean normal mice (*p<0.001, Figure 3A). Progressive rise in blood glucose levels was observed in db/db mice over 7 weeks of study period. There was no significant change in blood glucose levels in lean control mice over the 7 weeks of treatments (Figure 3A). Treatment with rosiglitazone normalized blood glucose levels in db/db mice after 2 weeks of treatment (*p<0.01, Figure 3A). There was no difference in blood glucose of lean mice on treatment with rosiglitazone when compared to untreated lean mice (Figure 3A).

(B) Effect of rosiglitazone on body weight

There was a significant increase in the body weight of 6 week old db/db mice compared to lean control mice (*p<0.01, Figure 3B). There was no difference in the body weight of lean mice over 7 weeks of study treatment. There was a significantly consistent increase in body weight in db/db mice compared to untreated db/db mice after rosiglitazone (*p<0.01, Figure 3B). Treatment with rosiglitazone did not have any effect on the body weight of lean mice when compared to untreated lean mice (Figure 3B).
(C) **Effect of rosiglitazone on food intake**

The daily food intake was significantly higher in *db/db* mice compared to lean mice at 6 weeks of age (*p*<0.001, Figure 4A). There was no difference in food intake of *db/db* mice after rosiglitazone treatment (Figure 4A). No difference was observed in food intake of lean mice treated with rosiglitazone compared to untreated lean mice.

(D) **Effect of rosiglitazone on water intake**

Water intake was significantly higher in *db/db* mice compared to lean control mice at the age of 6 weeks (*p*<0.001, Figure 4B). There was a significant decrease in water intake of *db/db* mice after rosiglitazone treatment compared to untreated *db/db* mice (*p*<0.0001, Figure 4B). No difference was observed in water intake of lean mice treated with rosiglitazone compared to untreated lean mice (Figure 4B).

(E) **Effect of rosiglitazone on absolute body fat**

The absolute body fat was increased in *db/db* mice compared to lean mice (*p*<0.01, Figure 5A). There was a significant increase in absolute body fat of *db/db* mice after treatment with rosiglitazone compared to untreated *db/db* mice (*p*<0.001, Figure 5A). No difference was observed in absolute body fat of lean mice treated with rosiglitazone compared to untreated lean mice (Figure 5A).

(F) **Effect of rosiglitazone on absolute lean mass**

There was no difference between absolute lean mass of *db/db* mice and lean mice (Figure 5B). Treatment with rosiglitazone did not demonstrate any difference in absolute lean mass of *db/db* mice compared to untreated *db/db* mice (Figure 5B). No difference was observed in absolute lean mass of lean mice treated with rosiglitazone (Figure 5B).

(G) **Effect of rosiglitazone on total body water**

There was no difference between total body water of *db/db* and lean mice (Figure 6A). Treatment with rosiglitazone did not demonstrate any difference in total body water of *db/db* mice compared to untreated
*db*/*db* mice (Figure 6A). No difference was observed in total body water of lean mice after treatment with rosiglitazone (Figure 6A).

**Plasma hormone and lipid parameters**

To assess the effect of hyperglycemia and rosiglitazone treatment on plasma hormonal in mice, plasma samples were collected at the end of the study from *db/db* and lean control mice fed normal chow or chow containing rosiglitazone. The following parameters were analyzed at the Mouse Metabolic Phenotyping Centre (Cincinnati, OH, USA) as described previously.

**(A) Effect of rosiglitazone on plasma insulin**

Plasma insulin levels were significantly elevated in *db/db* mice compared to their age-matched lean mice (*p<0.05, Table 2). Chronic treatment with rosiglitazone significantly reduced the plasma insulin levels in *db/db* mice compared to untreated *db/db* mice (#p<0.05, Table 2).

**(B) Effect of rosiglitazone on plasma adiponectin**

There was a significant increase in the plasma adiponectin levels in *db/db* mice compared to their age-matched lean mice (*p<0.05, Table 2). Chronic treatment with rosiglitazone significantly increased the plasma adiponectin levels in *db/db* mice compared to untreated *db/db* mice (#p<0.05, Table 2).

**(C) Effect of rosiglitazone on plasma leptin**

There was a significant increase in the plasma leptin levels in *db/db* mice compared to their age-matched lean mice (*p<0.05, Table 2). Chronic treatment with rosiglitazone significantly increased the plasma leptin levels in *db/db* mice compared to untreated *db/db* mice (#p<0.05, Table 2).
(D) Effect of rosiglitazone on plasma glucagon

Glucagon levels were significantly elevated in the plasma in db/db mice compared to their age-matched lean mice (*p<0.05, Table 2). Chronic treatment with rosiglitazone significantly decreased the plasma glucagon levels in db/db mice compared to untreated db/db mice (#p<0.05, Table 2).

(E) Effect of rosiglitazone on plasma triglyceride

Plasma triglyceride levels were significantly elevated in db/db mice compared to their age-matched lean mice (*p<0.05, Table 2). Chronic treatment with rosiglitazone significantly decreased the plasma triglyceride levels in db/db mice compared to untreated db/db mice (#p<0.05, Table 2).

(F) Effect of rosiglitazone on plasma cholesterol

There was no difference in plasma total cholesterol levels in db/db mice compared to their age-matched lean mice (Table 2). Chronic treatment with rosiglitazone has no significant effect on plasma cholesterol levels in db/db mice compared to untreated db/db mice (Table 2).

(v) Effect of rosiglitazone on ratio of heart weight to body weight

To determine any hypertrophic condition in diabetic mice, relative ratio of heart to their body weight was measured. There was no significant difference in heart weight to body weight ratio in db/db mice compared to lean mice (Figure 6B). Treatment with rosiglitazone did not demonstrate significant difference in heart to body weight ratio in both lean and db/db mice compared to untreated mice (Figure 6B).

Cardiac Protein expression of NEP, ACE2, ACE and ADAM 17

To investigate the effect of hyperglycemia and rosiglitazone treatment on protein expression, heart lysates from 14 week old db/db and age-matched lean mice were studied using western blot analysis.
(A) Cardiac ACE2

Western blot of cardiac ACE2 expression revealed two immunoreactive bands, an intense band at \( \approx 95 \text{ kDa} \) and a weak band at \( \approx 30 \text{ kDa} \) in (Figure 7).

(a) Effect of rosiglitazone in cardiac ACE2 (95kDa)

The protein expression of ACE2 (95kDa) was significantly increased in \( db/db \) mice compared to lean control mice (*\( p<0.001 \), Figure 8). Treatment with rosiglitazone did not show any difference in expression of ACE2 (95kDa) in \( db/db \) and lean mice (Figure 8).

(b) Effect of rosiglitazone in cardiac ACE2 (30kDa)

There was no difference in ACE2 (30kDa) expression in \( db/db \) mice compared to lean control mice (Figure 9). Treatment with rosiglitazone demonstrated no difference in ACE2 expression (30kDa) in \( db/db \) and lean mice Figure 9).

(B) Cardiac NEP

Western blot analysis of cardiac NEP protein expression revealed two immunoreactive bands, an intense band at \( \approx 100 \text{ kDa} \) and another band at \( \approx 65 \text{ kDa} \) in (Figure 10).

(a) Effect of rosiglitazone on cardiac NEP 100 kDa

The protein expression of NEP (100 kDa) was decreased in \( db/db \) mice compared to lean mice (*\( p<0.05 \), Figure 11). Treatment with rosiglitazone showed no difference in NEP expression (100kDa) in \( db/db \) and lean mice (Figure 11).
(b) Effect of rosiglitazone on cardiac NEP 65 kDa

The protein expression of NEP (65 kDa) was increased in db/db mice compared to lean mice (*p<0.01, Figure 12). Treatment with rosiglitazone showed no difference in NEP expression (65kDa) in db/db and lean mice (Figure 12).

(C) Effect of rosiglitazone on cardiac ACE

There was no difference in expression of ACE in db/db mice compared to lean control mice. (Figure 13). Treatment with rosiglitazone showed no difference in ACE expression in db/db and lean mice (Figure 13).

(D) Effect of rosiglitazone on cardiac ADAM 17

The protein expression of ADAM 17 increased in db/db mice compared to lean control mouse (Figure 14). Treatment with rosiglitazone showed no difference in ADAM 17 expression in db/db and lean mice (Figure 14).

Immunofluorescence Staining

To investigate the distribution patterns of NEP, ACE2 and ADAM 17 in the heart, immunofluorescence staining were performed on paraffin embedded heart sections obtained from 14 week old lean and db/db mice.

(A) Effect of rosiglitazone on cardiac ACE2

Immunostaining was obtained for ACE2 in the epicardium of the heart in lean control, db/db and db/db mice treated with rosiglitazone (Figure 15)

(B) Effect of pioglitazone on cardiac NEP

Immunostaining was obtained for NEP in the epicardium of the heart in lean control, db/db and db/db mice treated with rosiglitazone (Figure 16)
Effect of rosiglitazone on cardiac ADAM 17

Immunostaining was obtained for ADAM17 in the epicardium of the heart in lean control, \textit{db/db} and \textit{db/db} mice treated with rosiglitazone (Figure 17)

Summary

- Rosiglitazone normalized hyperglycemia in \textit{db/db} mice and did not affect blood glucose in lean control mice.
- Treatment with rosiglitazone significantly increased body weight and body fat in \textit{db/db} mice.
- Treatment with rosiglitazone has no effect on body weight and body fat in lean control mice.
- Two immunoreactive bands for ACE2 and NEP in the heart.
- Mature ACE2 upregulated in \textit{db/db} mice suggesting cardioprotective compensatory mechanism.
- Mature NEP (100 kDa) cleaved to form fragmented NEP (65kDa). This cleavage could be mediated by ADAM 17 enzyme.

Specific aim 2

To investigate the effect of pioglitazone on hyperglycemia and cardiac ACE2 and NEP expression.

Physiological and metabolic parameters

To determine the age dependent changes of diabetes and effect of pioglitazone in \textit{db/db} diabetic mice, the following parameters were measured weekly.

(A) Effect of pioglitazone on blood glucose levels

There was a significant increase in blood glucose levels in \textit{db/db} mice compared to lean control mice (*\textit{p}<0.001, Figure 18A). Treatment with pioglitazone normalized blood glucose levels in \textit{db/db} mice
(\#p<0.00, Figure 18A). There was no difference in blood glucose of lean mice treated with pioglitazone when compared to untreated lean mice (Figure 18A).

(B) Effect of pioglitazone body weight

There was a significant increase in the body weight of db/db mice compared to lean control mice (*p<0.001, Figure 18B). There was a significant increase in body weight in db/db mice compared to untreated db/db mice after pioglitazone treatment (#p<0.001, Figure 18B). Treatment with pioglitazone did not have any effect on the body weight of lean mice when compared to untreated lean mice (Figure 18B).

(C) Effect of pioglitazone on food intake

Daily food intake was significantly higher in db/db mice compared to lean mice (*p<0.001, Figure 19A). Pioglitazone treatment did not demonstrate any difference in food intake of both db/db and lean mice (Figure 19A).

(D) Effect of pioglitazone on water intake

Water intake was significantly higher in db/db mice compared to lean control mice (*p<0.001, Figure 19B). There was a significant decrease in water intake of db/db mice after pioglitazone treatment compared to untreated db/db mice (#p<0.001, Figure 19B). No difference was observed in water intake of lean mice treated with pioglitazone compared to untreated lean mice (Figure 19B).

(E) Effect of pioglitazone on absolute body fat

The absolute body fat was increased in db/db mice compared to lean control mice (*p<0.01, Figure 20A). Treatment with pioglitazone demonstrated a significant increase in absolute body fat of db/db mice compared to untreated db/db mice (#p<0.001, Figure 20A). There was no difference in absolute body fat of lean mice treated with pioglitazone compared to untreated lean mice (Figure 20A).
(F) Effect of pioglitazone on absolute lean mass

There was no difference between absolute lean mass in \(db/db\) mice and lean mice (Figure 20B). There was no significant difference in absolute lean mass of both lean and \(db/db\) mice after pioglitazone treatment (Figure 20B).

(G) Effect of pioglitazone on total body water

There was no difference between total body water in \(db/db\) mice and lean mice (Figure 21A). There was no significant difference in total body water of both lean and \(db/db\) mice after pioglitazone treatment (Figure 21A).

Effect of pioglitazone on ratio of heart weight to body weight

To determine any hypertrophic condition in diabetic mice, relative ratio of heart to their body weight was measured. There was no significant difference in heart weight to body weight ratio in \(db/db\) mice compared to lean mice (Figure 21B). There was no significant difference in heart to body weight ratio in \(db/db\) and lean mice after pioglitazone treatment (Figure 21B).

Cardiac Protein expression of NEP, ACE2, ACE and ADAM 17

To investigate the effect of hyperglycemia and pioglitazone treatment on protein expression, heart lysates from 18 week old \(db/db\) and age-matched lean mice were studied using western blot analysis.

(A) Cardiac ACE2

Western blot of cardiac ACE2 expression revealed two immunoreactive bands, an intense band at \(\approx 95\) kDa and a weak band at \(\approx 30\) kDa in (Figure 22).
(a) **Effect of pioglitazone on cardiac ACE2 (95 kDa)**

There was no difference in protein expression of ACE2 (95kDa) in \( db/db \) mice compared to lean control mice (Figure 23). Treatment with pioglitazone did not demonstrate a significant difference in protein expression of ACE2 (95kDa) in \( db/db \) mice compared to untreated \( db/db \) mice (Figure 23).

(b) **Effect of pioglitazone on cardiac ACE2 (30 kDa)**

There was no difference in protein expression of ACE2 (30kDa) expression in \( db/db \) mice compared to lean control mice (Figure 24). Treatment with pioglitazone demonstrated no difference in cardiac ACE2 expression (30kDa) in \( db/db \) mice compared to untreated \( db/db \) mice (Figure 24).

(B) **Cardiac NEP**

Western blot analysis of cardiac NEP expression revealed two immunoreactive bands, an intense band at \( \approx 100 \) kDa and another band at \( \approx 65 \) kDa in (Figure 25).

(a) **Effect of pioglitazone on cardiac NEP 100 kDa**

There was no difference in protein expression of NEP (100 kDa) in \( db/db \) mice compared to lean mice (Figure 26). Treatment with pioglitazone showed no difference in NEP expression (100kDa) in \( db/db \) and lean mice (Figure 26).

(b) **Effect of pioglitazone on cardiac NEP 65 kDa**

The protein expression of NEP (65 kDa) was upregulated in \( db/db \) mice compared to lean mice (*\( p<0.05 \), Figure 27). Treatment with pioglitazone showed no difference in NEP expression (65 kDa) in \( db/db \) and lean mice (Figure 27).
(C) Effect of pioglitazone on cardiac ACE

There was no difference in protein expression of ACE in \textit{db/db} mice compared to lean mice (Figure 28). Treatment with pioglitazone showed no difference in ACE expression in \textit{db/db} and lean mice (Figure 28).

**Immunohistochemical Staining**

To investigate the distribution patterns of NEP and ACE2 in the heart, immunostaining analyses were performed on heart sections obtained from perfused mice.

**(A) Effect of pioglitazone on cardiac ACE2**

Immunostaining was obtained for ACE2 in the epicardium of the heart in lean control, \textit{db/db} and \textit{db/db} mice treated with rosiglitazone (Figure 29).

**(B) Effect of pioglitazone on cardiac NEP**

Immunostaining was obtained for NEP in the epicardium of the heart in lean control, \textit{db/db} and \textit{db/db} mice treated with pioglitazone (Figure 30).

**Summary**

- Pioglitazone normalized hyperglycemia in \textit{db/db} mice and did not affect blood glucose on lean control mice.
- Treatment with pioglitazone significantly increased body weight and body fat in \textit{db/db} mice.
- Treatment with pioglitazone has no effect on body weight and body fat in lean control mice.
- Two immunoreactive bands for ACE2 and NEP in the heart.
- No difference in cardiac ACE2 and NEP expression in 18 week old mice. This suggests that ACE2 and NEP expression is age-dependent or cyclic in nature.
Specific Aim 3

To optimize the fluorescence based enzyme activity assay for cardiac and renal ACE2 and NEP. The effect of rosiglitazone and pioglitazone will also be investigated on cardiac ACE2 and NEP activity. In addition to studying the effect of hyperglycemia and thiazolidinediones on protein expression of RAS enzymes, it is important to study the activity with a sensitive and specific assay. We optimized and investigated the effect of some protease inhibitors on cardiac and renal ACE2 and NEP fluorescent based assays.

Optimization of ACE2 activity

The fluorogenic substrate MCA-APK (Dnp) is commonly used to measure ACE2 activity based on fluorescent signal produced to indicate activity. However, MCA-APK (Dnp) has been reported to be non-specific for measuring ACE2. Previous studies from the lab have reported other peptidases like PCP, PEP might cleave the substrate to produce fluorescent signal (199). We thus have introduced some inhibitors like Z-prolyl prolinal, amastatin, bestatin in the assay to inhibit interference by PCP, PEP, AP-A and AP-M. Enzyme activity was then determined by the fluorescence produced in absence and presence of the ACE2 specific inhibitor, MLN-4760. There are very few reports of ACE2 activity in the heart. We thus optimized the assay first in kidney lysates and then determine activity in the heart using similar assay conditions.

Renal ACE2 activity using Tris-HCl buffer (pH 7.4) containing ZnCl₂ and Lisinopril (Buffer A)

There was significant inhibition in total fluorescence in both lean (42% inhibition) and db/db (77% inhibition) kidney samples when incubated with MLN-4760 (*p<0.001, Figure 31A). There was some background fluorescence generated from lean and db/db samples after incubation with MLN-4760 (Figure 31A). This background indicates the presence of other proteases in the sample. ACE2 activity was measured as the hydrolysis of substrate in absence and presence of MLN-4760. There was a significant increase in ACE2 activity in db/db mice compared to lean mice (Figure 31B, *p<0.001).
Renal ACE2 activity using Buffer A containing ZPP, bestatin and amastatin (Buffer B)

Inhibition in total fluorescence was obtained in both lean (87% fluorescence) and db/db (98% inhibition) in kidney samples when incubated with MLN 4760 (*p<0.001, Figure 31A). The inhibition produced by MLN-4760 is thus enhanced by addition of these inhibitors as the total fluorescence was inhibited almost to the baseline. Moreover, the fluorescence produced in lean mice was lower than that produced in buffer A (887.757± 53.170 in buffer A vs 281.707± 19.957 in buffer B). ACE2 activity was measured as the hydrolysis of substrate in absence and presence of MLN-4760. There was a significant increase in ACE2 activity in db/db mice compared to lean mice (*p<0.001, Figure 31B).

Cardiac ACE2 enzyme activity optimization

Addition of the cocktail of the protease inhibitors (ZPP, amastatin, bestatin) to buffer A, improved the fluorogenic assay and produced more than 95% inhibition of renal ACE2 activity with MLN-4760. We repeated similar assay conditions for evaluation of cardiac ACE2 activity.

Cardiac ACE2 activity using Tris-HCl (pH 7.4) containing ZnCl$_2$ and lisinopril (Buffer A)

There was significant inhibition in ACE2 fluorescence in both lean (27% inhibition) and db/db (40%) heart samples when incubated with MLN-4760 (#p<0.001, Figure 32A). High background fluorescence is produced in both lean and db/db samples after incubation with MLN-4760 (Figure 32A). ACE2 activity was measured as hydrolysis of substrate in absence and presence of MLN-4760. There was a significant increase in ACE2 activity in db/db mice compared to lean mice (*p<0.001, Figure 32B, Buffer A).

Cardiac ACE2 activity using Buffer A containing ZPP, bestatin and amastatin (Buffer B)

Inhibition in ACE2 fluorescence by MLN-4760 was significant in both lean (86% inhibition) and db/db (80% inhibition) heart samples s (#p<0.001, Figure 32A, Buffer B). The inhibition of ACE2 fluorescence by MLN-4760 was enhanced by addition of protease inhibitors (Figure 32A, Buffer B). Fluorescence produced in both lean and db/db mice is lower than that those in buffer A. However, there was no difference
in ACE2 activity db/db mice compared to lean mice after addition of these inhibitors (Figure 32B). This might suggest the presence of other enzymes in the heart that influence the ACE2 activity assay.

**Effect of rosiglitazone using Tris HCl (pH 7.4), ZnCl₂ and Lisinopril (Buffer A)**

MLN-4760 demonstrated a significant decrease in fluorescence produced in all the groups (*p<0.05, Figure 33A). ACE2 activity was significantly increased in db/db mice compared to lean (#p<0.01 vs. lean control). Cardiac ACE2 activity was not affected in db/db mice after 7 weeks of rosiglitazone treatment (Figure 33B). There was no difference in ACE2 activity in lean mice treated with rosiglitazone compared to untreated lean mice (Figure 33B).

**Effect of rosiglitazone using buffer A containing ZPP, Bestatin and Amastatin (Buffer B)**

MLN-4760 demonstrated a significant decrease in fluorescence produced in all the four groups (*p<0.05, Figure 34A). Cardiac ACE2 activity was not affected in db/db mice after 7 weeks of rosiglitazone treatment (Figure 34B). There was no difference in ACE2 activity in lean mice treated with rosiglitazone compared to untreated lean mice (Figure 34B).

**Effect of pioglitazone on cardiac ACE2 enzyme activity in 18 week old mice**

In a separate study, we investigate the effect of pioglitazone on cardiac ACE2 in 18 week old mice. We used Buffer B to avoid interference from other proteases as indicated from above experiments. At 18 week of age, there was no difference in ACE2 activity between lean and db/db mice (Figure 39). This suggests that the expression of ACE2 is age-dependent in db/db mice. Treatment with pioglitazone for 10 weeks did not demonstrate any difference in ACE2 activity (Figure 39).

**Optimization of NEP activity**

NEP activity is measured with indirect coupled fluorometric method using a Succinyl-ala-ala-phe-methoxycoumarine (MCA) substrate. We optimized the NEP fluorogenic assay by introducing inhibitors for PCP, PEP, AP-A and AP-M to improve specificity and sensitivity of the assay. NEP activity was
measured by estimating the inhibition in hydrolysis of NEP substrate in presence or absence of the specific NEP inhibitor, thiorphan. There is limited data in the literature on cardiac NEP activity using this method. We thus optimized the assay in the kidney lysates.

**Renal NEP activity using Tris HCl (pH 7.4), ZnCl₂ and Lisinopril (Buffer A)**

Thiorphan produced significant inhibition of total fluorescence in lean (56%) and \(db/db\) (37%) kidney samples (*p<0.001, Figure 35A). Background fluorescence is produced in lean and \(db/db\) samples even after incubation with thiorphan (Figure 35A). NEP activity was measured by estimating the inhibition in hydrolysis of NEP substrate in presence or absence of thiorphan. NEP activity is significantly decreased in \(db/db\) mice compared to lean mice (*p<0.001, Figure 35B).

**Renal NEP activity using Buffer A containing ZPP, bestatin and amastatin (Buffer B)**

There was significant inhibition in NEP fluorescence by thiorphan in lean (80%) and \(db/db\) (51%) kidney samples (Figure 35A, *p<0.05). Although the inhibition of fluorescence by thiorphan enhanced by addition of these inhibitors, the background fluorescence in still present (Figure 35A). NEP activity was measured by estimating the inhibition in hydrolysis of NEP substrate in presence or absence of thiorphan. There was a significant decrease in NEP activity in \(db/db\) mice compared to lean mice in both buffer conditions (Figure 35B, *p<0.001).

**Cardiac NEP enzyme activity optimization**

Addition of the cocktail of the protease inhibitors (ZPP, amastatin, bestatin) to buffer A, improved the fluorogenic assay and produced ~30% more inhibition of renal NEP activity with thiorphan. We thus repeated similar assay conditions for evaluation of cardiac ACE2 activity.

**Cardiac NEP activity using Tris-Cl (pH 7.4) containing ZnCl² and lisinopril (Buffer A)**

There was a significant inhibition in NEP fluorescence in lean (51%) and \(db/db\) (34%) heart samples when incubated with thiorphan (*p<0.001, Figure 36A). Background fluorescence was produced in lean and
db/db samples incubated with thiorphan (Figure 36A). NEP activity was measured by estimating the inhibition in hydrolysis of NEP substrate in presence or absence of thiorphan. There was a significant decrease in NEP activity in db/db mice compared to lean mice (*p<0.001, Figure 36A).

**Cardiac NEP activity using Buffer A containing ZPP, bestatin and amastatin (Buffer B)**

Thiorphan demonstrated significant inhibition of NEP fluorescence in lean (50%) and db/db (22%) heart samples (*p<0.001, Figure 36A). Thus, the inhibition of total fluorescence by thiorphan was not enhanced by the addition of the cocktail of the protease inhibitors. There was no significant difference in inhibition produced by thiorphan in buffer B than buffer A (Figure 36A). NEP activity was measured by estimating the inhibition in hydrolysis of NEP substrate in presence or absence of thiorphan. There was a significant decrease in NEP activity in db/db mice compared to lean mice (*p<0.05, Figure 36B).

**Effect of rosiglitazone on cardiac NEP activity using Buffer A**

Incubation with thiorphan produced significant inhibition of fluorescence in all the four groups (*p<0.05, Figure 37A). NEP activity was significantly decreased in db/db mice compared to lean (*p<0.01, Figure 37B). Seven weeks of treatment with rosiglitazone has no effect on cardiac NEP activity in db/db mice (Figure 37B). No difference in NEP activity was seen in lean mice treated with rosiglitazone compared to untreated (Figure 37B).

**Effect of rosiglitazone using Buffer A containing ZPP, Bestatin and Amastatin (Buffer B)**

Incubation with thiorphan produced significant inhibition of fluorescence produced in all the four groups (*p<0.05, Figure 38A). NEP activity was significantly decreased in db/db mice compared to lean (*p<0.01, Figure 38B) Cardiac NEP activity was not affected in db/db mice after 7 weeks of rosiglitazone treatment (Figure 38B). No difference in NEP activity was seen in lean mice treated with rosiglitazone compared to untreated lean mice (Figure 38B).
Effect of pioglitazone on cardiac NEP enzyme activity in 18 week old mice

In a separate study, we investigate the effect of pioglitazone on cardiac NEP in 18 week old mice. We used assay buffer containing tris, zinc chloride, lisinopril as addition of inhibitors did not have a significant effect on the assay results. Thiorphan produced significant inhibition in fluorescence among all the four groups (*p<0.05, Figure 40A). At 18 week of age, there was no difference in NEP activity between lean and db/db mice (Figure 40 B). This suggests that the activity of NEP is age-dependent in db/db mice. Treatment with pioglitazone for 10 weeks did not demonstrate any difference in NEP activity (Figure 40B).

Summary

- Introduction of protease inhibitors for PCP, PEP, AP-A and AP-M in fluorogenic renal ACE2 activity assay improves specificity towards ACE2 activity.
- Cardiac ACE2 assay is influenced by other enzymes that confound the results of the assay when inhibitors are introduced.
- Renal and cardiac NEP assays are not affected by introduction of protease inhibitors for PCP, PEP, AP-A and AP-M.
- Rosiglitazone and pioglitazone have no effect on cardiac ACE2 and NEP activities.
VI. DISCUSSION

Leptin receptor deficient \textit{db/db} mice have been demonstrated to develop hyperglycemia, hyperinsulinemia, obesity, hypertension and cognitive dysfunction (200;201). Therefore, it is considered to be a good animal model to study the complication of type 2 diabetes and for screening for new antidiabetic medications. As expected, in the present study \textit{db/db} demonstrated hyperglycemia, hyperinsulinemia, obesity and increased body fat compared to age-matched littermates. Type 2 diabetes syndrome in \textit{db/db} mice is also characterized by hyperphagia and polydipsia. Increased food and water intake was demonstrated by \textit{db/db} mice in our studies. In addition, plasma hormones e.g. adiponectin, leptin, glucagon, triglyceride were significantly elevated in \textit{db/db} mice compared to their littermates. Previous studies in \textit{db/db} mice demonstrated normalization of hyperglycemia, water intake, plasma hormone parameters after rosiglitazone treatment (12). In agreement with our previous study, both rosiglitazone and pioglitazone treatment normalized hyperglycemia, water intake. Rosiglitazone and pioglitazone are insulin sensitizers and thus treatment lead to a decrease in plasma insulin, leptin, glucagon and triglycerides in \textit{db/db} mice.. In addition, adiponectin a hormone secreted from adipose tissue as a modulator for fatty acid oxidation is reduced with rosiglitazone in \textit{db/db} mice (202). Treatment with both rosiglitazone and pioglitazone demonstrated significant increase in body weight in \textit{db/db} mice. Increase in body mass index has been associated with increased risk of heart failure in both female and male patients (203). Several reports suggest that fluid retention after thiazolidinediones treatment leads to pressure overload on the heart and thus increases risk of heart failure (204). Our studies demonstrated no difference in total body water in mice treated with both rosiglitazone and pioglitazone between lean and \textit{db/db} mice. There was no difference in lean body mass of in both lean and \textit{db/db} mice after treatment. In addition, rosiglitazone and pioglitazone treatment has no effect on blood glucose and body weight of lean control mice
Our previous study showed decreased renal NEP protein expression in db/db mice (205). We have also shown that physical exercise training of db/db mice decreased hyperglycemia and increased renal NEP protein expression (206). We evaluated the cardiac expression of NEP in db/db mice. In addition, the effect of rosiglitazone and pioglitazone on cardiac NEP protein was also evaluated. Western blot analysis in heart lysates for NEP demonstrated two immunoreactive bands at 100kDa and 65 kDa in contrast with one only band in the kidney. To our knowledge this is the first report of two immunoreactive bands in the heart. This experiment was repeated more than 4 times and we obtained similar results in 14 and 18 week old mice. Since there was only one band in the kidney and two in the heart, this suggests that the expression of NEP is organ specific. The specificity of the NEP antibody was determined in WT and NEP KO mice. There was no band for NEP in the NEP-KO. This suggest the multiple bands obtained in the heart lysate is not due to technical artifacts. The 100 kDa immunoreactive band of NEP shows significant downregulation in 14 weeks old db/db mice compared to lean control. In the second study, there was no significant difference in the 100 kDa immunoreactive band of NEP heart lysates from 18 week old db/db mice compared to lean control lean. According to reports in various organs, this band represents the fully mature form of NEP (207;208). The degree of insulin resistance and hyperglycemia is worse by time. db/db mice follow an age-dependent progression in diabetes, with starts with early serve insulin resistance which is followed later by a defect in insulin secretion which will result in profound hyperglycemia. It has been shown before that there are age-dependent changes in metabolism, contractile function, and ischemic sensitivity in hearts from db/db mice (209). The difference in the data between 14 and 18 weeks might suggest that the expression of NEP in the heart might be age-dependent.

NEP is responsible for degradation of Ang I in addition to natriuretic peptides (210). Thus, standalone therapy with NEP inhibitors nullify the potential benefits of increased natriuretic peptides through increased levels of circulating Ang II (211-213). Despite there being no apparent benefits of NEP inhibition alone, combined NEP inhibition and ACE inhibition has demonstrated a decrease in blood pressure and cardiac hypertrophy in hypertensive rats (214;215). We have reported earlier an increase in plasma ACE activity
and Ang II content in \textit{db/db} mice (193;212;213). Downregulation in NEP could be thus in response to increased Ang II levels in the body as a cardioprotective measure.

Interestingly, the 65 kDa band of NEP shows significant upregulation in \textit{db/db} mice compared to lean control in both studies. This might suggest the possibility of the mature form (100kDa) of NEP being cleaved to form the 65kDa fragment in the heart. Thus, the downregulation in 100kDa band of NEP can be explained. It is required to ascertain the identity of both the bands as NEP. Amino acid sequence and mass spectrometric analysis can be performed in samples after separation of the two bands on a polyacrylamide gel. It is not known whether both forms of NEP in the heart are active, MALDI or SELDI mass spectrometric enzyme analysis could be performed after separation of the bands. There are numerous possibilities for the interaction of both forms of NEP with each other. Both forms might be enzymatically active or one of them might be more active than the other. It is also possible to speculate synergistic activity of both the forms.

Previous study demonstrated that treatment with rosiglitazone upregulates renal NEP expression and activity in \textit{db/db} mice (205). Rosiglitazone did not affect the cardiac expression of both forms of NEP (100kDa and 65KDa). In contrast, pioglitazone treatment demonstrated a downregulation in the expression of both the bands (100kDa and 65kDa). This differential effect of rosiglitazone and pioglitazone demonstrates a distinction in the action of both compounds on the same target. Several studies using rosiglitazone demonstrated either no effect or an increased risk of heart failure. In contrast, pioglitazone has been associated with a mild reduction in stroke and MI risk (216). It can be speculated that downregulation of cardiac NEP by pioglitazone is associated with cardiovascular benefit.

We also evaluated the cardiac ACE2 protein expression and activity in \textit{db/db} mice. In addition, the effect of rosiglitazone and pioglitazone on cardiac ACE2 was also evaluated. Western blot analysis of cardiac ACE2 demonstrated two immunoreactive bands at 95 kDa and 30 kDa in contrast with only one band (95 kDa) in the kidney. Similar to NEP expression in the heart, expression of ACE2 is also organ specific, there
are two distinct bands in the heart versus one in the kidney. The specificity of the ACE2 antibody was determined in WT and ACE2 KO mice. There was no band for ACE2 in the ACE2-KO. This suggest the multiple bands obtained in the heart lysate are not due to technical artifacts. The 95 kDa band of ACE2 is upregulated in db/db group compared to lean control in 14 week old mice. In 18 week old mice, there was no significant difference between 95kDa form of ACE2 in lean and db/db. Renal expression of ACE2 has been reported to be significantly higher in 8 week compared to 30 week old db/db mice (193). The results in heart samples thus suggest a similar age-dependent alteration in cardiac ACE2 expression. Upregulation of ACE2 could be a possible cardioprotective measure by the body. The 30 kDa band for ACE2 demonstrated no difference in ACE2 expression between lean and db/db mice in both the studies. Similar to NEP bands, identity of both forms of cardiac ACE2 could be confirmed with amino acid sequencing and mass spectrometric analysis after separation of the two bands on a polyacrylamide gel. MALDI or SELDI analysis of both forms of ACE2 could confirm whether both the bands of ACE2 are active.

The use of intramolecularly quenched fluorogenic substrate are widely used to measure enzyme activity in biological fluids (217). MCA-APK-Dnp is a fluorogenic substrate which is commonly used for measurement of ACE2 activity (218). Fluorescence is produced by the 7-methoxycoumarine (MCA) moiety after cleavage at Pro-Lys site separates the quenching 4-dinitiophenyl (Dnp). However, there were reports that this fluorescence produced represents 56% of total enzymatic activity of ACE2 at pH 7.5 (219). It has been suggested that fluorescence by such artificial substrates may be a result of peptidases other than ACE2 and thus produce inaccurate estimation of enzyme activity (220). Enzymes like prolyl endopeptidase (PEP), prolyl carboxypeptidase (PCP), aminopeptidases A and M might cause cleavage of the fluorogenic substrate (220). Alternative methods such as mass spectrometry based assays, use natural substrates and are more selective and specific (220). However, these methods are not easily accessible and are cost-prohibitive. Since, NEP and ACE2 activities were not evaluated before in db/db mice, we aimed to reevaluate the selectivity and specificity of the fluorogenic assay for renal and cardiac samples.
Our and other results showed increased renal ACE2 activity in db/db and Akita mouse models of diabetes (11;12). ACE2 activity was first performed in kidney lysate obtained from 18 week old mice using Tris-HCl buffer (pH 7.4) containing 0.15M NaCl, Zinc chloride and the selective ACE inhibitor, lisinopril (Buffer A). Specific ACE2 inhibitor, MLN-4760 was used to validate the specificity of the assay. Significant inhibition by MLN-4760 was observed in both lean and db/db mice (Figure 13A). As expected renal ACE2 activity was significantly increased in db/db mice compared to lean control. However, fluorescence was not completely inhibited in lean and db/db mice in the presence of MLN-4760 (Buffer A, Figure 13A). This suggest that other proteases in addition to ACE2, cleaves MCA-APK-Dnp and produce fluorescence. The percentage of fluorescence inhibited by MLN-47 in lean and db/db mice was significantly enhanced (95%) in the presence of specific inhibitors for PCP, PEP, APA and APM. This demonstrated that addition of inhibitors improves the specificity of the assay. Moreover, the total fluorescence produced in lean mice using buffer B was significantly reduced compared that produced using buffer A. The fluorescence produced in db/db mice was similar in both buffer conditions. This might suggest that the activity of PCP, PEP, AP-A and M are suppressed in db/db mice compared to lean. It’s noteworthy to mention that regardless of buffer conditions used, the renal expression of ACE2 was increased in db/db mice compared to lean. Whereas, in the heart there was different result in ACE2 activity with change in buffer conditions.

We next aimed to apply the same assay procedure in heart lysates. Inhibition produced by MLN-4760 incubation was significant in both lean and db/db mice. This inhibition was significantly reduced compared to that observed in the kidney samples. This suggests that there is much more ACE2 activity in the kidney than the heart. Background fluorescence was produced in the heart samples after MLN-4760 incubation suggesting interference of other enzymes in the assay. There was an increase in cardiac ACE2 activity in db/db mice compared to lean. Addition of PCP, PEP, AP-A and AP-M inhibitors produced significantly lower fluorescence than buffer A. This suggests that enzymes like PCP, PEP, AP-A and AP-M are involved in cleavage of substrate in the heart. Surprisingly, the fluorescence in db/db mice in buffer B was less than
lean mice in contrast to that observed in buffer A. There was no difference in cardiac ACE2 activity between lean and db/db mice. ACE2 activity in 18 week old mice demonstrated no difference between lean and db/db mice. This result corresponded to the western blot results for cardiac ACE 2 expression. Thus, alternative enzyme assay methods like MALDI mass spectroscopic analyses should be used for reliable measurement of ACE2 activity in heart lysates. Rosiglitazone treatment did not affect renal ACE2 expression and activity in db/db mice (12). In agreement with these data, Our results did not demonstrate any difference in cardiac ACE2 activity in db/db and lean mice treated with rosiglitazone and pioglitazone.

We also aimed to optimize the cardiac NEP fluorogenic assay. Similar buffer conditions like the ones used in ACE2 activity optimization assay were used. Previous studies using fluorogenic substrate demonstrated a decrease in renal NEP activity in db/db mice compared to lean (221). Assay was optimized in kidney lysates form 17 week old lean and db/db mice using inhibitors for PCP, PEP, AP-A and AP-M. The assay buffer containing Tris, NaCl, ZnCl2 and lisinopril was used. The NEP inhibitor ,thiorphan, was also used to confirm the NEP specificity in this assay. Thiorphan produced significant inhibition in both lean and db/db mice. Using buffer B, fluorescence was decreased in both lean and db/db mice. Inhibition with thiorphan was greater in buffer B conditions than buffer A. Renal and cardiac NEP activity was decreased in db/db mice in both buffer conditions compared to lean control. Overall fluorescence was decreased in buffer B compared to buffer A. NEP activity was decreased in db/db mice compared to lean in 14 week old mice.

Treatment with rosiglitazone in db/db mice demonstrated increase in renal NEP activity (205). Cardiac NEP activity is not affected in db/db mice with rosiglitazone treatment. However, treatment with pioglitazone demonstrated a decrease in NEP activity in 18 week old mice. This suggests a difference between actions of rosiglitazone and pioglitazone. Decrease in cardiac NEP by pioglitazone might have cardioprotective effect through the subsequent rise of natriuretic peptides. It would be interesting to investigate whether this effect of pioglitazone is through PPAR-γ agonism or through its effect in normalizing hyperglycemia.
Further mechanistic studies on cardiomyocytes can be conducted to study the mechanism of action for its direct action on NEP.

One thing to note in measurement of enzyme activity is the presence of multiple bands of ACE2 and NEP in heart lysate as demonstrated by western blot experiments. The identity and activity of individual forms of both cardiac ACE2 and NEP needs to be confirmed with amino acid sequencing and mass spectroscopy. The activities could be attributed to the presence of either of the forms or might be a synergistic effect. As mentioned, a MALDI technique for assessing activities of each of the forms is required.

We have previously reported the direct ability of ADAM 17 protein in cleaving and shedding membrane bound ACE2 (222). Studies in endothelial cell line demonstrate ADAM 17 mediated cleavage of NEP to form a soluble non-membrane bound form (223). Renal expression of ADAM 17 was upregulated in db/db mice in previous studies (12). Western blot analysis revealed that cardiac ADAM 17 is upregulated in db/db diabetic mice. ADAM 17 knockdown by RNA interference prevented development of cardiac hypertrophy and fibrosis in Ang II induced hypertensive rat model (224). ADAM 17 is upregulated with TNF-α in patients with decline in left ventricular systolic function (225). ADAM 17 is upregulated in patients with advanced congestive heart failure (226). ADAM 17 inhibition attenuated development of left ventricular hypertrophy (227). As mentioned before, there were two bands for NEP in the heart with two distinct forms. The mature band (100kDa) was downregulated in db/db mice and a fragment band of (65kDa) was upregulated (Figure 9 and 10). ADAM 17 could be responsible for the cleavage of the fully mature band with increased formation of cleaved NEP (65kDa).
VII. CONCLUSION

To the best of our knowledge this is the first time cardiac ACE2 and NEP expression has been studied in \textit{db/db} diabetic mice. Our study demonstrated a novel finding that there are two immunoreactive bands for ACE2 and NEP in the heart as opposed to one in the kidney. Upregulation in cardiac ADAM 17 might cause cleavage of mature NEP to form fragmented form in diabetic conditions. ACE2 upregulation in the heart could be a cardioprotective measure against hyperglycemic insult. Assessment of enzyme activity of NEP and ACE2 should however, be performed using more sophisticated methods like MALDI mass spectrometric methods.
Renal and cardiovascular dysfunction in db/db diabetic mice is associated with increased cardiac angiotensin converting enzyme 2 (ACE2) and neprilysin (NEP)

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The prevalence of diabetic kidney disease (DKD) has increased in the recent decades and is considered one of the main causes of ESRD. DKD is also a major risk factor for cardiovascular diseases. Proteinuria is now widely accepted as an independent risk factor for cardiovascular morbidity and mortality. The renin angiotensin system (RAS) plays an important role in regulating both the renal and cardiovascular systems. The deleterious actions of Ang II are antagonized by Ang (1-7), which is generated by ACE2 and NEP. ACE2 and NEP are multifunctional enzymes and their shedding in the urine have emerged as early biomarkers for DKD. ACE2 has been shown to have renoprotective and cardioprotective role in diabetic mice. In addition, a combination of AT1 receptor blockade and NEP inhibition, Sacubitril-Valsartan is used for management of heart failure. The aim of this study was to investigate whether shedding of urinary ACE2 and NEP could be a predictor of cardiovascular disease and index of intra cardiac ACE2 and NEP status in db/db diabetic mice. Radio-telemetry was used to measure blood pressure. Control and diabetic db/db mice (8 weeks) were treated with pioglitazone (20mg/Kg/day) for 10 weeks. Western blot, immunostaining and RAS enzyme assays were used to study renal, urinary and cardiac protein expression and activities. db/db mice are normotensive at the ages of 8-12 weeks. There were no significant differences in cardiac ACE2 and NEP between db/db and control mice. However, at 18 weeks, db/db mice developed albuminuria and hypertension. In addition, at this age there was a significant increase in urinary and cardiac ACE2, NEP expression and activity. Pioglitazone treatment of db/db diabetic mice normalized hyperglycemia and attenuated albuminuria. In addition, pioglitazone increased expression and activity of cardiac ACE2 whereas it decreased expression and activity of cardiac NEP compared to untreated db/db mice. Pioglitazone treatment could be used as a renoprotective and cardioprotective since it attenuated albuminuria, increased cardiac ACE2 and decreased cardiac NEP. Increased urinary ACE2 and NEP could be used to predict alteration of cardiac RAS status and possible risk of cardiovascular diseases.

Poster will be presented at the annual Kidney Week, American Society of Nephrology, New Orleans, November 2017
Effect of pioglitazone on the shedding of urinary angiotensin converting enzyme (ACE) 2 and neprilysin (NEP) in db/db mice and their role as urinary biomarkers for diabetic kidney disease

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Diabetic kidney disease (DKD) is one of the major causes of end-stage renal disease. Angiotensin (Ang) II is the major biological active peptide of the renin angiotensin system. Elevated levels of Ang II contribute to initiation and progression of DKD. The actions of Ang II could be antagonized by its conversion to the vasodilator Ang (1-7), partly generated by the action of ACE2 and NEP. Although there is an emergence of some urinary biomarkers such as angiotensinogen, kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL), there is still a need for new early biomarkers for DKD. The aim is to investigate the effect of hyperglycemia on urinary albuminuria, ACE2, NEP and KIM-1 and to test the hypothesis that ACE2 and NEP can be used as early biomarkers for DKD. 8 weeks old control and db/db diabetic mice were subjected to pioglitazone treatment (20 mg/kg/day) for 10 weeks. Metabolic and renal parameters were measured. Urine was collected for the evaluation of ACE2, NEP, KIM-1 protein expression and RAS activity. At 7 weeks old, there was no significant difference in urinary albumin, NEP and KIM-1 between db/db and control mice. However, western blot showed a significant increased shedding of urinary ACE2 fragment (60KDa and 70kDa immunoreactive bands) in db/db mice compared to controls. Although there was a prominent immunoreactive band for NEP in control mice at 9 weeks, there was no detectable immunoreactive band for ACE2. After the development of albuminuria in older db/db mice (9-17 week), there was a significant increase of full length (95KDa) and fragment of ACE2, NEP (90kDa) and KIM-1 (75KDa) compared to control mice. Urinary NEP and ACE2 activities significantly increased in 17 weeks db/db mice compared to controls. Pioglitazone normalized hyperglycemia and attenuated urinary albumin, glucose and ACE2 shedding. However, it has no effect on urinary NEP and KIM-1. In db/db diabetic mice, increased shedding of enzymatically active ACE2 precedes albuminuria. Depletion of tubular renal ACE2, NEP, could lead to accumulation of Ang II, with concomitant development of microalbuminuria. Urinary ACE2 and NEP could be used as biomarkers for DKD.

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105

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