Physical exercise training but not metformin attenuates albuminuria and shedding of ACE2 in type 2 diabetic db/db mice

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

By

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Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Hari K. Somineni ENTITLED “Physical exercise training but not metformin attenuates albuminuria and shedding of ACE2 in type 2 diabetic db/db mice” BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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Angiotensin II (Ang II), a potent vasoconstrictor cleaved from Ang I, is responsible for renal damage in diabetes. Angiotensin converting enzyme 2 (ACE2) is highly expressed in the kidney and has been shown to be renoprotective by degrading Ang II to Ang-(1-7). A Disintegrin and Metalloproteinases (ADAMs) were recently identified as an ectodomain sheddases of transmembrane proteins. ADAM17 mediated shedding of renal ACE2 could contribute to the pathogenesis of diabetic nephropathy. In our previous study, rosiglitazone treatment normalized hyperglycemia and improved renal injury by preventing ACE2 shedding. The aim of this study is to test the hypothesis that improved glucose homeostasis with exercise and/or metformin attenuates albuminuria, renal ADAM17 protein and prevents shedding of ACE2 in db/db mice. Seven week old normal and db/db mice were subjected to physical exercise training and/or metformin treatment (150 mg/kg/day) for 10 weeks. Exercised mice ran on a mouse forced exercise walking wheel system for 1 hr a day for 7 days a week at a speed of 8 meters/minute. At juvenile stages (6 week old), db/db mice demonstrated higher levels of blood glucose, urinary albumin and ACE2 excretion. Urinary ACE2 is enzymatically active and 20 kDa shorter as demonstrated by immunoblotting. Renal ADAM17 and ACE2 protein levels were significantly upregulated in db/db mice compared to non-diabetic controls. In diabetic kidney, upregulated ADAM17 and ACE2 proteins co-localized in the tubular cortex. However, physical exercise training significantly attenuated blood glucose, urinary albumin and ACE2 excretion of db/db mice throughout the study period, whereas metformin treatment was effective in lowering
hyperglycemia only in the initial stages of diabetes. The increased renal ADAM17 protein levels in \textit{db/db} diabetic mice were normalized by exercise training but not by metformin. In addition, exercise training reduced plasma triglycerides and enhanced insulin levels of \textit{db/db} mice. The combination of exercise and metformin was effective against lowering plasma glucagon. Results demonstrated a significant association between blood glucose, urinary albumin, plasma insulin, glucagon and triglycerides with urinary ACE2 excretion. In conclusion, co-localization of ADAM17 with ACE2 suggests a possible interaction in diabetic kidney. Exercise training with or without metformin prevented shedding of renal ACE2 by attenuating ADAM17 protein. Elevated plasma insulin by exercise could be responsible for improved glucose homeostasis, at least in partial. Urinary ACE2 could serve as a prognostic tool in the progression of kidney damage and its attenuation by exercise may partially contribute to its renal protection.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Diabetes</td>
<td>1</td>
</tr>
<tr>
<td>Diabetic nephropathy</td>
<td>3</td>
</tr>
<tr>
<td>The renin angiotensin system</td>
<td>5</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>6</td>
</tr>
<tr>
<td>Angiotensin II receptors</td>
<td>7</td>
</tr>
<tr>
<td>Angiotensin converting enzyme 2</td>
<td>8</td>
</tr>
<tr>
<td>Renoprotection of ACE2</td>
<td>8</td>
</tr>
<tr>
<td>Urinary markers for the diagnosis of diabetic nephropathy</td>
<td>9</td>
</tr>
<tr>
<td>Albuminuria</td>
<td>9</td>
</tr>
<tr>
<td>Recent discoveries in the field of biomarkers</td>
<td>10</td>
</tr>
<tr>
<td>Urinary RAS components as biomarkers</td>
<td>11</td>
</tr>
<tr>
<td>ADAM17</td>
<td>11</td>
</tr>
<tr>
<td>Benefits of physical exercise training in diabetes</td>
<td>13</td>
</tr>
<tr>
<td>Effects of exercise on RAS</td>
<td>15</td>
</tr>
<tr>
<td>Renoprotective role of exercise</td>
<td>15</td>
</tr>
<tr>
<td>Metformin</td>
<td>16</td>
</tr>
<tr>
<td>Renoprotective role of metformin</td>
<td>16</td>
</tr>
<tr>
<td>2. HYPOTHESIS AND SPECIFIC AIMS</td>
<td>19</td>
</tr>
<tr>
<td>3. MATERIALS AND METHODS</td>
<td>20</td>
</tr>
<tr>
<td>Animals</td>
<td>20</td>
</tr>
<tr>
<td>Physical exercise training and metformin treatment</td>
<td>20</td>
</tr>
<tr>
<td>Blood glucose measurement</td>
<td>21</td>
</tr>
<tr>
<td>Glucose tolerance test</td>
<td>21</td>
</tr>
</tbody>
</table>
Body composition measurement………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………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## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Effects of exercise and/or metformin on blood glucose</td>
<td>30</td>
</tr>
<tr>
<td>2.</td>
<td>Effects of exercise and/or metformin on body weight</td>
<td>31</td>
</tr>
<tr>
<td>3.</td>
<td>Effects of exercise and/or metformin on food intake</td>
<td>32</td>
</tr>
<tr>
<td>4.</td>
<td>Effects of exercise and/or metformin on water intake</td>
<td>33</td>
</tr>
<tr>
<td>5.</td>
<td>Effects of exercise and/or metformin on urine output</td>
<td>34</td>
</tr>
<tr>
<td>6.</td>
<td>Effects of exercise and/or metformin on absolute body fat</td>
<td>35</td>
</tr>
<tr>
<td>7.</td>
<td>Effects of exercise and/or metformin on absolute lean mass</td>
<td>36</td>
</tr>
<tr>
<td>8.</td>
<td>Effects of exercise and/or metformin on total body water</td>
<td>37</td>
</tr>
<tr>
<td>9.</td>
<td>Blood glucose levels at baseline</td>
<td>38</td>
</tr>
<tr>
<td>10.</td>
<td>Urinary albumin excretion at baseline</td>
<td>38</td>
</tr>
<tr>
<td>11.</td>
<td>Total protein excretion at baseline</td>
<td>39</td>
</tr>
<tr>
<td>12.</td>
<td>Urinary ACE2 activity at baseline</td>
<td>40</td>
</tr>
<tr>
<td>13.</td>
<td>Urinary ACE2 expression at baseline</td>
<td>41</td>
</tr>
<tr>
<td>14.</td>
<td>Correlation between urinary ACE2 and blood glucose levels at baseline</td>
<td>41</td>
</tr>
<tr>
<td>15.</td>
<td>Correlation between urinary ACE2 and albumin at baseline</td>
<td>42</td>
</tr>
<tr>
<td>16.</td>
<td>Blood glucose levels after 2 weeks of intervention</td>
<td>43</td>
</tr>
<tr>
<td>17.</td>
<td>Urinary albumin excretion after 2 weeks of intervention</td>
<td>44</td>
</tr>
<tr>
<td>18.</td>
<td>Total protein excretion after 2 weeks of intervention</td>
<td>45</td>
</tr>
<tr>
<td>19.</td>
<td>Urinary ACE2 activity after 2 weeks of intervention</td>
<td>46</td>
</tr>
<tr>
<td>20.</td>
<td>Urinary ACE2 expression after 2 weeks of intervention</td>
<td>47</td>
</tr>
<tr>
<td>21.</td>
<td>Correlation between urinary ACE2 and blood glucose after 2 weeks of intervention</td>
<td>47</td>
</tr>
<tr>
<td>22.</td>
<td>Correlation between urinary ACE2 and albumin after 2 weeks of intervention</td>
<td>48</td>
</tr>
<tr>
<td>23.</td>
<td>Blood glucose levels after 10 weeks of intervention</td>
<td>49</td>
</tr>
<tr>
<td>Number</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>24.</td>
<td>Effect of exercise and/or metformin on glucose tolerance</td>
<td>50</td>
</tr>
<tr>
<td>25.</td>
<td>Urinary albumin excretion after 10 weeks of intervention</td>
<td>51</td>
</tr>
<tr>
<td>26.</td>
<td>Total protein excretion after 10 weeks of intervention</td>
<td>52</td>
</tr>
<tr>
<td>27.</td>
<td>Urinary ACE2 activity after 10 weeks of intervention</td>
<td>53</td>
</tr>
<tr>
<td>28.</td>
<td>Urinary ACE2 expression after 10 weeks of intervention</td>
<td>54</td>
</tr>
<tr>
<td>29.</td>
<td>Correlation between urinary ACE2 and blood glucose after 10 weeks intervention</td>
<td>54</td>
</tr>
<tr>
<td>30.</td>
<td>Correlation between urinary ACE2 and albumin after 10 weeks of intervention</td>
<td>55</td>
</tr>
<tr>
<td>31.</td>
<td>Effects of exercise and/or metformin on renal ADAM17 expression</td>
<td>56</td>
</tr>
<tr>
<td>32.</td>
<td>Effects of exercise and/or metformin on renal Timp3 expression</td>
<td>57</td>
</tr>
<tr>
<td>33.</td>
<td>Effects of exercise and/or metformin on renal ACE2 expression</td>
<td>58</td>
</tr>
<tr>
<td>34.</td>
<td>Effects of exercise and/or metformin on renal ACE2 activity</td>
<td>59</td>
</tr>
<tr>
<td>35.</td>
<td>Correlation between urinary ACE2 and plasma glucagon</td>
<td>60</td>
</tr>
<tr>
<td>36.</td>
<td>Correlation between urinary ACE2 and plasma triglycerides</td>
<td>60</td>
</tr>
<tr>
<td>37.</td>
<td>Correlation between urinary ACE2 and plasma insulin</td>
<td>61</td>
</tr>
<tr>
<td>38.</td>
<td>Effects of exercise and metformin on urinary albumin of normal mice</td>
<td>62</td>
</tr>
<tr>
<td>39.</td>
<td>Effects of exercise and metformin on urinary ACE2 activity of normal mice</td>
<td>63</td>
</tr>
<tr>
<td>40.</td>
<td>PAS staining of renal tissue sections from treated and untreated mice</td>
<td>64</td>
</tr>
<tr>
<td>41.</td>
<td>Picro-sirius red staining of renal tissue sections</td>
<td>65</td>
</tr>
<tr>
<td>42.</td>
<td>Immunohistochemistry for ADAM17 in renal tissue sections</td>
<td>66</td>
</tr>
<tr>
<td>43.</td>
<td>Immunohistochemistry for ACE2 in renal tissue sections</td>
<td>67</td>
</tr>
<tr>
<td>44.</td>
<td>Co-localization of ACE2 and ADAM17 in cortical tubules</td>
<td>68</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Short-term effects of physical exercise training and/or metformin treatment on age dependent metabolic parameters</td>
<td>27</td>
</tr>
<tr>
<td>2.</td>
<td>Long-term effects of physical exercise training and/or metformin treatment on age dependent metabolic parameters</td>
<td>28</td>
</tr>
<tr>
<td>3.</td>
<td>Effects of physical exercise training and/or metformin treatment on plasma hormone and lipid parameters</td>
<td>29</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

Diabetes

Diabetes mellitus is a group of metabolic disorders characterized by elevated blood glucose (hyperglycemia) due to absolute or relative deficiencies in insulin secretion and/or action (Orozco et al., 2008). This pandemic disease has reached epidemic proportions worldwide and approximately 285 million people were believed to be suffering with diabetes in 2010 and this figure is estimated to reach 439 million by 2030 (Tramonti & Kanwar, 2012). Recent reports suggest that 26 million people in the United States are suffering from diabetes (Zhang, 2011) and this economic burden is expected to reach $490 billion by 2030 (Farag & Gaballa, 2011).

Classification of diabetes

The disease diabetes mellitus can be classified into four types as per the new classification system: type 1, type 2, gestational diabetes and other specific types (American Diabetes Association, 2013a). Type 1 diabetes mellitus or juvenile diabetes is characterized by beta cell destruction caused by an autoimmune process, usually leading to absolute deficiency of insulin secretion. The onset is usually acute, developing over a period of a few days to weeks. Type 2 diabetes mellitus is characterized by insulin resistance in peripheral tissue and defects in insulin secretion. Insulin resistance and hyperinsulinemia eventually lead to impaired glucose tolerance (American Diabetes Association, 2013b). Type 2 diabetes accounts for at least 90% of all cases of diabetes and is highly associated with a family history of diabetes, older age, obesity and lack of exercise. Gestational diabetes is a type of diabetes with high blood glucose levels during pregnancy. Gestational diabetes may eventually lead to type 2 diabetics (Massi-Benedetti, 2002). Other types of diabetes includes persons with genetic defects of beta-cell function also called MODY or maturity-onset diabetes in youth, persons with pancreatic dysfunction caused by
drugs, chemicals or infections, persons with diseases of the exocrine pancreas, such as pancreatitis, cystic fibrosis, trauma/pancreatectomy, and other genetic syndromes like Down’s syndrome, Klinefelter’s syndrome and Turner’s syndrome (American Diabetes Association, 2013b).

**Diagnosis of diabetes**

Based on the current criteria, diagnosis of diabetes is made, if any one of the following conditions is met: 1. Fasting (at least 8 hours) plasma glucose ≥ 126 mg/dL. 2. Two-hour plasma glucose ≥ 200 mg/dL, in 75 g oral glucose tolerance test (American Diabetes Association, 2010). 3. Glycosylated hemoglobin A1C (HbA1c) ≥ 6.5% (Alberti & Zimmet, 1998; American Diabetes Association, 2013a). In addition to these, there is an intermediate stage between the normoglycemia and diabetes known as prediabetic stage where impaired fasting glucose (the fasting glucose levels range from 110 mg/dL to 126 mg/dL) and impaired glucose tolerance are seen. However, mechanisms resulting in hyperglycemia vary from patient to patient, urging the need of developing new diagnostic approaches, prevention strategies, and therapeutic interventions.

**Complications of diabetes**

Chronic hyperglycemia results in the dysfunction of various organs such as the kidney, heart, nerves, eyes, and blood vessels. Complications associated with diabetes play an important role in determining the quality of life of patients. Diabetic complications could be divided into micro and macrovascular dysfunctions. Microvascular complications of diabetes include nephropathy, retinopathy and neuropathy (Massi-Benedetti, 2002). Macrovascular complications include cardiovascular and sexual dysfunction (Brownlee, 2005). Cardiovascular disease is a major contributor of morbidity and mortality in diabetic patients. However this condition is also known
to coexist with hypertension and dyslipidemia (Levey et al., 2009; Slinin et al., 2012; American Diabetes Association, 2013b).

**Diabetic nephropathy**

Diabetic nephropathy is one of the major microvascular complications of diabetes which eventually manifests into end-stage renal disease (ESRD) (Jim et al., 2012; Futrakul et al., 2006; Jawa et al., 2006). One-third of the diabetic population is prone to develop nephropathy and it represents the major cause of morbidity and mortality (Cooper, 1998). Diabetic nephropathy is classically defined by albuminuria and impaired renal function such as abnormal serum creatinine, creatinine clearance and glomerular filtration rate. This is predominantly seen in African Americans, Asians, and Native Americans (Young et al., 2003). In diabetes, high glucose-induced activated renin angiotensin system (RAS) and glomerular hyperfiltration are considered to be the primary causes underlying this implication (O'Bryan & Hostetter, 1997; Hostetter, 2003). Increased content of renal Ang II results in the depletion of glomerular nephrin and increased glomerular pore size (Giacchetti et al., 2005; Bichu et al., 2009), which makes several proteins to pass through easily. Polyol compounds generated from the excess glucose, glycation of tissue proteins, and generation of reactive oxygen species (ROS) by hyperglycemia are the other possible mechanisms implicated in tissue damage (Aronson, 2008; Shi et al., 2013). Further, excess glucose in diabetes reacts with several proteins and results in the formation of advanced glycation end-products (AGEs). AGEs mediate renal injury by producing ROS, activating protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) pathways (Cooper, 2004). In addition, AGEs acts through AGE receptors (RAGE) and promotes collagen deposition, inflammation, and
growth of extracellular matrix proteins ultimately leading to tissue fibrosis (Cooper, 2004). Further, ROS cause podocyte apoptosis, thus initiating kidney damage (Susztak et al., 2006).

Clinical manifestations of nephropathy include albuminuria (Gall et al., 1997), hypertension, impaired renal function (Tan et al., 2010) and a high incidence of cardiovascular morbidity and mortality (Wang et al., 1996). Diabetic nephropathy is generally characterized by mesangial matrix expansion, glomerular thickening of basement membrane, and proteinuria which are associated with chronic renal failure (Giunti et al., 2006; Forbes & Cooper, 2013). Previously it was believed that kidney disease is unidirectional. However, recent research reported that early therapeutic interventions may prevent or delay progression to ESRD. The biggest challenge for healthcare professionals is accurate and early detection of diabetic kidney disease. According to the recent guidelines from the American Diabetic Association, kidney disease can be categorized into 5 stages based on the glomerular filtration rate (Levey et al., 2009; Slinin et al., 2012; American Diabetes Association, 2013b).

Table 1: Stages of CKD

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
<th>GFR (mL/min/1.73 m² body surface area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Slight kidney damage with normal or increased filtration</td>
<td>More than 90</td>
</tr>
<tr>
<td>2</td>
<td>Mild decrease in kidney function</td>
<td>60 to 89</td>
</tr>
<tr>
<td>3</td>
<td>Moderate decrease in kidney function</td>
<td>30 to 59</td>
</tr>
<tr>
<td>4</td>
<td>Severe decrease in kidney function</td>
<td>15 to 29</td>
</tr>
<tr>
<td>5</td>
<td>Kidney failure</td>
<td>Less than 15 (or dialysis)</td>
</tr>
</tbody>
</table>

Even though considerable scientific effort has been made to identify patients at risk for the development of diabetic nephropathy, very little was achieved so far (Susztak & Bottinger, 2006;
Sharma et al., 2005). Appreciably, microalbuminuria is the only noninvasive technique available for the diagnosis of diabetic nephropathy. However, onset of diabetes is difficult to ascertain and so many patients diagnosed with high blood glucose already have microalbuminuria (Lee, 2005). Moreover, recent clinical studies questioned the reliability of albuminuria in anticipating the progression and prevention of ESRD (de Galan et al., 2009). Therefore, there is an urgent need for sensitive and specific biomarkers for better prediction of diabetic nephropathy. A substantial amount of evidence demonstrates that strict glycemic control (Tan et al., 2010) as well as blockade of RAS provides renoprotection (Lewis et al., 1993; Maschio et al., 1996; Hostetter, 2003).

The renin angiotensin system (RAS)

RAS is an endocrine or hormone system that regulates blood pressure, fluid balance, and tissue repair. RAS is found not only in the plasma but also in tissues like kidney, heart, vasculature, brain, retina, liver, pancreas, reproductive system, lymphatic and adipose tissue (Danser & Schalekamp, 1996; Paul et al., 2006; Baltatu et al., 2000; Bader et al., 2001; Bataller et al., 2003; Lau et al., 2004). When the blood volume drops, juxtaglomerular cells in the kidney secrete renin into the circulation. Renin plays a crucial role in RAS due to its rate-limiting activity on the precursor angiotensinogen (Weber, 2001; Zaman et al., 2002). The system begins by the release of angiotensinogen into circulation by the liver. An enzyme renin converts angiotensinogen to an inactive decapeptide angiotensin (Ang) I. Ang I is further converted to Ang II by angiotensin converting enzyme (ACE), which is predominantly found in the pulmonary circulation. However, ACE is also produced in the vascular endothelium of many tissues including kidneys, adrenal glands, brain and heart. Ang II thus formed act through Ang II type 1 receptor (AT1R) and regulate several activities such as vasoconstriction, sodium retention, aldosterone release,
hyperplasia, and hypertrophy. Further transformation of Ang II is carried out by angiotensin converting enzyme 2 (ACE2), a recently discovered homologue of ACE. ACE2, a monocarboxy peptidase degrades the octapeptide Ang II to a heptapeptide Ang-(1-7). Alterations within the RAS are considered to play an important role in the pathogenesis of diabetic complications, particularly diabetic renal disease and hypertension (Burnier & Zanchi, 2006).

**Angiotensin II (Ang II)**

Ang II is a potent vasoactive peptide generated by the actions of a dipeptidyl carboxypeptidase, ACE on Ang I. It is widely distributed in the kidney, heart, brain, adipose tissue, gonads, pancreas, and plasma (Nielsen et al., 2000; Navar & Nishiyama, 2004; Brewster & Perazella, 2004). Ang II is important in regulating salt, water, and vascular homeostasis (Belova, 2000). Being a positive regulator of RAS, Ang II is believed to play an important role in triggering hypertension, inflammation, oxidative stress and aldosterone release from adrenal glands (Schmidt-Ott et al., 2000; Mehta & Griendling, 2007). Moreover, it is implicated to have direct actions in the pathophysiology of renal and cardiovascular diseases such as cardiac hypertrophy and remodeling, heart failure, vascular thickening, atherosclerosis, and glomerulosclerosis in humans. Interestingly, concentrations of renal Ang II are approximately 1000 fold higher compared to their circulatory levels, suggesting the existence of an intrarenal RAS (Seikaly et al., 1990). However, in contrast to this, several reports demonstrate circulatory Ang II as a source Ang II in the kidney. Despite of these discrepancies, Ang II is implicated to play a crucial role in glomerular injury by several mechanisms. Most of the actions of Ang II are mediated by G-protein dependent pathways. However, activation of nicotinamide-adenine dinucleotide phosphate (NADPH) and generation of reactive oxygen species by Ang II are considered as the important mechanisms underlying inflammation and fibrosis (Mehta & Griendling, 2007).
**Ang II receptors**

Physiological actions of Ang II are mediated by the G-protein coupled receptors Ang II type 1 (AT1R) and Angiotensin II type 2 (AT2R) (Schmidt-Ott et al., 2000; Mehta & Griendling, 2007). Most of the known physiological and pathological actions of Ang II are mediated by AT1R (Schmidt-Ott et al., 2000; Mehta & Griendling, 2007) and hence they are considered crucial. The AT1R is a 359 amino acid protein belonging to the seven-membrane superfamily of G protein-coupled receptors and is predominantly distributed in the kidney, heart, liver, adrenals, brain, lung, and vasculature. Two isoforms, AT1A and AT1B sharing 95% amino acid sequence similarity with no differences in functionality and pharmacology were reported in rodents. However, only AT1A is characterized in humans (Crowley et al., 2007). AT1R receptors are up-regulated by conditions that increase Ang II such as dehydration and sodium deficiency (Barth & Gerstberger, 1999; Sanvitto et al., 1997; Chen et al., 2003), indicating that expression of AT1R is affected by its agonist Ang II. *db/db* mice have high levels of circulating Ang II (Senador *et al.*, 2009). So it is plausible that high expression of AT1R in response to high-circulating Ang II could lead to diabetes related complications in *db/db* diabetic mice. AT1R after getting activated by the agonist, Ang II, couple to Gaq/11, Gα12/13, Gβγ complexes (Ushio-Fukai et al., 1998) and results in the activation of protein kinase C (PKC) and the extracellular signal regulated kinase (ERK) pathway. These mechanisms are implicated in the maintenance of contraction as well as cellular growth. Further, it is also known to activate some of the downstream effectors including phospholipase C (PLC), phospholipase A2 (PLA2) and phospholipase D (PLD) (Ushio-Fukai et al., 1999).
**Angiotensin converting enzyme 2 (ACE2)**

ACE2 is a membrane-bound, monooxmo peptidase sharing 40% homology with ACE. However, it is also reported to exist as a soluble form (Donoghue et al., 2000; Feng et al., 2008). Although it exhibits sequential homology and similarity with ACE, ACE2 has different biochemical activities and is not arrested by conventional ACE inhibitors like captopril and lisinopril (Tipnis *et al*., 2000). Initially, ACE2 was thought to be restricted to human kidney, heart, and testis (Donoghue et al., 2000). Later, it has been characterized in various other tissues like brain, liver, lungs, adipose tissue, pancreas, and retina (Danser & Schalekamp, 1996; Paul *et al*., 2006; Baltatu *et al*., 2000; Bader *et al*., 2001; Bataller *et al*., 2003; Lau *et al*., 2004). ACE2 cleaves a single carboxy residue from Ang I and Ang II and generates Ang-(1-9) and Ang-(1-7) respectively. ACE2 is conceded to play a counter-regulatory role in RAS and consequently many studies demonstrated its involvement in cardiovascular diseases (Tipnis *et al*., 2000; Wysocki *et al*., 2006) as discussed below.

**Renoprotection of ACE2**

ACE2 is implicated to have positive effects in renal and cardiovascular disease (Monteiro *et al*., 2008; Alghamri *et al*., 2012; Wysocki *et al*., 2006). Distribution of ACE2 is 20-fold higher in the kidney compared to heart and is predominantly localized in the proximal tubules (Tikellis *et al*., 2003). ACE2 is shown to be renoprotective by degrading Ang II to Ang-(1-7), a biologically active peptide that contravene the negative effects of Ang II by interacting with the G protein-coupled receptor Mas (Tipnis *et al*., 2000; Passos-Silva *et al*., 2013).

A study conducted on STZ-induced diabetic rodent models reported reduced ACE2 mRNA and protein levels in diabetic conditions (Tikellis *et al*., 2003). Further, a study conducted in STZ-induced diabetic rats by Moon *et al*. demonstrated a negative correlation between glomerular
ACE2 protein and proteinuria (Moon et al., 2008). Decreased glomerular ACE2 was associated with enhanced albuminuria in db/db mice (Ye et al., 2006). Moreover, pharmacological inhibition of ACE2 in STZ diabetic mice resulted in increased albuminuria and declined renal functioning (Soler et al., 2007), whereas its deletion aggravated albumin excretion in the urine of an Akita mouse model of type 1 diabetes (Wong et al., 2007) which is reversed by the administration of human recombinant ACE2 (Oudit et al., 2010). Furthermore, overexpression of ACE2 in diabetic rodent models has been reported to ameliorate diabetic nephropathy (Liu et al., 2011; Nadarajah et al., 2012). In addition to the animal models, a study conducted on human subject’s demonstrated downregulation of renal ACE2 protein expression in diabetic patients with nephropathy (Reich et al., 2008).

**Urinary markers for the diagnosis of renal injury**

Urine is the most important tool for identifying biomarkers in non-invasive fashion (Thongboonkerd, 2008). Urine testing for biomarkers could substitute renal biopsy as safe and painless alternative. Common causes for urinary markers in diabetic nephropathy are kidney damage (glomerular injury), oxidative stress, inflammation, and vascular damage (Matheson et al., 2010). Enzymes and low-molecular weight proteins, characteristic of tubular and glomerular damage, are used as potential markers (De Carvalho et al., 2011).

**Albuminuria**

Albuminuria is a widely accepted and well-established marker for diabetic nephropathy. Microalbuminuria is a condition where urinary albumin excretion ranges from 30 to 300 mg/day and is considered as a first sign of renal impairment which is widely used as a clinical tool for the diagnosis of early nephropathy (Remuzzi et al., 2002; Barratt & Topham, 2007). However, microalbuminuria is not detectable by a standard urine dipstick test (Saeed et al., 2012; Barratt &
Topham, 2007). If the urinary albumin levels exceed 300 mg/day, condition is known as macroalbuminuria, overt proteinuria, or dipstick-positive proteinuria (Matheson et al., 2010; Saeed et al., 2012), which is evident after a significant kidney damage (Levey et al., 2009; Barratt & Topham, 2007). Even though albuminuria has been considered as a potent non-invasive biomarker, it is seen only after a significant glomerular damage (Barratt & Topham, 2007). Hence, there is a need for more specific, and reliable biomarker to predict diabetic nephropathy in early stages. In addition, ADVANCE (Action in Diabetes and Vascular disease: PreterAx and Diamicron-MR Controlled Evaluation) study reported that treating type 2 diabetic patients with pharmacological interventions (perindopril/indapamide) normalized albuminuria; however, they developed ESRD (de Galan et al., 2009). Continuing in this vein, due to its wide range (30 to 300 mg/day), recent clinical studies questioned the reliability of albuminuria in anticipating the progression and prevention of ESRD (de Galan et al., 2009; Haller et al., 2011). Therefore, there is a need for a more sensitive and specific marker for better prediction of diabetic nephropathy.

**Recent discoveries in the field of biomarkers**

For instance, N-acetyl β-glucosaminidase is a lysosomal enzyme derived from proximal tubular cells that are not filtered by the kidney under normal circumstances. Its excretion increases in circumstances that cause tubular injury (Basturk et al., 2006). Urinary microvesicle-bound dipeptidyl peptidase-IV, a membrane-associated peptidase enzyme secreted from the tubular epithelial cells, can be used as an early marker of renal damage (Sun et al., 2012). In addition, decreased podocyte number (Zheng et al., 2011) as well as stress-induced shedding of adiponectin hormone (Kadowaki & Yamauchi, 2005) in urine can be used to characterize the onset of diabetic nephropathy. Increased concentrations of liver-type fatty acid-binding protein
(Nielsen et al., 2010) and pigment epithelium-derived factor (PEDF) (Chen et al., 2010) in urine can be used to predict the development of nephropathy in type 1 and type 2 diabetic patients respectively.

**Urinary RAS components as biomarkers**

In recent years, Kobori and his associates have shown angiotensinogen, component of RAS in urine that could be used as a marker for intrarenal RAS status in STZ-induced diabetic mice as well as patients with hypertension and chronic kidney disease (CKD) (Kobori et al., 2009; Kobori & Urushihara, 2013; Kamiyama et al., 2012). Continuing in this vein, it is reported that urinary angiotensinogen precedes albuminuria and hence could be used as an early biomarker for diabetic nephropathy in type 1 diabetic patients (Saito et al., 2009). Apart from angiotensinogen, another component of RAS, ACE has been validated in the urine. The N-domain isoform of ACE observed at 90 kDa is associated with hypertension and the absence of this isoform was associated with normal blood pressure levels in human subjects suggesting that the N-domain isoform of ACE could serve as a urinary biomarker (Maluf-Meiken et al., 2012; Casarini et al., 2001).

**ADAM17**

A Disintegrin and Metalloproteases (ADAMs) are zinc-dependent, multidomain transmembrane proteins belonging to the adamalysin family of metalloproteinases (White, 2003). Metalloprotease domains of this membrane-anchored proteinase mediate ectodomain shedding, resulting in proteolytic release of various transmembrane proteins, growth factors and cytokines and the disintegrin domains are cysteine-rich involved in adhesive activities (Wolfsberg et al., 1993; Blobel et al., 1992). ADAM10 and ADAM17 also known as tumor necrosis factor-α-converting enzyme (TACE) are the most active sheddases of this family. ADAM17, sharing
close sequence similarities and potential structural features with ADAM10, was addressed to play a crucial role in various activities among all the ADAMs (Gooz, 2010).

ADAM17 is a cell surface protein found in many tissues like the kidney, heart, brain, and skeletal muscle (Black et al., 1997). Previous studies demonstrated the role of ADAM17 in a broad spectrum of diseases such as cancer, inflammation and alzheimer’s disease apart from diabetes (Kaneko et al., 2011; Gooz, 2010; Federici et al., 2005). A recent study using type 1 diabetic mouse model demonstrated that hyperglycemia results in the activation of renal ADAM17 (Ford et al., 2013). Alternatively, a study conducted on Ang II-infused mice demonstrated increased ADAM17 protein levels, suggesting the role of Ang II in activation and enhancement of ADAM17 (Lautrette et al., 2005). ADAM17 became a prime target for developing therapies due to its ability to shed a large variety of substrates, including ACE2. Lambert et al. demonstrated the role of ADAM17 in ectodomain shedding of ACE2 from stably transfected HEK293 cells and endogenously expressing Huh7 cells in vitro (Lambert et al., 2005). A Study conducted on CHO cells established the site of action of ADAM17 on ACE2 as Arg708_Ser709 bond (Lai et al., 2011). Further, Jia et al. showed the involvement of ADAM17 in ectodomain shedding of ACE2 in human airway epithelia (Jia et al., 2009). However, the biological importance of shedding events mediated by ADAM17 in vivo has not been fully elucidated. Due to its involvement in various deleterious activities, ADAM17 became a prime target for developing therapies. Biological inhibition of ADAM17 has been witnessed to ameliorate insulin resistance in obese mice by lowering tumor necrosis factor alpha (TNF-α) production from its precursor (Kaneko et al., 2011). Administration of WTACE2, a pharmacological inhibitor for ADAM17, is implicated to exert beneficial effects in chronic
kidney injury by attenuating glomerular and tubular lesions and interstitial fibrosis (Lautrette et al., 2005).

On the other hand, excitation of several cell signaling pathways resulting in dimeric to monomeric shift of ADAM17 was associated with increased ADAM17 and decreased tissue inhibitor of metalloproteinase 3 (Timp3), suggesting Timp3 as an endogenous inhibitor of ADAM17 (Xu et al., 2012). Continuing in this vein, recent studies suggest that deficiency of Timp3 results in increased ADAM17 activity and TNF-α production leading to diabetes in heterozygous insulin receptor mutant mice (Federici et al., 2005) and exacerbating diabetic nephropathy in the Akita, type 1 diabetic mouse model (Basu et al., 2012).

**Benefits of physical exercise training in diabetes**

Lifestyle intervention program is a cornerstone therapy for preventing (Knowler et al., 2002; Tuomilehto et al., 2001) or managing patients with type 2 diabetes (Sigal et al., 2006). Retrospective clinical trials validated exercise training as an important non-pharmacological strategy to prevent diabetes and obesity (Pan et al., 1997). However, the American Diabetes Association (ADA) and European Association for the study of diabetes (EASD) recommended initiation of a pharmacological treatment concurrently with lifestyle intervention to gain tight control over diabetes and related complications (Rhee et al., 2010; Nathan et al., 2009; 2012). From the perspectives of efficacy and economy, the Diabetes Prevention Program (DPP) and its Outcomes Study (DPPOS) demonstrated lifestyle intervention as a cost-effective strategy in preventing diabetes especially type 2 and reduced diabetes incidence by 58% in high risk patients (Knowler et al., 2002; Tuomilehto et al., 2001; Fradkin et al., 2012). Furthermore, a randomized clinical trial conducted in individuals with impaired glucose tolerance reported that interventions with exercise, diet, and both exercise and diet were associated with 46%, 31%, and 42%
reduction in the incidence of diabetes respectively (Pan et al., 1997), suggesting the efficacy of exercise alone in preventing type 2 diabetes.

150 minutes per week of moderate intensity exercise or 90 minutes per week of vigorous intensity exercise or an equivalent combination of the two is recommended to achieve therapeutic benefits in many chronic diseases, particularly type 2 diabetes (U.S. Department of Health and Human Services, 2008; American Diabetes Association, 2013a). Exercise is shown to reduce the incidence of diabetes by 46% in patients with impaired glucose tolerance, which is an intermediate stage between normal glucose tolerance and the disease diabetes. In addition, follow-up of the Finnish diabetes prevention study demonstrated reduced incidence of type 2 diabetes even 3 years after termination of the intervention (Lindstrom et al., 2006). Exercise training has been shown to attenuate hepatic glucose production (Minuk et al., 1981) and accentuate peripheral glucose utilization in human subjects by enhancing insulin mediated glucose transported type 4 (GLUT-4) translocation or by increasing 5’ adenosine monophosphate-activated protein kinase (AMPK) α2 activity (insulin independent) (Ivy, 1997; Musi et al., 2001). Physical exercise is known to enhance insulin-stimulated glucose disposal via several mechanisms such as increasing insulin sensitivity by changing the body composition (Yki-Jarvinen & Koivisto, 1983), stimulating muscle blood flow (Yki-Jarvinen & Koivisto, 1983) and GLUT-4 protein levels (Rodnick et al., 1990; Yki-Jarvinen & Koivisto, 1983), ameliorating insulin resistance, and glucose tolerance (Ivy, 1997). In a recent study on high-fat-fed mice, exercise has been shown to ameliorate glucose homeostasis and obesity via upregulation of plasma irisin, a hormone that stimulates browning and uncoupling protein 1 (UCP1) expression (Bostrom et al., 2012). In addition, moderate intensity exercise is associated with decreased inflammatory markers (interleukin-6; IL-6, tumor necrosis factor alpha; TNF
alpha and C-reactive protein; CRP) in healthy, older subjects (Colbert et al., 2004). Exercise training is implicated to prevent diabetic complications by lowering blood sugars, enhancing insulin sensitivity, and improving lipid profile (Arakawa, 1993; Zinman & Vranic, 1985).

**Effects of exercise training on RAS**

Hyperglycemia activates RAS and reactive oxygen species (ROS) and thus plays an important role in triggering inflammation. Several studies on animal models validated the beneficiary role of exercise on renal, brain, and cardiac RAS components (Pereira et al., 2009; Fernandes et al., 2011). Exercise training has been shown to degrade AT1R and Ang II protein levels and enhance ACE2 protein expression in the kidney of STZ-induced diabetic mice (Ciampone et al., 2011; Cunha et al., 2010). Regarding cardiac RAS, exercise training has been demonstrated to attenuate ACE and Ang II proteins and upregulate ACE2, Ang-(1-7) and AT2R protein levels in the heart of normotensive wistar rats (Fernandes et al., 2011). In addition, it is reported to enhance ACE2 protein expression in the brain of rabbits with chronic heart failure (Kar et al., 2010).

**Renoprotective role of exercise training**

Although several randomized clinical studies validated the beneficiary effects of exercise with or without diet restriction on the primary disease diabetes, effects of these interventions on the complications associated with it have not been extensively investigated. However, a very few studies addressed the renoprotective actions of exercise using animal models of diabetic nephropathy (Agarwal et al., 2012). Physical exercise is demonstrated to attenuate albuminuria, proteinuria, and glomerular sclerosis and maintain podocyte number in animal models of nephropathy (Kohzuki et al., 2001; Tufescu et al., 2008; Ishikawa et al., 2012). In addition, exercise training is unveiled to mitigate renal caspase-3 activity, mesangial matrix expansion and
tubulointerstitial fibrosis (Ghosh et al., 2009). Evidence suggests that exercise training improves renal injury in partial by lowering inflammation (Kasapis & Thompson, 2005; Ishikawa et al., 2012), oxidative stress (Ishikawa et al., 2012) and hypertension (Cardoso et al., 2012). Continuing in this vein, Boor et al. demonstrated the renoprotective effects of exercise could be partially mediated by alleviating plasma and renal advanced glycation end-products (Boor et al., 2009). However, clinical studies that investigated the beneficiary effects of exercise in chronic kidney disease are limited.

**Metformin**

Metformin is increasingly being used for the management of type 2 diabetes, especially after the adverse effects of thiazolidinediones became known. Retrospective clinical trials have shown metformin as the first line oral, anti-diabetic drug in treating type 2 diabetes (Rocha et al., 2012). Considering efficacy and its potential for cost-savings, Diabetes Prevention Program (DPP) and its Outcomes Study (DPPOS) demonstrated metformin as a marginally cost-saving strategy in preventing type 2 diabetes and was reported to reduce the incidence of diabetes by 31% in high risk patients (Knowler et al., 2002; Tuomilehto et al., 2001). Metformin acts by stimulating AMPK, resulting in the attenuation of hepatic glucose production and enhancement of peripheral glucose uptake (Scarpello & Howlett, 2008; Zhou et al., 2001). Upregulation of GLUT-1 and GLUT-4 plasma membrane concentrations were also implicated in the enhanced peripheral glucose uptake by metformin (Musi et al., 2002). However, metformin is potentially known to induce lactic acidosis.

**Renoprotective role of metformin**

A study conducted by UKPDS addressed that intensive glucose control with metformin, significantly reduced the risk of diabetes related outcomes in over-weight type 2 diabetic
patients. In particular, short-term administration of metformin attenuated albuminuria in type 2 diabetic patients (Amador-Licona et al., 2000). A study conducted by Zapecka et al demonstrated the renoprotective role of metformin could be mediated by reducing circulating amylin levels in type 2 diabetic patients (Zapecka-Dubno et al., 1999). Amylin is a peptide secreted by pancreatic beta cells, whose deposition in the kidney results in the severity of nephropathy (Gong et al., 2007). In addition, treatment with metformin significantly reduced plasma cholesterol and triglycerides (Amador-Licona et al., 2000) in type 2 diabetic patients, indicating the renoprotective efficacy of metformin, as hypercholesterolemia and hypertriglyceridemia were known to be associated with reduced kidney function even in nondiabetic healthy subjects (Maschio et al., 1989). As an extension, recent studies corroborated the renoprotective role of metformin in patients with CKD (Robinson-Cohen et al., 2009; Duong et al., 2012).

The incidence of type 2 diabetes and associated ravages were constantly increasing worldwide. Based on the retrospective clinical trials, first line recommendation for the prevention or management of type 2 diabetes is the combination of lifestyle (exercise training coupled with diet restriction to lose body weight) and metformin interventions. Alternatively, a study conducted by Pan et al. demonstrated that exercise alone is as effective as exercise plus diet in diabetics. However, no study was done until now in human subjects using the combination of exercise training and metformin treatment. In addition, effects of these interventions on the complications associated with diabetes are still under investigation. Therefore, in the current study, we investigated the individual as well as combined effects of exercise and metformin
during the early and late stages of diabetes and associated microvascular complications, diabetic nephropathy using type 2 diabetic mouse model.
2. HYPOTHESIS AND SPECIFIC AIMS

Hypothesis

Hyperglycemia induced shedding of renal ACE2 via ADAM17 is associated with the renal injury of \textit{db/db} mice. Improved glucose homeostasis with exercise and/or metformin prevents shedding of renal ACE2 and ameliorates renal injury in \textit{db/db} mice.

Specific aims

1. To test the hypothesis that, short-term physical exercise training and/or metformin treatment is associated with improved glucose homeostasis and decreased urinary albumin and ACE2 excretion of \textit{db/db} mice.

2. To test the hypothesis that, long-term physical exercise training and/or metformin treatment is associated with improved glucose homeostasis and decreased urinary albumin and ACE2 excretion of \textit{db/db} mice.

3. To test the hypothesis that, urinary ACE2 could be used as a biomarker for diabetic nephropathy and as a surrogate marker of diabetes.
3. MATERIALS AND METHODS

Animals

Five-week-old male db/db (BKS.Cgm +/+ Lepr<sup>db</sup>/J) mice and their age-matched non-diabetic littermates (db/m) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). The genetically diabetic mouse (db/db) has a mutation on the chromosome 4 that inhibits the expression of leptin receptor (Hummel et al., 1966). The syndrome of type 2 diabetes mellitus in db/db mice is similar to adult humans and is characterized by hyperinsulinemia, obesity, and progressive hyperglycemia. Animals were housed in standard cages at 22°C under a 12-hour light/12-hour dark cycle with ad libitum access to water and standard mouse chow. Wright State University Animal Care and Use Committee approved all experimental protocols.

Intervention with exercise and/or metformin

Seven-week-old mice were randomly assigned to 8 different groups: (1) Non-diabetic control (Normal) mice receiving regular water; (2) Normal mice receiving metformin in water (150 mg/kg/day; Spectrum laboratories, USA); (3) Normal mice receiving regular water and exercising daily (1 hour per day at a moderate intensity for 10 weeks); (4) Normal mice receiving metformin water and exercising daily; (5) db/db group receiving regular water; (6) db/db group receiving metformin water (150 mg/kg/day); (7) db/db group receiving regular water exercising daily and (8) db/db group receiving metformin water and exercising daily. Exercised mice ran on a mouse forced exercise walking wheel system (Lafayette Instrument, Lafayette, IN, USA). The mice began wheel-running for 1 hour a day, 7 days a week. Initial speed was set at 4 meters/minute and daily increased by 1 meter/minute and reached 8 meters/minute by the end of first week of training. In subsequent weeks, the mice were run for 1 hour a day at 8 meters/minute. Mice were run during the end of their dark cycle. All mice were monitored
weekly for blood glucose, body weight, food intake, water intake, and urine output. After 10 weeks of treatment, mice were euthanized by decapitation and trunk blood was collected in ice-chilled heparinized tubes. Plasma was immediately separated, centrifuged at 10,000 x g for 10 minutes at 4° C and stored at -80° C. Kidneys were collected in dry ice and stored at -80° C.

**Blood glucose measurement**

FreeStyle® Blood Glucose Test Strips & FreeStyle Lite® Blood Glucose Monitoring System 117 (Abbott Diabetes Care Inc, CA, USA) were used to measure blood glucose levels. A small cut was made on the tip of the tail vein to collect a drop of blood. Values were expressed in mg/dL.

**Glucose tolerance test**

An intra-peritoneal glucose tolerance test was performed in control normal, control db/db mice, and db/db mice subjected to exercise and/or metformin. Mice were fasted overnight for 16 hours and blood samples were collected from a cut made at the tip of the tail at 0, 30, 60, 90 and 120 minutes after a glucose load (I.P. injection of glucose in an aqueous solution, 1.5 g/kg). Samples were diluted in 300 µl water and 25 µl lysis buffer. Glucose concentration was determined using glucose oxidase/peroxidase reagent kit (Sigma, St. Louis, MO).

**Body composition measurement**

Body composition was measured using an ECHO MRI absolute body composition analyzer (Houston, TX, USA). Body weight of the mouse was determined. Instrument was calibrated and the mouse was placed in a transparent plastic cylinder and held in position with a plastic plunger to avoid any movements. This setup was placed inside the instrument and the measurements were taken.
**Urine collection**

For 24-hour urine collection, mice were housed individually in metabolic cages with a free access to food and water. A total of 20 µl of protease inhibitor (Roche Diagnostics, IN, USA) was used in each tube while collecting the urine. First collection was done at the end of the 12th hour and samples were stored at 4°C until the second collection. After the second collection at the 24th hour, samples were centrifuged at 10,000 x g for 3 minutes at 4°C. Then the supernatant was separated from the debris. Final volumes were recorded, aliquoted accordingly and stored at -80°C.

**Urinary albumin assay**

To monitor kidney function, quantitative estimation of urinary albumin was performed using a kit purchased from Bethyl Laboratories (Montgomery, TX, USA). Standards were diluted according to the kit’s protocol and samples (2 µl sample + 998 µl sample conjugate buffer) were prepared in 1:500 dilutions and added to a 96 well plate, incubated at RT for an hour. Then the plate was washed, 100 µl of diluted HRP conjugated secondary antibody (1:35000 in conjugate buffer) was added to each well and incubated for an hour at RT. Plate was washed again. TMB substrate was added, incubated for 15 minutes and the reaction was stopped using stop solution (2N H₂SO₄). Finally the absorbance was measured using Fusion® Packard plate reader at 450 nm.
Unknown urinary albumin concentrations were determined from a standard curve plotted using assay standards in the range 7.8-500 ng/ml.

**Urinary creatinine assay**

Urinary creatinine assays were performed using a kit purchased from Quidel (San Diego, CA, USA). The creatinine excretion rate in a normal individual is relatively constant. Thus, urinary creatinine levels are useful in detecting renal disease and estimating the extent of impairment of
renal function. The assay is based on modified Jaffe reaction where alkaline picrate forms a colored solution in presence of creatinine. Standards and urinary samples were prepared in 1:40 dilutions with distilled water and 50 µl were added to a 96 well plate. 150 µl of the working color solution was added to each well and incubated for 30 minutes at RT. The plate was read using Fusion® Packard plate reader at 490 nm. Unknown urinary creatinine concentrations were determined from a standard curve plotted using assay standards.

ACE2 activity
Urinary and renal ACE2 activities were measured by fluorometric test assay. The potentiality of ACE2 protein to cleave the fluorogenic substrate, 7-Mca-APK-(Dnp) was used to assess the activity of ACE2. Cleavage of this substrate at the C-terminal Lys residue by ACE2 removes the 2, 4-dinitrophenyl moiety that quenches the fluorescence of the 7-methoxycoumarin moiety, thus resulting in increased fluorescence. This emitted fluorescence was measured at excitation ($\lambda_{ex}$): 328 nm and emission ($\lambda_{em}$): 393 nm using a Fusion® Packard instrument. ACE2 activity was measured in presence of 10 mM lisinopril, an ACE inhibitor, to prevent any interference from ACE.

Western blot
Kidneys were taken out from -80°C and homogenized on ice in lysis buffer containing PMSF (Complete lysis M, Roche diagnostics, Mannheim, Germany). Tissue homogenates were centrifuged at 10,000 x g for 10 minutes at 4°C to remove cellular debris. Total protein content was determined in supernatant using BSA as a standard and BioRad reagent (BioRad, Hercules, CA, USA). 50 µg total protein samples were loaded and allowed to run on 10% SDS-PAGE gel for 1 hour. After electrophoresis, proteins were electrotransferred to an activated PVDF membrane (Millipore, MA, USA) with the help of a Mini Trans-Blot Electrophoretic Transfer
cell (BioRad, Hercules, CA, USA) at 72 V for 2 hours. Membranes were then blocked and probed with a polyclonal antibody directed against ACE2 (1:1000, R&D Systems, MN, USA), ADAM17 (1:500, Enzo Life Sciences, MI, USA) and Timp3 (1:200, Santa Cruz Biotechnology, CA, USA) respectively followed by incubation with respective secondary HRP conjugated antibody. Protein signals were detected by enhanced chemiluminescence reagent and analyzed using a ChemiDoc imaging system (BioRad, Hercules, CA, USA). The relative amounts of proteins of interest in kidney and urine were determined by normalizing to β-actin and creatinine respectively.

**Kidney histology**

One animal from each group was perfused at the end of the 10 weeks study. Kidneys were excised in formalin, after washing with PBS. Kidney sections (4 µm thick) were embedded in paraffin and stained with periodic acid Schiff base for histopathological observations at AML laboratories (Baltimore, MD, USA). These sections were then analyzed for glomerular hypertrophy and mesangial matrix expansion. 15 to 20 glomeruli from each group were observed. To investigate collagen deposits, kidney sections (4 µm thick) were stained with Picrosirius red using Weigert’s iron hematoxylin staining kit (ENG Scientific Inc, Clifton, NJ, USA). Sections were dewaxed with xylene and were sequentially treated with graded alcohols in order to rehydrate them. Slides were then subjected to Weigert’s iron hematoxylin solution for 8 minutes followed by tap water rinsing for 10 minutes. Then the slides were stained with Picrosirius red solution (5 g Direct Red 80 in 500 mL of saturated picric acid solution, Sigma-Aldrich). Coverslips were placed and viewed with a microscope. MetaMorph software (Molecular Devices, CA, USA) was used for quantitation.
Immunohistochemistry

Formalin fixed, paraffin-embedded kidney sections (4 µm thick) were air-dried and dewaxed with xylene and were sequentially treated with graded alcohols in order to rehydrate them. Then the slides were placed in a container holding 10mM sodium citrate buffer (pH 8.5), boiled for 30 minutes and incubated with methanol for about 20 minutes at -20°C for retrieving the antigen. Then the sections were blocked with 3% normal horse serum at 4°C to avoid nonspecific antibody binding. Sections were washed thrice and incubated either with primary polyclonal goat anti-ACE2 (1:150, R&D, MN, USA) or Timp3 (1:200, Santa Cruz, USA) or rabbit anti-ADAM17 (1:100, Enzo, USA) overnight at 4°C. Washings were repeated and the sections were incubated with biotinylated donkey anti-goat or anti-rabbit IgG secondary antibody conjugated with Cyanine 3 fluorescent dye. A drop of mounting medium was added; coverslips were placed and viewed with Olympus FV300 confocal microscope. MetaMorph software (Molecular Devices, CA, USA) was used for quantitation.

Plasma hormone and lipids measurement

Plasma samples collected at the end of the study were analyzed for insulin, glucose, adiponectin, leptin, glucagon, total cholesterol and triglyceride levels at the Mouse Metabolic Phenotyping Centre (Cincinnati, OH, USA). Plasma triglycerides were measured using commercially available assay kit (Randox Laboratories, UK). Plasma adiponectin concentration was measured with a mouse adiponectin ELISA kit (Millipore, St. Charles, MI, USA). Plasma levels of insulin and glucagon were measured using the Milliplex® MAP mouse metabolic hormone magnetic bead panel. Absorbance was measured using Luminex 200 (Millipore, Austin, TX). Plasma levels were calculated using standards provided with the Luminex kit.
**Statistical analysis**

The differences among groups were compared by Student’s unpaired two-tailed t-test. For more than two groups one-way ANOVA was used. All the valves are expressed as means ± SEM. For multiple comparisons between two or more groups, two-way ANOVAs were carried out followed by Bonferroni’s multiple comparison tests. Level of significance was set at \( p < 0.05 \). All the data was analyzed using Graph pad prism 5.01 and statistica software (v.10).
Table 1: Short-term effects of physical exercise training and/or metformin treatment on age dependent metabolic parameters

<table>
<thead>
<tr>
<th>Mice strain</th>
<th>Normal</th>
<th>db/db</th>
<th>db/db+M</th>
<th>db/db+E</th>
<th>db/db+E+M</th>
<th>Normal</th>
<th>db/db</th>
<th>db/db+M</th>
<th>db/db+E</th>
<th>db/db+E+M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (weeks)</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Duration of Treatment (weeks)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>20.5±0.4</td>
<td>29.7±0.8*</td>
<td>31.4 ± 1.3*</td>
<td>29.3±1.5*</td>
<td>30.5±0.7*</td>
<td>23.5±0.7</td>
<td>36.7±1.9*</td>
<td>40.0±1.4*</td>
<td>35.6±1.5*</td>
<td>36.4±1.9*</td>
</tr>
<tr>
<td>Food intake(^{†}) (g/day)</td>
<td>4.3±0.2</td>
<td>6.6±0.3*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>4.5±0.2</td>
<td>7.5±0.3*</td>
<td>7.3±0.5*</td>
<td>5.8±0.5**</td>
<td>6.1±0.9*(^{§})</td>
</tr>
<tr>
<td>Water intake (ml/day)</td>
<td>5.5±0.2</td>
<td>9.7±1.5*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>5.2±0.2</td>
<td>18.5±3.4*</td>
<td>18.7±1.8*</td>
<td>9.8±1.3**</td>
<td>11.1±1.1*(^{§})</td>
</tr>
<tr>
<td>Urine volume (ml/day)</td>
<td>1.0±0.0</td>
<td>2.6±0.2*</td>
<td>2.7±0.1*</td>
<td>2.4±0.2*</td>
<td>2.4±0.4*</td>
<td>1.2±0.2</td>
<td>11.6±1.1*</td>
<td>11.6±0.7*</td>
<td>2.7±0.5**</td>
<td>2.5±0.3*(^{§})</td>
</tr>
<tr>
<td>Absolute body fat (g)</td>
<td>3.4±0.2</td>
<td>13.5±0.5*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>4.2±0.7</td>
<td>20.9±0.8*</td>
<td>22.4±0.9*</td>
<td>20.0±0.9*</td>
<td>19.7±1.3*</td>
</tr>
<tr>
<td>Absolute lean mass (g)</td>
<td>14.3±1.2</td>
<td>13.5±0.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>16.9±1.7</td>
<td>14.5±0.6*</td>
<td>15.2±0.5</td>
<td>14.7±1.1*</td>
<td>15.6±1.7</td>
</tr>
<tr>
<td>Total body water (%)</td>
<td>58.9±3.7</td>
<td>41.7±3.5*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>55.1±4.1</td>
<td>32.4±2.8*</td>
<td>32.1±1.8*</td>
<td>33.7±2.4*</td>
<td>34.6±2.1*</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM. *p<0.05 vs age-matched normal mice and \(^{#}\), \(^{§}\)p<0.05 vs age-matched control db/db mice were considered statistically significant. ND means not determined. \(^{†}\) The amount of food spilled was minimal and was not accounted for the data presented in the table.
Table 2: Long-term effects of physical exercise training and/or metformin treatment on age dependent metabolic parameters

<table>
<thead>
<tr>
<th>Mice strain</th>
<th>Normal</th>
<th>db/db</th>
<th>db/db+M</th>
<th>db/db+E</th>
<th>db/db+E+M</th>
<th>Normal</th>
<th>db/db</th>
<th>db/db+M</th>
<th>db/db+E</th>
<th>db/db+E+M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (weeks)</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>17</td>
<td>17</td>
<td>17</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Duration of Treatment (weeks)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>20.5±0.4</td>
<td>29.7±0.8*</td>
<td>31.4±1.3*</td>
<td>29.3±1.5*</td>
<td>30.5±0.7*</td>
<td>28.4±0.8</td>
<td>39.5±2.0*</td>
<td>42.8±1.7*</td>
<td>41.7±2.0*</td>
<td>45.0±1.2*</td>
</tr>
<tr>
<td>Food intake† (g/day)</td>
<td>4.3±0.2</td>
<td>6.6±0.3*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>4.5±0.3</td>
<td>8.1±0.4*</td>
<td>7.7±0.2*</td>
<td>5.9±0.3*</td>
<td>6.2±0.2* S</td>
</tr>
<tr>
<td>Water intake (ml/day)</td>
<td>5.5±0.2</td>
<td>9.7±1.5*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>6.4±0.3</td>
<td>28.8±3.6*</td>
<td>26.4±1.6*</td>
<td>16.0±3.6*</td>
<td>14.0±0.6 S</td>
</tr>
<tr>
<td>Urine volume (ml/day)</td>
<td>1.0±0.0</td>
<td>2.6±0.2*</td>
<td>2.7±0.1*</td>
<td>2.4±0.2*</td>
<td>2.4±0.4*</td>
<td>1.2±0.2</td>
<td>17.3±6.1*</td>
<td>16.1±5.7*</td>
<td>4.2±1.5*</td>
<td>3.3±1.2 S</td>
</tr>
<tr>
<td>Absolute body fat (g)</td>
<td>3.4±0.2</td>
<td>13.5±0.5*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>5.5±0.6</td>
<td>22.4±1.3*</td>
<td>24.4±1.3*</td>
<td>20.1±1.6*</td>
<td>25.8±1.0*</td>
</tr>
<tr>
<td>Absolute lean mass (g)</td>
<td>14.3±1.2</td>
<td>13.5±0.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>18.9±2.6</td>
<td>14.9±1.8*</td>
<td>14.5±0.9*</td>
<td>15.1±1.3*</td>
<td>15.8±1.7*</td>
</tr>
<tr>
<td>Total body water (%)</td>
<td>58.9±3.7</td>
<td>41.7±3.5*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>57.7±4.6</td>
<td>33.6±3.4*</td>
<td>28.7±1.8*</td>
<td>29.6±2.1*</td>
<td>29.0±0.6*</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM. *p<0.05 vs age-matched normal mice and #, $p<0.05$ vs age-matched control db/db mice were considered statistically significant. ND means not determined. † The amount of food spilled was minimal and was not accounted for the data presented in the table.
### Table 3: Effects of physical exercise training and/or metformin treatment on plasma hormone and lipid parameters

<table>
<thead>
<tr>
<th>Mice strain</th>
<th>Normal</th>
<th>db/db</th>
<th>db/db+M</th>
<th>db/db+E</th>
<th>db/db+E+M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (weeks)</td>
<td>17</td>
<td>17</td>
<td>17</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Duration of Treatment (weeks)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Plasma insulin (ng/mL)</td>
<td>1.4±0.3</td>
<td>5.0±1.2*</td>
<td>4.5±0.7*</td>
<td>16.8±2.7*#</td>
<td>15.8±3.1*$</td>
</tr>
<tr>
<td>Plasma adiponectin (µg/mL)</td>
<td>13.5±0.8</td>
<td>6.6±0.6*</td>
<td>6.1±0.4*</td>
<td>8.1±1.1</td>
<td>8.2±1.1</td>
</tr>
<tr>
<td>Plasma leptin (ng/mL)</td>
<td>4.4±0.5</td>
<td>11.9±1.4*</td>
<td>14.5±2.2*</td>
<td>23.2±2.3*#</td>
<td>17.5±0.9*</td>
</tr>
<tr>
<td>Plasma glucagon (pg/mL)</td>
<td>14.8±3.5</td>
<td>101.5±6.8*</td>
<td>97.9±12.2*</td>
<td>64.2±3.6*</td>
<td>49.9±4.4$</td>
</tr>
<tr>
<td>Plasma triglyceride (mg/dL)</td>
<td>115.1±11.2</td>
<td>271.6±34.4*</td>
<td>386.8±38.8*@</td>
<td>137.2±25.3*#</td>
<td>95.4±7.8$</td>
</tr>
<tr>
<td>Plasma cholesterol (mg/dL)</td>
<td>115.3±6.8</td>
<td>113.9±8.7</td>
<td>113.4±4.8</td>
<td>112.8±8.6</td>
<td>107.1±5.8</td>
</tr>
</tbody>
</table>

Values represent mean±SEM. *p<0.05 vs age-matched normal mice and @, #, $p<0.05 vs age-matched control db/db mice were considered statistically significant. ND means not determined.
Figure 1: Blood glucose levels of normal mice, db/db mice, db/db treated with metformin, db/db treated with exercise and db/db treated with exercise plus metformin during the period of study. Repeated measures two-way ANOVA using a Bonferroni’s posthoc test showed that exercise training alone and in combination with metformin attenuated blood glucose levels of treated db/db mice consistently throughout the study, $p<0.001$ vs untreated db/db mice. Metformin reduced the blood glucose levels significantly during the first 2 weeks of treatment and had no effect later, $p<0.05$ vs untreated db/db mice. Data are represented as mean ± SEM of group size (n=10).
Figure 2: Body weights of normal, db/db, db/db + metformin, db/db + exercise and db/db + exercise + metformin groups during the 10 weeks period of study. Repeated measures two-way ANOVA using a Bonferroni’s posthoc test showed that there was no significant difference in body weights between treated and untreated db/db mice. Data are represented as mean ± SEM of group size (n=10).
Figure 3: Food intake of normal, $db/db$, $db/db +$ metformin, $db/db +$ exercise and $db/db +$ exercise + metformin groups during the 10 weeks period of study. Repeated measures two-way ANOVA using a Bonferroni’s posthoc test showed that physical exercise training alone and in combination with metformin caused a significant decrease in food intake of treated $db/db$ mice throughout the study whereas, treatment with metformin had no effect, $p<0.05$ vs untreated $db/db$ mice. Data are represented as mean ± SEM of group size (n=10).
Figure 4: Water intake of normal and db/db controls and db/db mice treated with metformin, exercise and exercise plus metformin during the 10 weeks period of study. Repeated measures two-way ANOVA using a Bonferroni’s posthoc test showed that physical exercise training alone and in combination with metformin caused a significant decrease in water intake of treated db/db mice throughout the study whereas treatment with metformin had no effect, $p<0.05$ vs untreated db/db mice. Data are represented as mean ± SEM of group size (n=10).
**Figure 5**: 24-hour urine output measurement from normal and *db/db* controls and *db/db* mice treated with metformin, exercise and exercise plus metformin during the 10 weeks period of study. Repeated measures two-way ANOVA using a Bonferroni’s posthoc test showed that physical exercise training alone and in combination with metformin caused a significant decrease in the urine output of treated *db/db* mice throughout the study whereas, treatment with metformin had no effect, *p*<0.05 vs untreated *db/db* mice. Data are represented as mean ± SEM of group size (n=10).
Figure 6: Percent body fat of normal and db/db controls and db/db mice treated with metformin, exercise and exercise plus metformin during the 10 weeks period of study. Repeated measures two-way ANOVA using a Bonferroni’s posthoc test showed that none of the treatments had effect on percent fat mass of db/db + metformin, db/db + exercise, db/db + exercise + metformin groups. Data are represented as mean ± SEM of group size (n=10).
Figure 7: Percent lean mass of normal and db/db controls and db/db mice treated with metformin, exercise and exercise plus metformin during the 10 weeks period of study. Repeated measures two-way ANOVA using a Bonferroni’s posthoc test showed that none of the treatments had effect on percent lean mass of db/db + metformin, db/db + exercise, db/db + exercise + metformin groups. Data are represented as mean ± SEM of group size (n=10).
Figure 8: Percent body water of normal and db/db controls and db/db mice treated with metformin, exercise and exercise plus metformin during the 10 weeks period of study. Repeated measures two-way ANOVA using a Bonferroni’s posthoc test showed that none of the treatments had effect on percent body water of db/db + metformin, db/db + exercise, db/db + exercise + metformin groups. Data are represented as mean ± SEM of group size (n=10).
Figure 9: Blood glucose levels in 6 weeks old db/db mice and their age-matched normal mice. One-way ANOVA showed a significant increase in the blood glucose levels of db/db mice compared to normal mice. *p<0.01 vs age-matched non-diabetic controls. Each bar represents mean ± SEM of group size (n=10).
Figure 10: Urinary albumin excretion in 6 weeks old db/db mice and their age-matched normal mice. One-way ANOVA showed a significant increase in albuminuria of db/db mice compared to normal mice. *p<0.001 vs age-matched non-diabetic controls. Each bar represents mean ± SEM of group size (n=10).

Figure 11: Urinary total protein excretion in 6 weeks old db/db mice and their age-matched normal mice. One-way ANOVA showed no difference in the total protein excretion of db/db mice compared to normal mice. Each bar represents mean ± SEM of group size (n=10).
Figure 12: Urinary ACE2 activity in 6 weeks old db/db mice and their age-matched normal mice. One-way ANOVA showed a significant increase in the urinary ACE2 activity of db/db mice compared to normal mice. *p<0.01 vs age-matched non-diabetic controls. Each bar represents mean ± SEM of group size (n=10).
Figure 13: Urinary ACE2 expression in 6 weeks old db/db mice and their age-matched normal mice. Student’s unpaired two tailed t-test showed a significant increase in the urinary ACE2 excretion of db/db mice compared to non-diabetic controls. *p<0.05 vs age-matched normal mice. Each bar represents mean ± SEM of group size (n=10).
Figure 14: Correlation between blood glucose levels and urinary ACE2 excretion in 6 weeks old db/db mice and their age-matched normal mice. Pearson correlation factor analysis showed a significant positive correlation between blood glucose levels and urinary ACE2 excretion. r=0.60, *p<0.05 vs age-matched normal mice. Each bar represents mean ± SEM of group size (n=10).

Figure 15: Correlation between urinary albumin and ACE2 excretion in 6 weeks old db/db mice and their age-matched normal mice. Pearson correlation factor analysis showed a significant positive correlation between urinary albumin and ACE2 excretion. r=0.65, *p<0.05 vs age-matched normal mice. Each bar represents mean ± SEM of group size (n=10).
Figure 16: At the age of 9 weeks, db/db mice had high levels of blood glucose compared to their age-matched normal mice. One-way ANOVA showed that, 2 weeks after the commencement of treatment there is a significant decrease in the blood glucose levels of db/db + metformin, db/db + exercise and db/db + exercise + metformin mice compared to untreated db/db mice. *p<0.0001 vs age-matched normal mice, †p<0.001, ‡p<0.0001, §p<0.0001 vs control db/db mice. Each bar represents mean ± SEM of group size (n=10).
**Figure 17:** At the age of 9 weeks, urinary albumin excretion of *db/db* mice is significantly higher compared to normal mice. One-way ANOVA showed that after the commencement of 2 weeks treatment, urinary albumin excretion of *db/db* + metformin, *db/db* + exercise and *db/db* + exercise + metformin groups are significantly less than control *db/db* group. *p*<0.0001 vs age-matched normal mice, †*p*<0.001, ‡*p*<0.0001, §*p*<0.0001 vs control *db/db* mice. Each bar represents mean ± SEM of group size (n=10).
Figure 18: At the age of 9 weeks, total protein excretion of $db/db$ mice is significantly higher compared to normal mice. One-way ANOVA showed that after the commencement of 2 weeks treatment, exercise training alone and in combination with metformin lowered urinary total protein excretion in treated $db/db$ diabetic mice compared to untreated $db/db$ mice. Treatment with metformin had no effect on urinary total protein excretion. *$p<0.0001$ vs age-matched normal mice, #$p<0.001$, $^\$p<0.001$ vs control $db/db$ mice. Each bar represents mean ± SEM of group size (n=10).
Figure 19: At the age of 9 weeks, $db/db$ mice demonstrated higher levels of urinary ACE2 excretion compared to normal mice. One-way ANOVA showed that 2 weeks after treatment commenced there is a significant decrease in urinary ACE2 excretion in $db/db +$ metformin, $db/db +$ exercise and $db/db +$ metformin + exercise mice compared to untreated $db/db$ mice. *$p<0.0001$ vs age-matched normal mice, **$p<0.0001$, ***$p<0.0001$, ****$p<0.0001$ vs control $db/db$ mice. Each bar represents mean ± SEM of group size (n=10).
**Figure 20**: Urinary ACE2 expression in 9 weeks old db/db mice. One-way ANOVA showed that, 2 weeks after the commencement of treatment there is a significant decrease in urinary ACE2 expression of db/db + metformin (M), db/db + exercise (E) and db/db + exercise + metformin (E+M) mice compared to control db/db mice (C). *p<0.001, †p<0.001, §p<0.0001 vs control db/db mice. Each bar represents mean ± SEM of group size (n=10).
**Figure 21:** Correlation between blood glucose levels and urinary ACE2 excretion in 9 weeks old normal, untreated and treated *db/db* mice. Pearson correlation factor analysis showed that there is a significant positive correlation between blood glucose levels and urinary ACE2 excretion. \( r = 0.73, \ *p<0.001 \). Each bar represents mean ± SEM of group size (n=10).

**Figure 22:** Correlation between urinary albumin and ACE2 excretion in 9 weeks old normal, untreated and treated *db/db* mice. Pearson correlation factor analysis showed that there is a significant positive association between urinary albumin and ACE2 excretion. \( r = 0.65, \ *p<0.001 \). Each bar represents mean ± SEM of group size (n=10).
Figure 23: At the age of 17 weeks, db/db mice had significantly higher levels of blood glucose compared to normal mice. One-way ANOVA showed that after the commencement of 10 weeks treatment, physical exercise training alone and in combination with metformin significantly decreased blood glucose levels in db/db mice compared to untreated db/db mice. However, 10 week treatment with metformin had no effect on blood glucose of treated db/db mice compared to control diabetic mice. *p<0.0001 vs age-matched normal mice, #p<0.0001, $p<0.0001 vs control db/db mice. Each bar represents mean ± SEM of group size (n=10).
Figure 24: Intra-peritoneal glucose tolerance test was performed in 17 weeks old normal mice, control \( db/db \) mice and \( db/db \) mice subjected to exercise training and/or metformin for 10 weeks. One-way ANOVA of area under curve showed that exercise training with or without metformin significantly improved glucose tolerance in \( db/db \) mice compared to untreated \( db/db \) mice. *\( p<0.001 \) vs age-matched normal mice, \( \# p<0.0001 \), \( \$ p<0.0001 \) vs control \( db/db \) mice. Each bar represents mean ± SEM of group size (n=6).
**Figure 25:** At 17 weeks of age, urinary albumin excretion of *db/db* mice is significantly higher compared to normal mice. One-way ANOVA showed that 10 weeks after the commencement of treatment, physical exercise alone and in combination with metformin lowered albumin excretion significantly in treated *db/db* diabetic mice compared to untreated *db/db* mice. Chronic treatment with metformin for 10 weeks had no effect on albuminuria. *p<0.0001* vs age-matched normal mice, *#p<0.0001, $p<0.0001* vs control *db/db* mice. Each bar represents mean ± SEM of group size (n=10).
**Figure 26:** At 17 weeks of age, total protein excretion of $db/db$ mice is significantly higher compared to normal mice. One-way ANOVA showed those 10 weeks after the commencement of treatment, physical exercise alone and in combination with metformin significantly lowered urinary total protein excretion significantly in treated $db/db$ diabetic mice compared to untreated $db/db$ mice. Chronic treatment with metformin for 10 weeks had no effect on urinary total protein excretion. $^*p<0.0001$ vs age-matched normal mice, $^#p<0.0001$, $^$p<0.0001 vs control $db/db$ mice. Each bar represents mean ± SEM of group size (n=10).
Figure 27: At the age of 17 weeks, db/db mice excreted significantly higher levels of urinary ACE2 compared to normal mice. One-way ANOVA showed that 10 week training with physical exercise training alone and in combination with metformin attenuated urinary ACE2 excretion in db/db mice compared to untreated db/db mice. In contrast, 10 week treatment with metformin had no effect on urinary ACE2 excretion of treated db/db mice compared to control diabetic mice. *p<0.0001 vs age-matched normal mice, #p<0.0001, $p<0.0001 vs control db/db mice. Each bar represents mean ± SEM of group size (n=10).
Figure 28: Urinary ACE2 expression in 17 weeks old db/db mice. One-way ANOVA showed that after the commencement of 10 weeks treatment, exercise training (E) alone and in combination with metformin (E+M) lowered urinary levels of ACE2 excretion in treated db/db diabetic mice compared to untreated db/db mice (C). Treatment with metformin for 10 weeks had no effect on urinary ACE2 expression of treated db/db mice (M). #p<0.01, $p<0.01 vs control db/db mice. Each bar represents mean ± SEM of group size (n=10).
**Figure 29:** Correlation between blood glucose levels and urinary ACE2 excretion in 17 weeks old normal, untreated and treated *db/db* mice. Pearson correlation factor analysis showed a significant positive correlation between blood glucose levels and urinary ACE2 excretion. \( r = 0.75, \ast p < 0.0001 \). Each bar represents mean ± SEM of group size (n=10).

![Graph showing correlation between blood glucose levels and urinary ACE2 excretion](image)

**Figure 30:** Correlation between urinary albumin and ACE2 excretion in 17 weeks old normal, untreated and treated *db/db* mice. Pearson correlation factor analysis showed a significant positive association between urinary albumin and ACE2 excretion. \( r = 0.84, \ast p < 0.0001 \). Each bar represents mean ± SEM of group size (n=10).
Figure 31: Renal ADAM17 protein expression in 17 weeks old normal, untreated and treated db/db mice. One-way ANOVA showed increased renal ADAM17 protein expression in db/db diabetic mice compared to their age-matched non-diabetic mice. 10 weeks exercise training alone and in combination with metformin attenuated ADAM17 protein in kidneys of treated db/db mice compared to untreated db/db mice. In contrast, 10 week treatment with metformin had no effect on renal ADAM17 protein levels of treated db/db mice compared to untreated diabetic mice. \#p<0.01, \$p<0.01 vs control db/db mice. Each bar represents mean ± SEM of group size (n=10).
**Figure 32:** Renal Timp3 protein expression in 17 weeks old normal, untreated and treated *db/db* mice. One-way ANOVA showed that there was no difference in renal Timp3 protein levels among normal, *db/db*, *db/db* + metformin, *db/db* + exercise and *db/db* + exercise + metformin groups. Each bar represents mean ± SEM of group size (n=10).
**Figure 33:** Renal ACE2 protein expression in 17 weeks old normal, untreated and treated *db/db* mice. One-way ANOVA showed increased renal ACE2 protein expression in *db/db* diabetic mice compared to their age-matched non-diabetic mice. After the commencement of 10 weeks treatment, there was no difference in renal ACE2 expression in *db/db, db/db* + metformin, *db/db* + exercise and *db/db* + exercise + metformin groups. *p*<0.001 vs age-matched non-diabetic mice. Each bar represents mean ± SEM of group size (n=10).
**Figure 34:** Renal ACE2 activity in 17 weeks old normal, untreated and treated db/db mice. One-way ANOVA showed a significant increase in the renal ACE2 activity of db/db mice compared to their age-matched non-diabetic mice. After the commencement of 10 weeks treatment, there was no difference in the renal ACE2 activity of db/db, db/db + metformin, db/db + exercise and db/db + exercise + metformin groups. *p<0.0001 vs age-matched normal mice. Each bar represents mean ± SEM of group size (n=10).
Figure 35: Correlation between plasma glucagon and urinary ACE2 excretion in 17 weeks old normal, untreated and treated db/db mice. Pearson correlation factor analysis showed a significant positive correlation between plasma glucagon levels and urinary ACE2 excretion. \( r = 0.66, \) \( p < 0.001 \). Each bar represents mean ± SEM of group size (n=10).
**Figure 36:** Correlation between plasma triglycerides and urinary ACE2 excretion in 17 weeks old normal, untreated and treated *db/db* mice. Pearson correlation factor analysis showed a significant positive correlation between plasma triglyceride levels and urinary ACE2 excretion. \( r=0.75, *p<0.001 \). Each bar represents mean ± SEM of group size (n=10).

![Plasma triglycerides vs. urinary ACE2 excretion](image)

**Figure 37:** Correlation between plasma insulin and urinary ACE2 excretion in 17 weeks old normal, untreated and treated *db/db* mice. Pearson correlation factor analysis showed a significant negative correlation between plasma insulin levels and urinary ACE2 excretion. \( r = -0.54, *p<0.05 \). Each bar represents mean ± SEM of group size (n=10).
**Figure 38:** Urinary albumin excretion in 17 weeks old normal untreated, normal + metformin and normal + exercise mice. One-way ANOVA showed those 10 weeks after the commencement of treatment, there is a significant increase in urinary albumin excretion of normal + exercise mice compared to their age-matched untreated normal mice. However, treatment with metformin for 10 weeks had no effect on urinary albumin levels. $p<0.0001$ vs age-matched normal mice. Each bar represents mean ± SEM of group size (n=10).
**Figure 39:** Urinary ACE2 excretion in 17 weeks old normal untreated, normal + metformin and normal + exercise groups. One-way ANOVA showed no difference in urinary ACE2 excretion of untreated normal mice, normal mice treated with metformin and normal mice trained with exercise. Each bar represents mean ± SEM of group size (n=10).
Figure 40: Representative PAS stained photomicrographs from A) Normal, B) db/db, C) db/db + metformin, D) db/db + exercise, E) db/db + exercise + metformin mice are shown along with graphs displaying the magnitude of mesangial expansion. Original magnification: x200. There was a significant increase in the mesangial matrix and glomerular surface areas of db/db diabetic mice compared to their age-matched non-diabetic mice. 10 weeks exercise training alone and in combination with metformin attenuated mesangial matrix expansion and glomerular surface area of treated db/db mice compared to untreated db/db mice. In contrast, 10 week treatment with metformin had no effect on the relative mesangial matrix and glomerular surface areas of db/db mice. *p<0.0001 vs age-matched normal mice, #p<0.0001, §p<0.0001 vs control db/db mice. Each bar represents mean ± SEM of group size (n=no of glomeruli=20).
**Figure 41:** Representative Picro-sirius stained photomicrographs from A) Normal, B) db/db, C) db/db + metformin, D) db/db + exercise, E) db/db + exercise + metformin mice are shown along with graphs displaying the magnitude of collagen deposits. Original magnification: x200.

Collagen deposition was significantly increased in db/db diabetic mice compared to their age-matched non-diabetic mice. 10 week training with physical exercise alone and in combination with metformin attenuated collagen deposition of treated db/db mice compared to untreated db/db mice. In contrast, 10 week treatment with metformin had no effect on collagen deposits of db/db mice. *p<0.0001 vs age-matched normal mice, #p<0.0001, $p<0.0001 vs control db/db mice. Each bar represents mean ± SEM of group size.
**Figure 42:** Representative photomicrographs from A) Normal, B) db/db, C) db/db + metformin, D) db/db + exercise, E) db/db + exercise + metformin mice are shown along with graphs displaying the magnitude of intensity of staining for ADAM17 protein. Original magnification: x200. ADAM17 immunostaining was significantly increased in 17 week db/db mice compared to their age-matched non-diabetic mice. After the commencement of 10 weeks treatment, staining was significantly decreased in db/db + exercise and db/db + exercise + metformin groups compared to untreated db/db and db/db + metformin mice. Metformin had no effect on renal ADAM17 protein expression in db/db mice compared to untreated db/db group. *p<0.0001 vs age-matched normal mice, #p<0.0001, $p<0.0001$ vs control db/db mice. Each bar represents mean ± SEM of group size.
Figure 43: Representative photomicrographs from A) Normal, B) db/db, C) db/db + metformin, D) db/db + exercise, E) db/db + exercise + metformin mice are shown along with graphs displaying the magnitude of intensity of staining for ACE2 protein. Original magnification: x200. Expression patterns of ACE2 protein was significantly decreased in glomeruli and increased in tubules of db/db mice compared to normal mice. Glomerular ACE2 protein was significantly increased in 17 wks old db/db mice subjected to exercise training with or without metformin. However, treatment with metformin alone had no effect on glomerular ACE2 protein expression compared to control db/db mice. Alternatively, no difference was seen in the tubular ACE2 protein expression of control db/db mice and db/db mice subjected to exercise training with or without metformin. *p<0.0001 vs age-matched normal mice, #p<0.0001, $p<0.0001 vs control db/db mice. Each bar represents mean ± SEM of group size.
**Figure 44:** Double-immunofluorescence staining of ACE2 (green; left) and ADAM17 (red; middle) in cortical tubules of *db/db* mice. Original magnification: x200. Merge of both images (yellow; right) demonstrates co-localization of ACE2 and ADAM17 in the tubular cortex of diabetic *db/db* mice.
4. RESULTS

4.1. General physiologic, metabolic and body composition parameters

To examine the age-dependent changes in db/db mice, the following parameters were monitored weekly.

a) Blood glucose: At 6 weeks of age db/db mice exhibited significantly higher levels of blood glucose compared to normal mice. Indeed, there was a progressive increase in blood glucose levels of db/db mice over time (Figure 1, *p<0.01, unpaired-t test). Moreover, db/db mice had impaired glucose tolerance in an intra-peritoneal glucose tolerance test (Figure 24, *p<0.001). However, these differences were not seen in normal mice during the study period.

b) Body weight: At 6 weeks, db/db mice weighed significantly more than the normal mice (Table 1, *p<0.001). In fact, there was a consistent upregulation in the body weight of db/db mice over time and with age when compared to normal mice (Table 1&2, *p<0.01, unpaired-t test).

c) Food intake: At 6 weeks, db/db mice demonstrated a significant increase in the food intake compared to normal mice (Table 1, *p<0.001). Similarly, food intake of db/db mice was significantly more at 9 weeks and 17 weeks (Table 1&2, *p<0.01, unpaired-t test). However, no difference was observed in the food intake of normal mice over time.

d) Water intake: At 6 weeks, db/db mice demonstrated significantly higher levels of water intake compared to normal mice (Table 1, *p<0.001). Similarly, water intake of db/db mice increased significantly at 9 weeks and 17 weeks (Table 1&2, *p<0.001, unpaired-t test). However, no difference was observed in the water intake of normal mice over time.

e) Urine output per day: At 6 weeks, db/db mice excreted significantly higher volumes of urine compared to age-matched normal mice (Table 1, *p<0.001, unpaired-t test). In fact, there was a
consistent increase in 24-hour urine volumes of db/db mice over time (Table 1&2, *p<0.001). However, no difference was observed in the urine output of normal mice over time.

f) **Absolute body fat:** At 6 weeks, absolute body fat of db/db mice was significantly high compared to normal mice (Table 1, *p<0.001, unpaired-t test). Moreover, there was a consistent increase in the absolute body fat of db/db mice over time (Table 1&2, *p<0.001).

g) **Absolute lean mass:** db/db mice exhibited a significantly lower content of absolute lean mass at 6 weeks, 9 weeks and 17 weeks compared to their age-matched normal mice (Table 1&2, *p<0.001, unpaired t-test).

h) **Total body water:** db/db mice exhibited a significantly lower content of total body water compared to normal mice in the juvenile stages (6 weeks) (Table 1, *p<0.001). Decrease in the total body water content was also seen in 9 and 17 week old db/db mice (Table 1&2, *p<0.001).

4.2. **Plasma hormone and lipid parameters**

To assess the effect of hyperglycemia on hormonal changes in type 2 diabetes, following parameters were measured.

a) **Plasma insulin:** One of the important characteristics of db/db mice is hyperinsulinemia. Plasma insulin levels were significantly elevated in 17 week old db/db mice compared to age-matched normal mice (Table 3, *p<0.0001).

b) **Plasma leptin:** There was a significant increase in the plasma leptin levels of 17 week old db/db mice compared to normal mice (Table 3, *p<0.001).

c) **Plasma glucagon:** Glucagon levels were significantly elevated in the plasma of 17 week old db/db mice compared to normal mice (Table 3, *p<0.0001).

d) **Plasma adiponectin:** Levels of plasma adiponectin was significantly decreased in 17 week old db/db mice compared to age-matched normal mice (Table 3, *p<0.0001).
e) **Plasma triglycerides**: Plasma triglyceride levels were significantly elevated in 17 week old db/db mice compared to their age-matched normal mice (Table 3, *p*<0.0001).

f) **Plasma cholesterol**: At 17 weeks, there was no difference in the plasma cholesterol levels of db/db mice compared to age-matched normal mice (Table 3).

### 4.3. Assessment of renal function

To investigate the effect of hyperglycemia on diabetic nephropathy, urinary albumin and total protein excretions were measured in 24-hour urine samples.

a) **Urinary albumin**: There was a significant increase in the urinary albumin excretion of 6 week old db/db mice compared to normal mice (Figure 10, *p*<0.001). With the progression of age, urinary albumin levels were up-regulated consistently in db/db mice compared to their age-matched normal mice. Indeed, there was an age-dependent increase in albumin excretion of db/db diabetic mice (Figure 17&25, *p*<0.0001).

b) **Urinary total protein**: We observed no differences in the total protein excretion of diabetic and non-diabetic mice at juvenile stages (6 weeks) (Figure 11). However, with the progression of age there was a significant increase in the urinary total protein excretion of db/db mice compared to their age-matched normal mice. In addition, there was an age-dependent increase in total proteinuria of db/db mice (Figure 18&26, *p*<0.0001).

### 4.4. Enzyme activities measurement

To evaluate the effect of hyperglycemia on ACE2 activity, 24-hour urine samples and whole kidney lysates were analyzed using a fluorogenic substrate.

a) **Urinary ACE2 activity**: To demonstrate the activity of ACE2 in urine, 24-hour urinary samples were incubated with Mca-APK (Dnp) fluorogenic substrate. In fact, there was a significant increase in the urinary ACE2 activity of 6 week old db/db mice compared to age-
matched normal mice (Figure 12, *p<0.01). Similar trend was observed in the urine samples from 9 week and 17 week old mice (Figure 19&27, *p<0.01).

b) **Renal ACE2 activity:** To investigate the protective role of ACE2 in diabetic nephropathy, renal ACE2 activity was measured in the whole kidney lysate using Mca-APK (Dnp) fluorogenic substrate. There was a significant increase in renal ACE2 activity of 17 week old \( db/db \) mice compared to age-matched normal mice (Figure 34, *p<0.0001).

### 4.5. Protein expression of urinary ACE2, renal ADAM17, Timp3 and ACE2

To investigate the effect of hyperglycemia on shedding of renal ACE2, whole kidney lysates from 17 week old \( db/db \) and normal mice were analyzed using immunoblot analyses.

a) **Urinary ACE2 protein expression:** Urinary ACE2 protein expression was significantly increased in 6 week, 9 week and 17 week old \( db/db \) mice compared to their age-matched normal mice as confirmed from the western blot analyses (Figure 13,20&28, *p<0.05).

b) **Renal ADAM17 protein expression:** ADAM17 protein content was significantly upregulated in 17 week old \( db/db \) mice compared to age-matched normal mice as confirmed from the western blot results (Figure 31, *p<0.001).

c) **Renal Timp3 protein expression:** No difference was observed in renal Timp3 protein levels of 17 week old diabetic and non-diabetic mice (Figure 32).

d) **Renal ACE2 protein expression:** Immunoblot analyses demonstrated that ACE2 protein expression was significantly increased in the whole kidney lysate of 17 week old \( db/db \) mice compared to their age-matched normal mice (Figure 33, *p<0.001).
4.6. Histopathology

To investigate the distribution patterns of ADAM17 and ACE2 in the kidney, immunostaining analyses were performed on the kidney sections obtained from perfused mice. In addition, to evaluate the renal pathologies, PAS and Picro-sirius red stained kidney sections were analyzed.

a) Immunostaining for renal ADAM17: In concordance with immunoblot analyses, staining for ADAM17 was significantly high in the kidney of 17 week old db/db mice compared to age-matched normal mice (Figure 42, *p<0.0001).

b) Immunostaining for renal ACE2: Immunostaining result showed a significantly increased and decreased staining for ACE2 in the tubular and glomerular kidney of 17 week old db/db mice compared to age-matched normal mice respectively (Figure 43, *p<0.0001).

c) PAS staining: PAS stained kidney sections from 17 week old db/db mice demonstrated a significantly increased glomerular surface area and expanded mesangial matrix compared to age-matched normal mice (Figure 40, *p<0.0001).

d) Picro-sirius red staining: Collagen deposition was significantly increased in the kidney of 17 week old db/db mice compared to age-matched normal mice (Figure 41, *p<0.0001).

4.7. Correlation of urinary ACE2 with blood glucose and albuminuria

To investigate the potentiality of urinary ACE2 as a risk marker in diabetes, it was correlated with the following parameters.

a) With blood glucose: To identify statistically significant relationship between urinary ACE2 and blood glucose, Pearson correlations were calculated between these variables in normal and four different groups of diabetic db/db mice when they were 6 week old. There was a significant and positive correlation between urinary ACE2 and the blood glucose levels (Figure 14, r = 0.60, *p<0.05).
b) **With albuminuria**: To identify statistically significant relationship between urinary ACE2 and albumin excretion, Pearson correlations were calculated between these variables in normal and four different groups of diabetic *db/db* mice when they were 6 week old. There was a significant and positive correlation between urinary ACE2 and albuminuria (Figure 15, $r = 0.65$, $p<0.05$).

### 4.8. Short term intervention with exercise, metformin and combination

To evaluate the short term effects of lowering hyperglycemia on type 2 diabetes related metabolic parameters, juvenile (7 week) *db/db* mice were subjected to exercise training and/or metformin treatment for 2 weeks.

a) **Effect of exercise, metformin and combination on blood glucose**: Short term intervention with exercise and/or metformin significantly lowered blood glucose levels in *db/db* mice compared to untreated *db/db* mice (Figure 16, $@p<0.0001$, $#p<0.0001$, $^wp<0.0001$). Blood glucose lowering effect was seen as early as 1 week after the initiation of treatment. However, normal mice subjected to exercise and/or metformin intervention did not show a difference in their blood glucose levels compared to control normal mice.

b) **Effect of exercise, metformin and combination on body weights**: Neither exercise trained nor metformin treated *db/db* mice exhibited difference in their body weights compared to untreated *db/db* mice (Table 1).

c) **Effect of exercise, metformin and combination on food intake**: Exercise training alone and in combination with metformin for 2 weeks attenuated food intake of *db/db* mice (Table 1, $^wp<0.05$, $^wp<0.01$). However, administration of metformin for 2 weeks had no effect compared to control *db/db* mice. Similarly, no difference was observed in the food intake of untreated and treated normal mice.
d) **Effect of exercise, metformin and combination on water intake:** Exercise training alone and in combination with metformin for 2 weeks attenuated water intake of db/db mice (Table 1, \(^{#p<0.05,}^{\$p<0.05}\)). However, administration of metformin for 2 weeks had no effect compared to control db/db mice. No difference was observed in water intake of untreated and treated normal mice.

e) **Effect of exercise, metformin and combination on urine output:** Exercise training alone and in combination with metformin for 2 weeks attenuated 24-hour urinary output of db/db mice (Table 1, \(^{#p<0.001,}^{\$p<0.001}\)). However, administration of metformin to db/db mice for 2 weeks had no effect compared to control db/db mice. Similarly, no difference was observed in the urinary output of untreated and treated normal mice.

f) **Effect of exercise, metformin and combination on absolute body fat:** Short term intervention with exercise and/or metformin for 2 weeks had no effect on the absolute body fat of db/db mice as well as normal mice (Table 1).

g) **Effect of exercise, metformin and combination on absolute lean mass:** No differences were noticed in the absolute lean mass of db/db mice and normal mice that are subjected to exercise training and/or metformin treatment for 2 weeks (Table 1).

h) **Effect of exercise, metformin and combination on total body water:** Intervention with exercise and/or metformin for 2 weeks had no effect on the total body water content of db/db mice as well as normal mice (Table 1).

4.9. **Effect of exercise, metformin and combination on renal function**

To investigate the effect of lowering hyperglycemia by exercise and/or metformin on the renal function, following parameters were measured.
a) **Urinary albumin**: Physical exercise for 2 weeks significantly attenuated urinary albumin excretion of trained *db/db* mice compared to control *db/db* mice. Indeed, short term treatment with metformin improved albuminuria of *db/db* mice (Figure 17, $p<0.0001$).

b) **Urinary total protein excretion**: Physical exercise for 2 weeks significantly attenuated urinary total protein excretion of trained *db/db* mice compared to control *db/db* mice (Figure 18, $p<0.001$). However, administration of metformin for 2 weeks had no effect on the urinary total protein excretion of in *db/db* mice compared to untreated *db/db* mice (Figure 18).

**4.10. Effect of exercise, metformin and combination on urinary ACE2 activity**

a) **Urinary ACE2 activity**: Diabetic *db/db* mice subjected to exercise training and/or metformin treatment for 2 weeks demonstrated a significant decrease in their urinary ACE2 excretion compared to untreated *db/db* mice (Figure 19, $p<0.0001$).

**4.11. Effect of exercise, metformin and combination on urinary ACE2 protein expression**

a) **Urinary ACE2 protein expression**: Urinary ACE2 protein expression was significantly attenuated in 9 week old *db/db* mice subjected to exercise training and/or metformin treatment for 2 weeks compared to untreated *db/db* mice (Figure 20, $p<0.0001$).

**4.12. Correlation of urinary ACE2**

To investigate the potentiality of urinary ACE2 as a risk marker in type 2 diabetes, it was correlated with the following parameters from 9 week old control normal, control *db/db* and *db/db* mice subjected to exercise and/or metformin for 2 weeks.

a) **With blood glucose**: To identify statistically significant relationship between urinary ACE2 and blood glucose, Pearson correlations were calculated between these variables in the total
groups of 9 week old mice after 2 weeks intervention with exercise and/or metformin. Urinary ACE2 was significantly and positively correlated with the blood glucose levels (Figure 21, r = 0.73, p<0.001).

b) With albuminuria: To identify statistically significant relationship between urinary ACE2 and albumin excretion, Pearson correlations were calculated between these variables in the total groups of 9 week old mice after 2 weeks intervention with exercise and/or metformin. Urinary ACE2 was significantly and positively correlated with albuminuria (Figure 22, r = 0.65, p<0.001).

4.13. Long term intervention with exercise, metformin and combination

To evaluate the long term effect of lowering hyperglycemia on type 2 diabetes related metabolic and renal parameters, exercise training and/or metformin treatment was continued for 10 weeks.

a) Effect of exercise, metformin and combination on blood glucose: Chronic intervention with physical exercise with or without metformin significantly lowered blood glucose levels of intervened db/db mice compared to control db/db mice (Figure 23, #p<0.0001, $p<0.0001). In fact, blood glucose lowering effect of exercise was consistent during the study period. In contrast, chronic treatment with metformin for 10 weeks had no effect on blood glucose levels of treated db/db mice compared to untreated db/db mice (Figure 23). However, normal mice subjected to exercise and/or metformin intervention did not show a difference in their blood glucose levels compared to untreated normal mice. In addition, physical exercise training with or without metformin ameliorated glucose utilization in intervened db/db mice compared to untreated diabetic mice (Figure 24, #p<0.0001, $p<0.0001). Alternatively, metformin treatment had no effect (Figure 24).
b) *Effect of exercise, metformin and combination on body weights*: Intervention with exercise and/or metformin for 10 weeks had no effect on the body weights of intervened *db/db* mice compared to untreated *db/db* mice. Similarly, no difference was seen in the body weights of intervened and control normal mice (Table 2).

c) *Effect of exercise, metformin and combination on food intake*: Exercise training with or without metformin for 10 weeks attenuated food intake of intervened *db/db* mice compared to untreated *db/db* mice (Table 2, \(^{#}p<0.01, \^{\$}p<0.01\)). On the otherhand, metformin treatment of *db/db* mice for 10 weeks had no effect. Similarly, no difference was seen in the food intake of exercise trained and metformin treated normal mice compared to control normal mice.

d) *Effect of exercise, metformin and combination on water intake*: Exercise training alone and in combination with metformin for 10 weeks attenuated water intake of *db/db* mice (Table 2, \(^{#}p<0.01, \^{\$}p<0.01\)). However, administration of metformin for 10 weeks had no effect compared to untreated *db/db* mice. Similarly, no difference was seen water intake of exercise trained and metformin treated normal mice compared to control normal mice.

e) *Effect of exercise, metformin and combination on urine output*: Chronic intervention with exercise alone and in combination with metformin attenuated 24-hour urinary output of *db/db* mice (Table 2, \(^{#}p<0.001, \^{\$}p<0.001\)). Treatment of *db/db* mice with metformin for 10 weeks had no effect on 24-hour urinary volumes compared to untreated *db/db* mice. Similarly, no difference was seen in the urinary output of exercise trained, metformin treated and control normal mice.

f) *Effect of exercise, metformin and combination on absolute body fat*: Chronic intervention with exercise and/or metformin for 10 weeks had no effect on the absolute body fat of *db/db* mice as well as normal mice (Table 2).
g) **Effect of exercise, metformin and combination on absolute lean mass:** No differences were seen in the absolute lean mass of *db/db* mice and normal mice that are subjected to exercise training and/or metformin treatment for 10 weeks compared to their respective controls (Table 2).

h) **Effect of exercise, metformin and combination on total body water:** Intervention with exercise and/or metformin for 10 weeks had no effect on the total body water content of *db/db* mice as well as normal mice (Table 2).

### 4.14. Effect of exercise, metformin and combination on plasma hormone parameters:

To examine the effect of lowering hyperglycemia on type 2 diabetes related plasma hormonal and lipid changes, measurements of the following were carried out using plasma from untreated and treated *db/db* mice.

a) **Plasma insulin levels:** Chronic intervention with exercise training alone and in combination with metformin for 10 weeks, significantly increased plasma insulin levels of *db/db* mice compared to untreated *db/db* mice (Table 3, $^#p<0.0001$, $^p<0.0001$). However, administration of metformin for 10 weeks had no effect on plasma insulin levels of treated *db/db* mice compared to untreated *db/db* mice (Table 3).

b) **Plasma leptin levels:** Physical exercise for 10 weeks significantly increased the plasma leptin levels of trained *db/db* mice compared to control *db/db* mice (Table 3, $^#p<0.05$). Interestingly, administration of metformin prevented this effect of exercise in *db/db* mice. On the other hand, treatment with metformin alone had no effect compared to untreated *db/db* mice.

c) **Plasma adiponectin levels:** Chronic intervention with exercise and/or metformin had no effect on the plasma adiponectin levels in intervened *db/db* mice compared to untreated *db/db* mice (Table 3).
d) **Plasma triglyceride levels**: Physical exercise with or without metformin significantly reduced plasma triglyceride levels of trained *db/db* mice compared to control *db/db* mice (Table 3, \(^\#p<0.0001, ^\$p<0.0001\)). Metformin treatment significantly increased plasma triglyceride levels of treated *db/db* mice compared to untreated *db/db* mice (Table 3, \(^\wp<0.05\)).

e) **Plasma glucagon levels**: Neither exercise training nor metformin treatment improved plasma glucagon levels of *db/db* mice individually. However, combination of exercise and metformin significantly reduced plasma glucagon levels of intervened *db/db* mice compared to untreated *db/db* mice (Table 3, \(^\Sp<0.0001\)).

f) **Plasma cholesterol levels**: No differences were seen in the plasma cholesterol levels of exercise trained, metformin treated and untreated *db/db* (Table 3).

### 4.15. Effect of exercise, metformin and combination on renal function

To investigate the chronic effect of lowering hyperglycemia by exercise and/or metformin on the renal function, following parameters were measured.

a) **Urinary albumin**: Physical exercise training with or without metformin for 10 weeks significantly attenuated urinary albumin excretion in intervened *db/db* mice compared to untreated *db/db* mice (Figure 25, \(^\#p<0.0001, ^\$p<0.0001\)). However, there was a significant increase in the urinary albumin levels of normal mice subjected to exercise training compared to control normal mice (Figure 38, \(^\#p<0.0001\)). On the otherhand, administration of metformin for 10 weeks had no effect on the urinary albumin levels of treated and untreated diabetic and non-diabetic mice (Figure 24).

b) **Urinary total protein excretion**: Physical exercise training with or without metformin for 10 weeks significantly attenuated urinary total protein excretion of intervened *db/db* mice compared
to untreated db/db mice (Figure 26, \(^p<0.0001\), \(^p<0.0001\)). However, metformin treatment for 10 weeks had no effect on the total protein excretion of treated db/db mice compared to untreated db/db mice (Figure 26). On the other hand, no difference was seen in the urinary total protein levels of intervened and control normal mice.

4.16. Effect of exercise, metformin and combination on enzyme activities

a) **Urinary ACE2 activity:** Diabetic db/db mice subjected to exercise training with or without metformin for 10 weeks demonstrated a significant decrease in their urinary ACE2 activity compared to untreated db/db mice (Figure 27, \(^p<0.0001\), \(^p<0.0001\)). In contrast, administration of metformin for 10 weeks had no effect on the urinary ACE2 activity of 17 week old treated db/db mice compared to untreated db/db mice (Figure 26). Similarly, no difference was seen in the urinary ACE2 excretion of exercise trained, metformin treated and control normal mice (Figure 39).

b) **Renal ACE2 activity:** No differences were seen in the renal ACE2 activity of 17 week old control db/db mice and age-matched db/db mice intervened with exercise and/or metformin for 10 weeks (Figure 34). Similarly, no difference was seen in the renal ACE2 activity of exercise trained, metformin treated and control normal mice.

4.17. Effect of exercise, metformin and combination on protein expression of Urinary ACE2, renal ADAM17, Timp3 and ACE2

To investigate the effect of lowering hyperglycemia on shedding of renal ACE2, 24-hour urine sample and whole kidney lysates from 17 week old db/db mice subjected to exercise and/or metformin were analyzed using immunoblot analyses.
a) **Urinary ACE2 protein expression:** Physical exercise training with or without metformin for 10 weeks significantly attenuated urinary ACE2 protein excretion in 17 week old trained db/db mice compared to untreated db/db mice (Figure 28, $^p<0.01$, $^\$p<0.01$). In contrast, chronic treatment with metformin had no effect on urinary ACE2 protein levels of treated db/db mice compared to untreated db/db mice (Figure 28).

b) **Renal ADAM17 protein expression:** Physical exercise training with or without metformin for 10 weeks significantly reduced ADAM17 protein expression in the kidney of 17 week old db/db mice compared to their age-matched untreated db/db mice (Figure 31, $^p<0.01$, $^\$p<0.01$). Alternatively, treatment with metformin for 10 weeks had no effect on the renal ADAM17 protein content of treated db/db mice compared to untreated db/db mice (Figure 31).

c) **Renal Timp3 protein expression:** No difference was seen in renal Timp3 protein levels of 17 weeks old control db/db mice and db/db mice subjected to exercise training, metformin treatment and exercise plus metformin for 10 weeks (Figure 32).

d) **Renal ACE2 protein expression:** Immunoblot analyses demonstrated no differences in renal ACE2 protein expression of 17 week old db/db mice subjected to exercise and/or metformin compared to untreated db/db mice (Figure 33).

4.18. Histopathology

To investigate the effect of lowering hyperglycemia on the distribution pattern of ADAM17 and ACE2 proteins in the kidney, immunostaining analyses were performed on the kidney sections obtained from perfused mice that are subjected to exercise and/or metformin for 10 weeks. In addition, to evaluate the effect of lowering hyperglycemia on the renal pathologies, PAS and Picro-sirius red stained kidney sections were analyzed.
a) **Immunostaining for renal ADAM17:** In concordance with the western blot analyses, staining for ADAM17 was significantly reduced in exercise trained *db/db* mice compared to untreated *db/db* mice (Figure 42, \(^p<0.0001\), \(^p<0.0001\)). Metformin treatment had no effect on renal ADAM17 protein levels in *db/db* mice compared to untreated *db/db* mice (Figure 42).

b) **Immunostaining for renal ACE2:** Glomerular ACE2 protein was significantly increased in 17 weeks old *db/db* mice subjected to physical exercise training with or without metformin for 10 weeks (Figure 43, \(^p<0.0001\), \(^p<0.0001\)). However, treatment with metformin alone had no effect on glomerular ACE2 protein expression compared to untreated *db/db* mice. No difference was seen in the tubular ACE2 protein expression of untreated *db/db* mice and *db/db* mice subjected to exercise and/or metformin for 10 weeks (Figure 43).

c) **PAS staining:** Chronic intervention with exercise training alone and in combination with metformin reduced glomerular surface area significantly and attenuated mesangial expansion in *db/db* mice (Figure 40, \(^p<0.0001\), \(^p<0.0001\)). This effect was not seen in metformin treated *db/db* mice compared to untreated *db/db* mice (Figure 40).

d) **Picro-sirius red staining:** Physical exercise training alone and in combination with metformin for 10 weeks was associated with significantly reduced collagen deposits in *db/db* mice compared to untreated *db/db* mice (Figure 41, \(^p<0.0001\), \(^p<0.0001\)). This effect was not seen in metformin treated *db/db* mice compared to untreated *db/db* mice (Figure 41).

### 4.19. Correlation of urinary ACE2

To investigate the potentiality of urinary ACE2 as a risk marker in type 2 diabetes, it was correlated with the results obtained from 17 week old control normal, *db/db* and *db/db* mice subjected to exercise and/or metformin for 10 weeks.
a) *With blood glucose:* To identify statistically significant relationship between urinary ACE2 and blood glucose, Pearson correlations were calculated between these variables in the total groups of 17 week old mice after 10 weeks intervention with exercise and/or metformin. Urinary ACE2 was significantly and positively correlated with the blood glucose levels (Figure 29, $r = 0.75, p<0.0001$).

b) *With albuminuria:* To identify statistically significant relationship between urinary ACE2 and albumin excretion, Pearson correlations were calculated between these variables in the total groups of 17 week old mice after 10 weeks intervention with exercise and/or metformin. Urinary ACE2 was significantly and positively correlated with albuminuria (Figure 30, $r = 0.84, p<0.0001$).

c) *With plasma glucagon:* To identify statistically significant relationship between urinary ACE2 and plasma glucagon levels, Pearson correlations were calculated between these variables in the total groups of 17 week old mice after 10 weeks intervention with exercise and/or metformin. Urinary ACE2 was significantly and positively correlated with plasma glucagon (Figure 35, $r = 0.66, p<0.001$).

d) *With plasma triglycerides:* To identify statistically significant relationship between urinary ACE2 and plasma triglyceride levels, Pearson correlations were calculated between these variables in the total groups of 17 week old mice after 10 weeks intervention with exercise and/or metformin. Urinary ACE2 was significantly and positively correlated with plasma triglyceride levels (Figure 36, $r = 0.75, p<0.001$).

e) *With plasma insulin:* To identify statistically significant relationship between urinary ACE2 and plasma insulin levels, Pearson correlations were calculated between these variables in the total groups of 17 week old mice after 10 weeks intervention with exercise and/or metformin.
Urinary ACE2 was significantly and negatively correlated with plasma insulin levels (Figure 37, $r = -0.54, p<0.05$).
5. DISCUSSION

In our previous study, we reported that therapeutic intervention with the insulin sensitizer rosiglitazone, normalized hyperglycemia and improved renal damage by attenuating ACE2 shedding. However, due to its potential side effects, this drug is no longer available in the market. Hence, in the current study, we investigated the effects of physical exercise training and/or metformin on glucose homeostasis and associated renal alterations in \(db/db\) type 2 diabetic mice. Though several studies have shown the beneficiary effects of exercise with or without diet restriction on the primary disease diabetes (Knowler et al., 2002; Tuomilehto et al., 2001; Pan et al., 1997; Fradkin et al., 2012), effects of these interventions on the complications associated with diabetes have not been extensively investigated. In addition, mechanisms underlying the positive effects of exercise on glucose homeostasis remain poorly understood. Both physical exercise and metformin are first-line interventions for the management of type 2 diabetes. We thus investigated the effects of exercise training and/or metformin treatment on glucose homeostasis, albuminuria, renal ADAM17, ACE2 shedding, and renal pathology. In addition, we also focused on the effects of exercise and/or metformin on some of the plasma risk factors in diabetes.

In our previous study using \(db/db\) mice, we showed that hyperglycemia is evident before the onset of hypertension and plays a pivotal role in triggering adverse effects (Senador et al., 2009), thus we speculate that lowering hyperglycemia is crucial in preventing long-term complications that might lead to end organ damage. In the current study, 6 week old \(db/db\) mice had significantly increased blood glucose levels compared to normal mice. At this early stage, \(db/db\) mice excreted higher levels of albumin and ACE2 in the urine, but no differences were observed in their total urinary protein excretion levels. Albuminuria is a widely accepted and well-
established marker for diabetic nephropathy, which is considered as one of the major microvascular complications of diabetes that eventually manifests into ESRD (Jim et al., 2012). High blood glucose associated glomerular hyperfiltration and activated RAS are considered as primary causes underlying this implication (Kobori et al., 2007). Increased renal Ang II has been shown to cause depletion of glomerular nephrin, thus increasing the glomerular pore size (Giacchetti et al., 2005; Bichu et al., 2009). In addition, Ang II can enhance intraglomerular pressure by constricting both afferent and efferent arteriole, thus exhilarating albumin excretion into urine (Remuzzi & Bertani, 1998). However, onset of diabetes is difficult to ascertain and so many patients diagnosed with high blood glucose already have microalbuminuria (Lee, 2005). Moreover, recent clinical studies questioned the reliability of albuminuria in anticipating the progression and prevention of ESRD (de Galan et al., 2009). Alternatively, normal mice subjected to exercise training excreted significantly higher levels of urinary albumin with no differences in their blood glucose and renal morphologies. Therefore, there is a need for a more sensitive and specific marker for the better prediction of diabetic nephropathy.

Increased ACE2 activity and protein in the urine of db/db diabetic mice is in close resemblance with diabetic patients (Xiao et al., 2012; Mizuiri et al., 2011), suggesting the syndrome of diabetic nephropathy in db/db mouse model reflects human diabetics with nephropathy. It has been suggested before that the origin of urinary ACE2 protein could be at least partly from the plasma or derived from the kidney (Lew et al., 2006), the organ where ACE2 is predominantly localized (Donoghue et al., 2000). However, as we reported earlier, there is no detectable ACE2 activity in the plasma of normal non-diabetic and db/db mice (Chodavarapu et al., 2013); therefore we believe in the kidney as the source of urinary ACE2 in db/db mice, which is in agreement with recent findings in diabetic patients (Xiao et al., 2012). In fact, our results
demonstrated a strong association between urinary albumin and ACE2 suggesting ACE2 as a non-invasive biomarker for diabetic nephropathy. This observation was recently evaluated in type 2 diabetic human subjects (Park et al., 2013). It is presumed that albuminuria is an indication of glomerular damage (Ye et al., 2006) and urinary ACE2 is a reflection of tubular damage (Chodavarapu et al., 2013). Even though mechanisms responsible for urinary albumin and ACE2 are quite contrasting, we correlated urinary ACE2 with albuminuria to explore its potential use as a new, additional marker of renal injury.

Physical exercise with or without metformin significantly lowered the blood glucose levels consistently and improved glucose tolerance of db/db mice. Blood glucose lowering effect of exercise was seen as early as 1 week after the initiation of treatment. In comparison, metformin treatment was effective lowering hyperglycemia in the early stages of diabetes, but failed with the progression of disease severity in late stages. Exercise mediated improvements in glucose homeostasis could at least be partially attributed to the elevated plasma insulin levels. Physical exercise training increased plasma insulin levels by almost three folds in db/db mice. Although type 2 diabetes is characterized by hyperinsulinemia, enhanced insulin levels may not be sufficient to overcome the tissue resistance to insulin. Based on our findings, we speculate that, further increase in the plasma insulin levels during exercise training could be able to counteract the resistance exhibited by various tissues and it could be one of the mechanisms behind improved glycemic control in exercised db/db mice. However, in contrast to our observation, a study conducted on type 2 diabetic subjects reported decreased blood glucose and plasma insulin levels with exercise (Musi et al., 2001), which could be explained by increased insulin sensitivity. Insulin sensitivity can be enhanced by changing the body composition (Yki-Jarvinen & Koivisto, 1983), stimulating muscle blood flow (Yki-Jarvinen & Koivisto, 1983) or GLUT-4
protein levels (Rodnick et al., 1990; Yki-Jarvinen & Koivisto, 1983). We noticed no differences in the body composition between exercised and sedentary diabetic and non-diabetic mice. However, we cannot rule out the possibility of enhanced insulin mediated GLUT-4 translocation from an intracellular pool to the plasma membrane of muscle and AMPK α2 activation in exercised diabetic mice (Scarpello & Howlett, 2008; Musi et al., 2002; Ivy, 1997; Musi et al., 2001; Hughes et al., 1993). These improvements in exercised db/db mice were not associated with changes in body weight and composition. Since there are only very few studies that observed beneficial effects of physical activity alone without diet modifications (to lose weight) in diabetes, findings from our study highlight the notion that physical exercise alone can be effective in managing diabetes type 2. This, in turn, is supported by a randomized clinical trial conducted on individuals with impaired glucose tolerance (Pan et al., 1997). Physical exercise alone and in combination with metformin was consistently effective in attenuating albuminuria and proteinuria, reflecting its positive effects against diabetic nephropathy. This observation is in agreement with the previous reports (Kohzuki et al., 2001; Tufescu et al., 2008; Ishikawa et al., 2012). However, exercise-induced albuminuria/proteinuria is often reported in normal as well as diabetic human subjects (Kornhauser et al., 2012; Koh et al., 2011; Heathcote et al., 2009), which could be attributed to the intensity and duration of exercise (Saeed et al., 2012). This is the reason why exercise should be well-controlled and some of the factors like maximal oxygen consumption (VO2), body composition, and sex should be considered while designing the exercise protocols. Indeed, exercise training attenuated ACE2 excretion in the urine of db/db mice. Since ACE2 is considered to be renoprotective, prevention of urinary ACE2 excretion, in addition to the improved albumin excretion rate, suggests the protective role of exercise in kidney damage. Improvements in albuminuria and ACE2 excretion were seen as early as 2
weeks after the initiation of treatment. To our knowledge, this is the first report showing that improved kidney function during exercise training is associated with a significant decrease in ACE2 excretion into urine.

Apart from its usage as a biomarker, a thorough urinalysis demonstrated that urinary ACE2 is enzymatically active and 20 kDa shorter. Since the kidney is the source of urinary ACE2; we speculated that 20 kDa shorter fragment of ACE2 in the urine of db/db mice is due to its ectodomain shedding from the kidney. ADAM17 protein that is implicated in the ectodomain shedding of ACE2 in vitro, was significantly increased in the kidney of db/db mice. In fact, our finding is in agreement with the recent report demonstrating increased renal ADAM17 protein expression in human subjects with renal disease (Melenhorst et al., 2009). Although it is reported that ADAM17 is predominantly localized to distal renal tubules (Lautrette et al., 2005), our results showed a strong staining in both proximal and distal cortical tubules and glomeruli but not in the medulla of db/db mice. This discrepancy may be due to differences in the species, age, or the severity of the disease. Further, immuno-double staining results demonstrated the co-localization of ADAM17 and ACE2 proteins in the tubular cortex under diabetic conditions. In fact, the increased renal ADAM17 protein expression in db/db mice was significantly decreased by exercise training. This is the first study reporting effect of physical exercise training on ADAM17, a metalloproteinase that is implicated in many chronic diseases (Kaneko et al., 2011; White, 2003).

In spite of some reports suggesting decreased ACE2 in diabetes (Reich et al., 2008; Tikellis et al., 2003), our previous studies showed a significant increase in renal ACE2 protein expression in db/db mice (Chodavarapu et al., 2013). In fact, plasma ACE activity and Ang II content were high in 8 week old db/db mice compared to normal mice (Senador et al., 2009) and this suggests
that the deleterious renal effects of Ang II is counterregulated by upregulating ACE2. As we reported earlier, renal ACE2 activity of 8 week old db/db mice is significantly higher than 31 week old db/db mice. Based on these observations, we speculate that, with progression of the disease (age), the kidney is unable to maintain ACE2 levels due to escalating ADAM17 protein. As a consequence of increased renal ADAM17 protein and shedding of ACE2, db/db mice had a significantly increased glomerular surface area, glomerular and tubular basement membrane thickening, expanded mesangial matrix and collagen deposits. Strong staining for collagen was noticed in the interstitium, mesangial cells, and glomerular as well as tubular basement membranes, suggesting the involvement of both glomerular and tubular damage in the renal injury of db/db mice. In agreement with previous reports (Ghosh et al., 2009), physical exercise training blunted mesangial expansion and glomerular surface area of db/db mice. Reduced glomerular and tubular basement membrane thickening and attenuation of glomerular and tubular collagen deposits by exercise suggests that the beneficiary effect of exercise in renal injury is accompanied with the improvements in both glomerular and tubular pathologies in db/db mice. Since ACE2 is considered as renoprotective, ADAM17-induced shedding of ACE2 is an important contributor to the pathogenesis of diabetic nephropathy. Basing on all these considerations, it is tempting to speculate that attenuation of ADAM17 by exercise training in the kidney is responsible for attenuating ACE2 shedding into urine of db/db diabetic mice and this could be considered as a renoprotective mechanism. In contrast, administration of metformin did not attenuate renal ADAM17 and ACE2 shedding and had no effect on the renal pathologies of diabetic mice. However, considering albuminuria and urinary ACE2 as risk markers of diabetic nephropathy, we might presume that metformin treatment could have beneficiary actions on diabetic renal pathologies during the initial stages. Unaltered levels in the expression and
activity of renal ACE2 between control, metformin-treated, and exercise-trained diabetic db/db mice needs further investigation.

Continuing in this vein, we focused on the possible mechanisms that could be responsible for the upregulation of renal ADAM17 protein levels in db/db mice. Previously, it has been shown that several cell signaling pathways excitation results in dimeric to monomeric shift of ADAM17. This is associated with increased ADAM17 and decreased Timp3, suggesting Timp3 as an endogenous inhibitor of ADAM17 (Xu et al., 2012). Also, recent studies reported that deficiency of Timp3 results in increased ADAM17 activity, exacerbating diabetic nephropathy in Akita type 1 diabetic mice (Basu et al., 2012). In contrast to these findings, we observed no differences in renal Timp3 protein levels among non-diabetic and diabetic db/db mice, highlighting the involvement of different mechanisms in the activation of ADAM17 in type 1 and type 2 diabetes. However, basing on the recent findings (Lautrette et al., 2005; Ford et al., 2013), hyperglycemia and activated Ang II could be possible reasons behind enhanced ADAM17 protein in the kidney of db/db mice.

In addition to its positive effects on glucose homeostasis and renal pathologies, physical exercise training exerted beneficiary actions on metabolic abnormalities associated with type 2 diabetes. Plasma analysis showed db/db mice had significantly increased levels of plasma glucose, glucagon, and triglycerides and decreased levels of adiponectin. However, physical exercise training significantly attenuated plasma triglyceride levels of db/db mice. Previously, exercise training has been shown to improve renal function of chronic kidney disease patients by lowering plasma triglycerides, at least in partial (Toyama et al., 2010). Alternatively, metformin had no effect on the plasma glucose, glucagon, insulin or adiponectin. In fact, metformin treatment was associated with a significant increase in triglyceride levels of db/db mice, which in contrast, has
been shown to attenuate plasma triglyceride levels in rodent models as well as type 2 diabetic patients (Wulffele et al., 2004; Tessari & Tiengo, 2008). A combination of physical exercise training and metformin significantly decreased the plasma glucagon concentrations. Furthermore, we correlated these plasma risk factors with urinary ACE2 excretion to strengthen our notion of using urinary ACE2 as a surrogate marker for diabetes.

Based on these results, it is tempting to speculate that physical exercise is capable of improving the complications of type 2 diabetes with minimal or no adverse side effects. Since exercise training has been shown to exert pronounced effects in type 2 diabetes and associated complications, physical training programs should be widely adopted into the medical care system. Though metformin treatment is also considered as a cost-effective intervention with minimal potential side effects in managing type 2 diabetes, our results demonstrate that it may be effective only during the initial stages of diabetes, or where the severity of the disease is less pronounced. This novel finding is in consensus with the recent report from DPP/DPPOS study demonstrating life style interventions were effective in reducing the incidence of diabetes by 71% in human subjects with the age of 60 years, whereas administration of metformin exerted no effects. However, treatment with metformin had beneficial effects in participants 25 to 44 years old (Fradkin et al., 2012).
6. CONCLUSION

Diabetic nephropathy is one of the major microvascular complications of diabetes that eventually manifests into ESRD. Ang II, a potent vasoconstrictor cleaved from Ang I, is responsible for renal damage in diabetes. ACE2 is highly expressed in the kidney and has been shown to be renoprotective by degrading Ang II to Ang-(1-7). Several studies demonstrated that pharmacological inhibition or deletion of ACE2 worsened albuminuria and glomerular sclerosis in diabetes. A Disintegrin and Metalloproteinases (ADAMs) were recently identified as an ectodomain sheddases of transmembrane proteins. ADAM17 mediated shedding of renal ACE2 is an important contributor to the pathogenesis of diabetic nephropathy.

In the current study, we demonstrated that 6 week old type 2 diabetic db/db mice developed hyperglycemia and excreted more amounts of urinary albumin and ACE2. Microalbuminuria is a widely accepted clinical sign of renal dysfunction in patients with diabetes. One of the aims of this study was to evaluate urinary ACE2 as a non-invasive biomarker for diabetic nephropathy. Urinary ACE2 was significantly and positively correlated with albuminuria, suggesting its potential to be an early biomarker. Indeed, renal ADAM17 and ACE2 protein levels were significantly enhanced in db/db mice. Increased ADAM17 and ACE2 proteins co-localized in the cortical tubules suggesting possible interaction in the diabetic kidney. Hence, in the present study, we hypothesized that lowering hyperglycemia would prevent or delay the progression of diabetic nephropathy by preventing renal ACE2 shedding mediated by ADAM17.

Moderate intensity exercise training improved glucose homeostasis, attenuated albuminuria and ACE2 excretion as early as 2 week after the initiation of treatment. In addition, physical exercise decreased renal ADAM17 protein levels and ameliorated renal pathologies in trained db/db mice compared to control db/db mice. Interestingly, our results suggest that metformin was effective
in the initial stages of diabetes and had no effect in the later stages where the disease progress is more severe. Indeed, administration of metformin for 10 weeks had no effect on the albuminuria, renal ADAM17 protein, and shedding of ACE2. Further, our results demonstrated a significant association between blood glucose, urinary albumin, plasma insulin, glucagon, and triglycerides with urinary ACE2 excretion, suggesting urinary ACE2 as an alternate marker for diabetes. In addition, urinary ACE2 could be used as a screening tool in assessing the effectiveness of therapeutic interventions. Since exercise training has been shown to exert pronounced effects in type 2 diabetes and associated complications, with no compromising side effects, physical training programs should be widely adopted into the medical care system.
Diabetic nephropathy is one of the major microvascular complications of diabetes which eventually manifests into end stage renal disease. Alteration in renin-angiotensin system (RAS) is considered to be the primary cause underlying this implication. Emerging evidence suggests that the biological actions of Ang II may be opposed by the formation of Ang (1-7), partly generated by the actions of ACE2 and neprilysin (NEP). NEP is a zinc-containing metallopeptidase catalyzing the conversion of Ang I to Ang-(1-7), a potent vasodilator, thus counteracting the deleterious effects of Ang II. In our previous studies it has been shown that renal NEP is down-regulated and ACE2 is up-regulated in type 2 diabetic (db/db) mice. The goal of the present study is to explore the role of renal NEP and ACE2 in the pathogenesis of diabetic nephropathy in the STZ mouse model of type 1 diabetes. Diabetes was induced by five consecutive injections of STZ (50 mg/kg, i.p.). Urine was collected in presence of protease inhibitor for measuring albumin, creatinine and total protein contents. STZ diabetic mice exhibited hyperglycemia, microalbuminuria and renal hypertrophy. Renal NEP activity, detected via formation of Ang-(1-7) (m/z 899), was significantly reduced by 30% in diabetic mice. Renal ACE2 activity, measured using the fluorogenic test assay, was found to be unaltered in diabetic and control mice. Western blot analysis demonstrated decreased renal NEP, nephrin and unaltered ACE2 protein expression in diabetic mice, which was further supported by immunohistochemical staining. In conclusion, decreased NEP coupled with depletion of nephrin in diabetes could possibly play a crucial role in the development of diabetic nephropathy.
APPENDIX B

Upregulation of Tumor necrosis factor \(\alpha\)-converting enzyme (TACE) Protein Expression in \(db/db\) Mice is reversed by Rosiglitazone


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TACE, also known as ADAM17, is involved in the ectodomain shedding of several membrane bound proteins. Angiotensin converting enzyme 2 (ACE2), homologue of angiotensin converting enzyme (ACE), is known to be renoprotective by increasing the degradation of vasoactive peptide Angiotensin II (Ang II) to vasodilator peptide Ang-(1-7). It has been shown before that TACE mediates regulated ectodomain shedding of ACE2. PPAR\(\gamma\) agonist, rosiglitazone, is known to impart renoprotection by attenuating albuminuria. However, the exact mechanism of renoprotection is not clear. Our previous results and others showed that renal ACE2 protein expression is increased during early stages of diabetes in \(db/db\) mice. We also demonstrated increased urinary ACE2 excretion in \(db/db\) mice. The goal of this study is to test the hypothesis that TACE is upregulated in \(db/db\) diabetic mice and treatment with rosiglitazone imparts renoprotection by attenuating the shedding of renal ACE2 via downregulating TACE protein expression. Male 6 week \(db/db\) mice were fed rosiglitazone (20mg/kg/day) for 10 weeks. Metabolic and urinary parameters were monitored weekly. Kidney lysate and urine was used to perform western blot and ACE2 activity (pmols/h/\(\mu\)g protein) respectively. \(db/db\) mice demonstrated glucose intolerance, hyperglycemia, and albuminuria at a very early age. Rosiglitazone treatment normalized blood glucose levels, improved glucose tolerance and decreased albuminuria in treated \(db/db\) mice. Western blot showed increased renal TACE protein expression of \(db/db\) mice compared to controls \((p<0.05)\). Rosiglitazone treatment significantly decreased renal TACE protein expression in \(db/db\) mice compared to untreated mice \((p<0.05)\). In addition, \(db/db\) mice demonstrated a 7 fold increase in urinary ACE2 activity compared to controls. Treatment with rosiglitazone significantly attenuated and normalized ACE2 activity in treated \(db/db\) mice. In conclusion, TACE is upregulated in \(db/db\) mice and normalizing hyperglycemia with rosiglitazone could impart renoprotection by decreasing renal TACE protein expression and shedding of renal ACE2.

Poster presented in American Heart Association Meeting – High Blood Pressure Research (HBPR); Washington. D.C. 2012
APPENDIX C

Insulin Normalizes Angiotensin Converting Enzyme 2 (ACE2) and Attenuates Albuminuria in Type 1 Diabetic Akita Mice


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Diabetic nephropathy (DN) is a microvascular complication of diabetes that is clinically diagnosed by a progressive increase in albuminuria. Alterations within renin angiotensin system balance contribute to the pathogenesis of diabetic kidney disease. Angiotensin converting enzyme 2 (ACE2), a metallocarboxypeptidase, has a renoprotective role due to its ability to form Angiotensin (1-7) [Ang-(1-7)] by degrading Angiotensin II (Ang II). Accumulating evidence shows that strict glycemic control attenuates diabetic kidney damage. Therefore, the aim of this study is to test the hypothesis that normalizing hyperglycemia with insulin will reduce albuminuria by increasing ACE2 in Akita diabetic mice. Type 1 diabetic Akita mice and their wild type (WT) littermates were used. Metabolic parameters were monitored weekly. Urine was collected over 24 hours to measure urinary albumin, total protein and ACE2 activity. Akita mice developed significant hyperglycemia compared to WT mice. There was a significant increase in urinary albumin excretion in Akita mice compared to WT mice. In addition, Akita mice demonstrated a significant increase in renal and urinary ACE2 activity compared to WT mice (p<0.05). Western blot revealed upregulation of renal ACE2 and downregulation of renal ACE protein expression in Akita mice compared to WT mice. Treatment with insulin implants (LinβitR) for 20 weeks significantly decreased hyperglycemia in Akita mice. Insulin treatment significantly decreased urinary albumin excretion as well as renal and urinary ACE2 activity in Akita mice. Further, insulin administration, downregulated renal ACE2 and upregulated renal ACE protein expression in Akita mice. In conclusion, normalizing hyperglycemia in Akita mice with insulin decreased ACE2 protein expression and activity.

Poster presented in American Heart Association Meeting – High Blood Pressure Research (HBPR); Washington. D.C. 2012
Diabetic nephropathy is one of the major microvascular complications of diabetes which eventually manifests into end stage renal disease. Alteration in renin-angiotensin system (RAS) is considered to be the primary cause underlying this implication. Emerging evidence suggests that the biological actions of Ang II may be opposed by the formation of Ang (1-7), partly generated by the actions of ACE2 and neprilysin (NEP). NEP is a zinc-containing metallopeptidase catalyzing the conversion of Ang I to Ang-(1-7), a potent vasodilator, thus counteracting the deleterious effects of Ang II. In our previous studies it has been shown that renal NEP is down-regulated and ACE2 is up-regulated in type 2 diabetic (db/db) mice. The goal of the present study is to explore the role of renal NEP and ACE2 in the pathogenesis of diabetic nephropathy in the STZ mouse model of type 1 diabetes. Diabetes was induced by five consecutive injections of STZ (50 mg/kg, i.p.). Urine was collected in presence of protease inhibitor for measuring albumin, creatinine and total protein contents. STZ diabetic mice exhibited hyperglycemia, microalbuminuria and renal hypertrophy. Renal NEP activity, detected via formation of Ang-(1-7) (m/z 899), was significantly reduced by 30% in diabetic mice. Renal ACE2 activity, measured using the fluorogenic test assay, was found to be unaltered in diabetic and control mice. Western blot analysis demonstrated decreased renal NEP, nephrin and unaltered ACE2 protein expression in diabetic mice, which was further supported by immunohistochemical staining. In conclusion, decreased NEP coupled with depletion of nephrin in diabetes could possibly play a crucial role in the development of diabetic nephropathy.

Poster presented in Ohio Physiological Society Meeting (OPS); Dayton 2012
APPENDIX E

Downregulation of Renal Neprilysin in Streptozocin (STZ) Diabetic Mice

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Diabetic nephropathy is one of the major microvascular complications of diabetes which eventually manifests into end stage renal disease. Alteration in renin-angiotensin system (RAS) is considered to be the primary cause underlying this implication. Emerging evidence suggests that the biological actions of Ang II may be opposed by the formation of Ang (1-7), partly generated by the actions of ACE2 and neprilysin (NEP). NEP is a zinc-containing metallopeptidase catalyzing the conversion of Ang I to Ang-(1-7), a potent vasodilator, thus counteracting the deleterious effects of Ang II. In our previous studies it has been shown that renal NEP is down-regulated and ACE2 is up-regulated in type 2 diabetic (db/db) mice. The goal of the present study is to explore the role of renal NEP and ACE2 in the pathogenesis of diabetic nephropathy in the STZ mouse model of type 1 diabetes. Diabetes was induced by five consecutive injections of STZ (50 mg/kg, i.p.). Urine was collected in presence of protease inhibitor for measuring albumin, creatinine and total protein contents. STZ diabetic mice exhibited hyperglycemia, microalbuminuria and renal hypertrophy. Renal NEP activity, detected via formation of Ang-(1-7) (m/z 899), was significantly reduced by 30% in diabetic mice. Renal ACE2 activity, measured using the fluorogenic test assay, was found to be unaltered in diabetic and control mice. Western blot analysis demonstrated decreased renal NEP, nephrin and unaltered ACE2 protein expression in diabetic mice, which was further supported by immunohistochemical staining. In conclusion, decreased NEP coupled with depletion of nephrin in diabetes could possibly play a crucial role in the development of diabetic nephropathy.

*Poster presented in Research Forum; WSU, OH 2012*
APPENDIX F

Behavioral Differences in Type II Diabetic Mice Treated with Exercise and Metformin

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Diabetes and mental health disorders have a high co-morbidity of making their study a priority. Furthermore, the effects of standard therapeutics on these disorders are not well understood. Metformin (a biguanide) is the most commonly prescribed medication for type II diabetics. It treats insulin resistance primarily by controlling the amount of glucose in circulation. In addition, exercise is commonly prescribed to diabetic patients in order to improve weight loss, and improve peripheral neuropathy. Exercise is also recommended for those with mental health disorders, most commonly depression and anxiety. Previous work in the lab using a leptin deficient mouse model (db/db) has demonstrated deficits in multiple behavioral tests. The current experiment was completed to differentiate the effects of exercise and metformin on behavioral outcomes.

*Poster presented in Research Forum; WSU, OH 2012*
APPENDIX G

Physical Exercise Training and Metformin Treatment Attenuated Albuminuria and Shedding of ACE2 in db/db Mice

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Angiotensin II (Ang II), a potent vasoconstrictor cleaved from Ang I, is responsible for the renal damage in diabetes. Studies have shown that the strict glycemic control and blockade of renin angiotensin system attenuates diabetic kidney damage. Angiotensin converting enzyme (ACE) 2 is highly expressed in the kidney and has been shown to be renoprotective by degrading Ang II to Ang-(1-7). We have shown previously increased urinary ACE2 excretion in db/db type 2 diabetic mice which contributes to the pathogenesis of diabetic nephropathy. We tested the hypothesis that physical exercise training and metformin treatment improve glucose homeostasis and attenuate albuminuria in db/db mice. We also investigated whether there is a correlation between urinary albumin excretion and shedding of ACE2 in diabetic mice. Six weeks old normal and db/db mice were subjected either to physical exercise training and/or metformin treatment (150 mg/kg/day) for 10 weeks. The exercise groups were run on a mouse forced exercise walking wheel system for 1 hour a day for 7 days a week at a speed of 8 m/min. Exercise training significantly lowered blood glucose, urinary albumin and ACE2 excretion ($p<0.05$) in db/db mice. Furthermore, exercise training lowered food and water intake of db/db mice, but had no effect on their body weights. Metformin treatment decreased hyperglycemia, urinary albumin and ACE2 excretion ($p<0.05$) only during the first 2 weeks of treatment. Further, urinary ACE2 correlated positively with, albuminuria ($r=0.84; p<0.0001$), glycemia ($r=0.75; p<0.0001$), plasma glucagon ($r=0.66; p<0.001$) and triglycerides ($r=0.75; p<0.0001$). In addition, exercise training reduced plasma triglycerides and enhanced insulin levels in db/db mice. In conclusion, physical exercise training attenuated microalbuminuria and shedding of renal ACE2 throughout the study. However, metformin was only effective during the initial stages of diabetes.

Poster presenting at American Diabetic Association Meeting (ADA); Illinois 2013
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