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Impact of Diabetes on ACE/ACE2 Balance and Angiotensin II Type 1 Receptor Expression in db/db Diabetic Mice

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IMPACT OF DIABETES ON ACE/ACE2 BALANCE AND ANGIOTENSIN II TYPE 1 RECEPTOR EXPRESSION IN \textit{db/db} DIABETIC MICE

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

By

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B.Pharm., North Gujarat University, Gujarat, India 2006

2009
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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY MALAV MADHU ENTITLED "IMPACT OF DIABETES ON ACE/ACE2 BALANCE AND ANGIOTENSIN II TYPE 1 RECEPTOR EXPRESSION IN \(db/db\) Diabetic Mice" BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT

Madhu, Malav. M.S., Department of Pharmacology and Toxicology, Wright State University, 2009. Impact of Diabetes on ACE/ACE2 Balance and Angiotensin II Type 1 Receptor Expression in db/db Diabetic Mice.

Alterations in the renin-angiotensin system (RAS) are considered to be crucial for the development of diabetic complications like hypertension and nephropathy. Our previous work demonstrated role of AT1 receptors (AT1R) in the development of hypertension in db/db diabetic mice. The aim of this study was to test the hypothesis that there is upregulation of renal AT1R and imbalance in renal ACE/ACE2 homeostasis in db/db mice. In addition, we hypothesize that treatment with an anti-hyperglycemic or an AT1R blocker will correct this imbalance. Five week old control and db/db mice were housed in metabolic cages for 24 hour collection of urine. At early age of 5 weeks, db/db mice were obese and hyperglycemic. Urinary albumin excretion was also significantly high in db/db mice. Changes in RAS were evaluated using enzyme activities, western blots and immunohistochemistry. There was a significant increase in urinary ACE2 activity and ACE2 content in db/db mice at 5 weeks. There was a significant increase in plasma ACE activity and Ang II content in db/db mice compared to controls at 8 weeks. Western blot analysis showed significant increase in AT1R protein expression in 8, 18 and 31 week db/db mice compared to controls. There was upregulation of ACE2 and down-regulation of ACE in kidney to compensate the effects of high plasma Ang II. To study the effect of reduction in blood glucose and AT1R blockade, mice were treated
with metformin and losartan for 12 weeks. Chronic treatment with metformin (150 mg/kg/day) and losartan (10 mg/kg/day) significantly decreased urinary albumin and protein excretion. Metformin improved blood glucose and glucose tolerance db/db mice, but did not affect renal expression of ACE, ACE2 and AT1R. Although chronic losartan treatment did not alter blood glucose levels, it improved the morphology of pancreatic islets. There was a significant increase in renal AT1R protein expression and decrease in renal ACE2 protein expression following losartan treatment. Losartan treatment significantly increased urinary ACE2 activity. Western blot of concentrated urine from 8 week db/db mice revealed immunoreactive bands of ACE, ACE2 and AT1R protein.

Conclusion: 1) There is upregulation in renal AT1R protein expression in db/db mice. 2) Chronic metformin treatment significantly reduces blood glucose and microalbuminuria in db/db mice without affecting ACE/ACE2 balance. 3) Chronic losartan treatment had no effect on blood glucose, but it up-regulates renal AT1R and down-regulates renal ACE2. 4) Enzyme activity and western blot shows increased excretion of ACE2 in the urine of db/db mice. These data show that urinary ACE and ACE2 provide good index of intra-renal RAS status and could be used in early diagnosis and prognosis of diabetic renal disease.
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INTRODUCTION

Diabetes

Diabetes mellitus is a chronic metabolic disorder resulting in hyperglycemia and disturbances of carbohydrate, fat, and protein metabolism. Diabetes is occurring at an epidemic rate in the United States and other western countries (Mokdad et al., 2001). In the year 2000, there were approximately 171 million people with diabetes worldwide; estimates for 2030 suggest that the prevalence of diabetes will increase to 366 million (Wild et al., 2004). According to Centers for Disease Control and Prevention, the risk of death among individuals with diabetes is almost twice that of individuals who do not have diabetes of similar age (USNDFS, 2005). For individuals born in 2000, the risk for developing diabetes is 33% for males and 39% for females. Diabetes is a huge burden on our healthcare system (USNDFS, 2005). The total estimated cost of diabetes in 2007 was 174 billion in US only (Ettaro et al., 2004). One in 5 healthcare dollars in the US is spent caring for someone with diagnosed diabetes (Ettaro et al., 2004). The exact cause of diabetes is still unknown and many factors like obesity, genetics, diet, environment, individual lifestyle are believed to play part in the pathogenesis. There are 3 types of diabetes: 1) Type 1 diabetes mellitus caused by beta-cell destruction that leads to absolute insulin deficiency, hyperglycemia and ketosis, 2) Type 2 diabetes mellitus characterized by insulin resistance and relative insulin deficiency and 3) Gestational diabetes which occurs in 5-10% pregnant women with no previous history of diabetes. Of all diabetics,
90 to 95% have type 2 diabetes. Kidney disease, heart disease, blindness, nervous system disease, dental diseases are some of the complications of diabetes.

**Diabetes, hypertension and kidney disease**

Diabetic nephropathy (DN) is a leading cause of end-stage renal disease (ESRD) that requires renal dialysis or kidney transplantation (USRDS, 2003). When kidney disease is caught late, ESRD usually follows. Nearly 44% of all new patients enrolled in ESRD treatment programs have diabetic background (USRDS, 2003). The number of people in the United States with ESRD has doubled between 1991 and 2001. In the year 2000, expenditures for about 400,000 patients with ESRD in the United States totaled about $20 billion, meaning that the cost to manage ESRD for each patient in the year 2000 was $50,000 (Rodby, 2004). According to United States Renal Data System, diabetes mellitus is the single largest cause of ESRD requiring chronic dialysis or kidney transplantation in the US.

Hyperglycemia may lead to nephropathy by a number of mechanisms (Larkins & Dunlop, 1992). Sustained hyperglycemia of diabetes causes microvascular dysfunction, which contributes to the development of ESRD (Futrakul et al., 2006; Jawa et al., 2006). Many transmembrane proteins translocate glucose into the cells (Brosius & Heilig, 2005). Intracellular glucose and its metabolites give rise to vasoactive peptides and elevate intraglomerular pressure (Wautier & Schmidt, 2004). Advanced glycation end-products mediate renal tissue injury by producing reactive oxygen species (ROS) (Cooper, 2004) and by initiating signaling events through activation of protein kinase C, mitogen activated protein kinase and nuclear factor-κ of activated B cells. This would be followed
by over activity of tumor growth factor-β and thereby alter expression of extracellular matrix proteins (Jakus & Rietbrock, 2004). ROS can then cause apoptosis of podocytes, thus initiating kidney damage (Susztak et al., 2006). Moreover, acute elevations in glomerular filtration rate (GFR) were observed in patients in response to glucose infusion (Christiansen et al., 1981).

Circulating leptin levels are increased in patients with obesity and type 2 diabetes (Nyholm et al., 1997). High serum leptin levels cause an increase in sympathetic tone which then leads to hypertension and other related complications (Considine et al., 1996; Haynes, 2005). It has also been reported that hypertensive individuals have high leptin levels and are at high risk of developing diabetes (Adamczak et al., 2000).

Hypertension is twice as common in type 2 diabetics and is responsible for many complications of diabetes (Sowers et al., 2001). Kidney plays an important role in the development of hypertension. In fact, kidney disease is both a cause and a consequence of hypertension (Paul et al., 2006). One study reports that normotensive rats receiving a kidney from genetically hypertensive donor rats develop hypertension (Navar, 2005). On the contrary, genetically hypertensive rats who have had bilateral nephrectomy and receive a kidney from normotensive rats exhibit a reduction in blood pressure (Navar, 2005). Increase in renal vascular resistance, induced by hyperglycemia, can lead to diminished GFR. Many patients at risk of hypertension manifest reduced renal blood flow and elevated filtration fraction even before development of hypertension (Navar, 2005).

The first sign of renal damage in diabetes is the presence of persistent microalbuminuria (24-hour urinary albumin excretion of 30 to 300 mg/day), hypertrophy of epithelial cells
and thickening of glomerular basement membrane (Cooper, 1998). This stage is known as incipient nephropathy. 20% to 40% of patients show this symptom 10 to 15 years after the onset of diabetes (Lee, 2005). Unfortunately, the onset of type 2 diabetes is often difficult to ascertain and so when a patient presents for the first time with type 2 diabetes, they may already have microalbuminuria. In type 2 diabetes, the microalbuminuria is seldom reversible and is probably a sign of endothelial dysfunction (Ritz, 2003).

Progression to proteinuria (urinary albumin excretion >300 mg/day) occurs in patients 15 to 20 years following the onset of diabetes and this stage is known as overt nephropathy (Lee, 2005). Following this, the creatinine clearance start to decline at an average rate of 10 to 12 mL/min/year in untreated patients and hypertension develops (Parving et al., 2001).

Optimization of glycemic control helps to reduce onset of DN (Hasslacher et al., 1989). In this study, we want to study the effect of metformin, a glucose sensitizer, on renal outcomes of DN. A variety of anti-hypertensive therapies produce beneficial effects in reducing proteinuria (Cooper & Johnston, 2000). However not all antihypertensive agents are the same in terms of delaying the progression of renal disease in diabetic patients. The renin-angiotensin system plays an important role in the pathogenesis of kidney disease. A substantial amount of evidence demonstrates that blockade of the renin-angiotensin system provides renoprotection (Hostetter, 2003; Lewis et al., 1993; Maschio et al., 1996).

**Renin-angiotensin system (RAS)**

The renin-angiotensin system (RAS) has been implicated in the pathophysiology of the diabetic renal disease (Andersen et al., 2000; Lewis, 2002). The functional roles of RAS
in the regulation of blood flow, sodium, bicarbonate and water transport, cell growth and differentiation have also been clarified. There is evidence of presence of RAS in pancreas of human (Tahmasebi et al., 1999), mouse (Leung et al., 1998) and dog (Chappell et al., 1992). RAS plays a key role in renal injury (Taal & Brenner, 2000; Hollenberg & Raij, 1993). It is one of the major targets in the treatment of DN (Ye et al., 2004; Ye et al., 2006; Tikellis et al., 2003). Drugs that interrupt RAS cascade are widely used in the management of diabetes and its complications. Additionally, weight gain and obesity are believed to activate RAS in humans (Barton et al., 2003).

The RAS has long been recognized to play a crucial role in the regulation of blood pressure and electrolyte balance (see diagram 1). Angiotensinogen (AGT) is converted to angiotensin I (Ang I) by the enzyme renin secreted by kidney. Ang I has little or no biological activity. Ang I is further catabolized by ACE to the biologically active peptide angiotensin II (Ang II) (Skeggs, Jr. et al., 1956). Ang II is found in circulation, in tissues and even in cells making it an endocrine, paracrine and intracrine peptide (Kumar et al., 2007). Ang II has been shown to cause vasoconstriction, renal tubule sodium re-absorption, growth promotion, cellular dedifferentiation, increased aldosterone secretion, polydipsia and increased sympathetic outflow (Giacchetti et al., 2005). Ang II directly contributes to the progression of chronic kidney disease, including DN (Lewis et al., 1993; Maschio et al., 1999; Parving et al., 2001). In renal interstitial fluid, the concentration of Ang II is 1000 times higher than plasma (Seikaly et al., 1990). That is why kidney is a very important organ when studying hypertension and diabetes. Recently, an ACE-related carboxypeptidase (ACE2) was identified and cloned (Donoghue et al., 2000). ACE2 primarily cleaves Ang II into angiotensin 1-7 (Ang 1-7)
and less preferably degrades Ang I to angiotensin 1-9 (Ang 1-9) (Donoghue et al., 2000; Li et al., 2005; Eriksson et al., 2002). Interestingly, these products of ACE2 cleavage appear to have effects that oppose those of ANG II (Haulica et al., 2003). Thus, the vasoconstrictor/proliferative or vasodilator/anti-proliferative actions of RAS are primarily driven by ACE-ACE2 balance.
Abbreviations: AMP, aminopeptidase; AT1, Ang II type 1 receptor; AT2, Ang II type 2 receptor; Mas, Ang(1–7) receptor Mas; D-Amp, dipeptidyl-aminopeptidase; IRAP, insulin-regulated aminopeptidase; PCP, prolyl-carboxypeptidase; PEP, prolyl-endopeptidase; NEP, neutral endopeptidase and (P)RR, Renin/prorenin receptor.

Diagram 1: A schematic representation of the renin-angiotensin system cascade
Angiotensin converting enzyme (ACE)

ACE was discovered in plasma in 1956 by Leonard T. Skeggs (Skeggs, Jr. et al., 1956). ACE is a monomeric, membrane bound, zinc and chloride dependent di-peptidyl carboxypeptidase (Riordan, 2003). ACE was also found to be present in other organs such as kidney, heart, pancreas and brain (Nash, 1992). ACE plays a crucial role in RAS; it catalyzes the cleavage of decapeptide Ang I to produce a potent vasoconstrictor Ang II, by removal of carboxy terminal peptides. ACE also less preferably hydrolyzes the inactive Ang (1-9) to produce vasodilator Ang (1-7) (Tschope et al., 2002). Ang II is a potent vasoconstrictor and mediates its effects through AT1 and AT2 receptors (Higuchi et al., 2007). Pathological activation of tissue ACE with resulting increase in local Ang II produces deleterious effects on kidney and heart during organ remodeling (Dzau et al., 2002). ACE promotes degradation of bradykinin, which acts as a vasodilator via stimulation of nitric oxide (Carey & Siragy, 2003). The kidney, under the regulation of Ang II and aldosterone, maintains the electrolyte balance in the body. A recent study on ACE expression revealed that higher ACE was associated with an increased diabetic pathologies like renal dysfunction and high blood pressure (Ye et al., 2004; Senador et al., 2009). High ACE leads to high production of Ang II followed by increased blood pressure and subsequent kidney damage due to hyperfiltration. Inhibition of ACE has been shown to decrease systemic blood pressure, albuminuria and glomerular capillary pressure (Mathiesen et al., 1991; Ahmad et al., 1997; Lewis et al., 1993). Aside from lowering blood pressure, ACE inhibitors have found to improve the prognosis of patients with congestive heart failure and myocardial infarction (Garg & Yusuf, 1995).
Angiotensin converting enzyme 2 (ACE2)

ACE2, a new homologue of ACE, was identified and cloned by two separate groups in the year 2000 (Donoghue et al., 2000; Tipnis et al., 2000). ACE2 displays 42% sequence homology and 62% sequence similarity with NH2-terminal catalytic domain of ACE and, like ACE, is a type 1 integral membrane protein (Tipnis et al., 2000). ACE2 is not inhibited by the ACE inhibitors captopril and lisinopril (Tipnis et al., 2000). Unlike ACE, ACE2 levels in plasma are very low and this could be due to less shedding of ACE2 from plasma membrane of endothelial cells (Rice et al., 2006). Our previous study shows that ACE2 activity is not detectable in the plasma of diabetic and control mice (Elased et al., 2006). ACE2 was first found to be present in kidneys, heart and testes but later studies show its widespread distribution (Donoghue et al., 2000; Tipnis et al., 2000; Hamming et al., 2004). In kidneys, ACE2 is particularly found in apical membranes of the proximal tubules and in podocytes (Soler et al., 2008). ACE2 is a carboxy-peptidase that primarily cleaves vasoconstricting Ang II to vasodilator Ang (1-7). Ang (1-7) acts on Mas, a G-protein coupled receptor, to exert its vasodilatory action (Pinheiro et al., 2004) and thus ACE2 acts in a counter-regulatory manner with ACE, keeping production and actions of Ang II in check. Furthermore, ACE2 activity has been detected in urine of healthy subjects (Warner et al., 2005) and sheep (Shaltout et al., 2007). Studies have shown that ACE2 protein expression and activity changes in diabetic kidneys (Wysocki et al., 2006; Ye et al., 2006). It has been shown that Ang II can upregulate ACE and downregulate ACE2 (Koka et al., 2008). Administration of MLN-4760, a specific ACE2 inhibitor, worsens albuminuria in db/db mice together with increased glomerular expression of fibronectin (Ye et al., 2006). Moreover, ACE2 null mice develop a
progressive, age-dependent cardio-myopathy with increased oxidative stress, collagenase levels and hypertrophy together with increased urinary albumin excretion (Oudit et al., 2007). Whereas, generation of double mutant ACE-ACE2 null mice prevented cardiac dysfunction suggesting cardio-protective role of ACE2 (Crackower et al., 2002). Another study reports that ACE2 null mice do not develop cardiac complications but they show high plasma Ang II levels and increased pressor sensitivity after Ang II infusion (Gurley et al., 2004). Thus, ACE2 counteracts pressor activity of Ang II by its degradation and by production of Ang (1-7) having depressor activity.

**Angiotensin II type 1 receptor (AT1R)**

One of the aims of the present study is to investigate the effect of losartan, an AT1R blocker (ARB), on kidney function and intra-renal RAS in db/db diabetic mice. The actions of Ang II are mediated by AT1 and AT2 receptors which, invariably mediate opposite functions (Carey & Padia, 2008). Most of the effects of Ang II, such as hypertension, atherosclerosis and heart failure, are mediated by AT1R, a seven transmembrane G-protein coupled receptor (Higuchi et al., 2007). AT1Rs are expressed in kidney, heart, adrenal gland, brain, lung and adipose tissue (de et al., 2000). Studies indicate that AT1R signaling in endothelial cells induce endothelial dysfunction (Nakashima et al., 2006). Dysfunctional endothelium is characterized by less production of nitric oxide, accelerated vasoconstriction, smooth muscle proliferation and a prothrombotic state (Watanabe et al., 2005). Ang II has been shown to reduce baroreflex sensitivity by interacting with AT1R, contributing to the development of hypertension (Gao et al., 2005). By its action on AT1R, Ang II causes generation of oxidative radicals
and promotes inflammatory processes like atherosclerosis and vascular aging (Fyhrquist & Saijonmaa, 2008).

A recent study reported that Ang II infusion causes decrease in plasma adiponectin, an insulin sensitizer via AT1R in rats (Ran et al., 2006). Suppression of adiponectin may be a mechanism whereby Ang II impairs glucose tolerance and explains why hypertensive patients are at more risk of developing diabetes. In fact, treatment with ARBs reduce new onset of diabetes by 15-30% (Aguilar & Solomon, 2006). Our previous study showed that, losartan treatment reduced blood pressure without affecting glucose handling in \textit{db/db} mice (Senador et al., 2009). Blockade of AT1R reduces cardiovascular complications in animal models of type 1 and type 2 diabetes (Nielsen et al., 2003;Amazonas & Lopes De Faria, 2006).

It has been reported that Ang II suppresses glucose induced insulin release from isolated islets and pre-treatment of islets with losartan restores the effect of glucose. This effect of Ang II is partly because of reduction in (pro)insulin biosynthesis (Lau et al., 2004). A recent study showed up regulation of AT1 receptor together with over expression Ang II content in cavernous tissue of type 1 diabetic rats suggesting its possible role in erectile dysfunction (Yang et al., 2009). In present study, we will investigate whether there is upregulation of renal AT1 receptor protein expression in \textit{db/db} diabetic mice.

**Changes in RAS homeostasis in diabetes**

The balance between Ang II and Ang (1-7), reflecting ACE and ACE2 activities, respectively, is considered as physiologically significant ratio (Huentelman et al., 2004). Over activity of the RAS has been identified as an important determinant in the etiology
of diabetes, heart disease and therefore represents a major target for therapy. In young 
\textit{db/db} diabetic mice, the pattern of ACE and ACE2 expression in the renal cortical 
tubules was characterized by decrease in ACE and an increase in ACE2 (Ye \textit{et al.}, 2004). 
In diabetic rat and human kidneys, ACE is redistributed towards glomeruli and away 
from proximal tubules (Anderson \textit{et al.}, 1993). Furthermore, glomerular immunostaining 
for ACE2 decreases and that of ACE increases in \textit{db/db} mice at the age of 8 weeks (Ye \textit{et al.}, 2006). At this age, \textit{db/db} mice excrete more albumin in urine without obvious signs 
of renal pathology (Sharma \textit{et al.}, 2003). Furthermore, increased ACE expression was 
related to ACE2 inhibition in diabetic mice, suggesting that the ACE2-Ang (1-7)-Mas 
axis may play a renoprotective role by negatively regulating the ACE-Ang II- AT1 axis 
(Soler \textit{et al.}, 2007). Kidney also sequesters Ang II from circulation via AT1R mediated 
mechanisms (Navar, 2005). Therefore, sustained increase in plasma Ang II, as seen in 
diabetes, results in more uptake of Ang II by kidneys.

\textbf{Diagnosis of diabetic nephropathy}

The biggest challenge for healthcare professionals is accurate and early detection of 
diabetic kidney disease. Considerable scientific effort has been dedicated to identify 
patients at risk for the development of diabetic nephropathy (Susztak & Bottinger, 2006). 
There are some hurdles in diagnosis of kidney disease. Standard method for the diagnosis 
of diabetic nephropathy is renal biopsy which is a painful process and can suffer from 
sampling errors (Sharma \textit{et al.}, 2005). Detection of albumin in urine is the only 
noninvasive technique available for the diagnosis of diabetic nephropathy. Unfortunately 
the onset of diabetes is difficult to ascertain and so many patients diagnosed with high 
blood glucose already have microalbuminuria (Lee, 2005). Microalbuminuria is seldom
reversible in type 2 diabetics (Ritz, 2003). The problem with albuminuria as a disease marker is twofold. Microalbuminuria is a poor predictor of diabetic nephropathy (Perkins et al., 2007); proteinuria (macroalbuminuria), which is a strong predictor, only develops at advanced stages of diabetic nephropathy, when little can be done to prevent the development of ESRD. Furthermore, the immunoassay to measure albuminuria can detect only immunoreactive forms of albumin where as immunounreactive forms are not detectable by this conventional method (Comper & Osicka, 2005). An undiagnosed and untreated microalbuminuria progresses to proteinuria and at this point little can be done to stop progression of nephropathy. An early diagnosis is necessary for early treatment and to prevent further kidney damage.

A lot of research is going on to find a better biomarker for kidney disease. For instance, N-acetyl β-glucosaminidase is a lysosomal enzyme derived from proximal tubular cells which is not filtered by kidney under normal circumstances. Its excretion increases in circumstances that cause tubular injury (Basturk et al., 2006). Smad1 is a transcription factor for mesangial matrix expansion. Its excretion increases in early stage of diabetic nephropathy and decreases with olmesartan treatment (Mima et al., 2008). Angiotensinogen, which is also a part of RAS, is also a potential candidate (Kobori et al., 2009). Urine testing for biomarkers could substitute renal biopsy as safe and painless alternative.

Aggressive control of hyperglycemia and hypertension is very important in the management of diabetic nephropathy. Effectiveness of ACEIs and ARBs illustrate the contribution of RAS in development of diabetes and its complications. Nevertheless, the effect of these medications on renal and urinary RAS remains a matter of debate. To
clarify the issue, we studied the effect of an anti-diabetic, metformin and an anti-
hypertensive, losartan on renal RAS using $db/db$ mice as a model of type 2 diabetes.
HYPOTHESIS AND SPECIFIC AIMS

Hypothesis:

There is upregulation of AT1 receptor protein expression and imbalance in ACE/ACE2 homeostasis in the kidney of \(db/db\) mice. Treatment with an anti-hyperglycemic or an AT1 receptor blocker will correct this imbalance.

Specific aims:

1. To test the hypothesis that there is upregulation of renal AT1 receptor protein expression in \(db/db\) mice.

2. To test the hypothesis that early treatment with metformin or losartan will correct the imbalance of ACE and ACE2.

3. To test the hypothesis that urinary ACE2 could be used as an early biomarker for diabetic nephropathy.
MATERIALS AND METHODS

Animals:

Five week old db/db mice of the strain BKS.cg-m +/+ Lepr<sup>db</sup>/J and their age matched non-diabetic littermates were used. Animals were purchased from The Jackson Laboratory (Bar harbor, ME). Mice were housed individually at 22° C with a 12:12-hour light-dark cycle (6:30-18:30, lights on). Mice were maintained on a standard pellet diet with tap water available ad libitum. Cages were examined daily to assess the health of the animals. The obese (db/db) mouse strain has a point mutation in diabetes (db) gene encoding the leptin receptor gene. At early age, they serve as a good model of type 2 diabetes, characterized by hyperinsulinemia, obesity and progressive hyperglycemia. All experimental protocols were approved by WSU Animal Care and Use Committee.

1. Chronic treatment with metformin and losartan

Five to 8 week db/db and control mice were randomly assigned to metformin and losartan treatment groups. Each group consisted of 8 mice. Losartan group received 10 mg/kg/day losartan dissolved in drinking water for 12 weeks. Metformin group received 150 mg/kg/day metformin in drinking water for 12 weeks. After 12 weeks of treatment, mice were euthanized by decapitation trunk blood was collected in ice-chilled heparin washed tubes. Plasma was immediately separated by centrifugation at 10,000 x g for 10 minutes at 4° C and stored at -80° C. Tissues were collected in dry ice and stored at -80° C.
2. Western blot analysis

Control and db/db mice were sacrificed by decapitation and kidneys were quickly removed and homogenized on ice in phosphate buffered saline (PBS) containing protease inhibitor (Complete lysis M, Roche diagnostics, Mannheim, Germany). Tissue homogenates were centrifuged at 10,000 x g for 10 mins 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). Fifty microliter of tissue lysate was added to 50 µl sample loading buffer (8% SDS, 125 mmol/L Tris-HCl, pH-6.8, 20% glycerol, 0.02% bromophenol blue, 100 mmol/L dithiothreitol) and boiled for 10 minutes. Approximately 50 µg protein was loaded to 8% or 10% SDS-PAGE gel and separated by electrophoresis. Proteins on gel were then transferred (with Bio-Rad transfer apparatus, Hercules, CA) to a 0.2 µm PVDF membrane (Millipore, MA). The membrane was blocked for 1 hour with 10% non-fat milk made in 10 mM Tris buffered saline with Tween 20 (TBS-T) at room temperature (R.T.). For analysis of ACE, ACE2 and AT1, membranes were probed with respective antibodies (Santa Cruz, CA) made in 5% non-fat milk in TBS-T for 2 days at 4° C. Dilution for ACE and ACE2 antibodies was 1:200 while for AT1R antibody it was 1:500. The membranes were then washed with TBS-T buffer 3 times for 5 minutes at R.T. Then membranes were incubated with horse radish peroxidase (HRP) conjugated donkey anti-rabbit secondary antibody (Jackson Immunoresearch, PA) made in TBS-T buffer with 1:40,000 dilution for 1 hour at R.T. Blots were detected using SuperSignal chemiluminescent substrate (Pierce, Rockford, IL) and visualized in Fujifilm image analyzer (LAS-3000 Image Quant, Sunnyvale, CA). ACE, ACE2 and AT1 have
molecular weights 195 kDa, 90 kDa, and 43 kDa respectively. The relative amounts of proteins of interest were determined by normalizing to β-actin.

3. Plasma renin activity

Renin activity assays were performed using a kit purchased from DiaSorin (Stillwater, MN). Mice were sacrificed by decapitation and trunk blood was collected in ice chilled, heparin washed tubes as described above. Plasma samples stored at -80° C were thawed and 100 µl plasma was transferred to 0.5 mL uncoated tubes. One microliter of PMSF and 10 µl of maleate generation buffer were added to plasma samples and mixed well. Fifty microliter of this mixture was transferred to ice chilled tubes. One set of tubes was incubated at 37° C and the other set was incubated at 4° C for 18 hours. After incubation, 50 µl of samples, standards, blank and controls were transferred to appropriately marked angiotensin I antibody coated tubes. Then 500 µl of tracer-buffer solution was added to each tube followed by gentle vortex and incubated for 3 hours at room temperature. All tubes except ones for total count were decanted after incubation. Each tube was then counted in gamma counter for 1 minute. Renin activity was expressed as ng/mL/hr of generated Ang I.

4. Urine collection and concentration

Mice were put in metabolic cages for 24 hour collection of urine with free access to food and water. A total of 15 µl of protease inhibitor (Roche Diagnostics, IN) was added to each sample during 24 hour collection. Urine samples were centrifuged at 1,000 x g for 5 minutes at 4° C to remove debris. Urine was then aliquoted and stored at -80° C. For concentration, frozen urine samples were thawed and mixed with protease inhibitor
(Sigma-Aldrich, MO) in a dilution of 1:1000. Samples were then concentrated in ultrafiltration chambers with a semi-permeable membrane (Millipore, MA) at 4° C. Concentrated samples were mixed with protease inhibitor (Sigma-Aldrich, MO) in a dilution of 1:100. The sample concentrates were then aliquoted and stored at -80° C for later use.

5. ACE activity

ACE activity assays were performed using a kit purchased from Alpco Diagnostics (Salem, NH). Mice were sacrificed by decapitation. Plasma, kidneys and urine were collected and processed as described above. Kidney lysate (80-100 µg) or plasma samples (10 µl) were incubated with 100 µl HEPES buffer containing synthetic substrate, 3H-hippuryl-glycyl-glycine, at 37° C for 1 hour. For evaluation of urinary ACE activity, 50µl of urine samples were incubated with 100 µl of the 3H-hippuryl-glycyl-glycine at 37° C for 1 hour. Incubation was followed by acidification with 50 µl 1N HCl to stop the reaction. Tritiated hippuric acid was separated from unreacted substrate by extraction with 1.5 mL scintillation cocktail and measured in beta counter (Packard 18TR Liquid Scintillation Analyzer). ACE activity was expressed as Units/µg protein or Units/Liter as previously described (Neels et al., 1982). One unit (U/L) of ACE activity is defined as the amount of enzyme required to release 1 µmol hippuric acid per minute per liter of sample at 37° C.

6. ACE2 activity

ACE2 activity was measured using fluorogenic substrate 7-Mca-APK(Dnp) which is specific for ACE2 as described before (Vickers et al., 2002; Guy et al., 2003) with some
modification. Fluorescence of Mca is quenched by Dnp group until cleavage by ACE2. After cleavage fluorescence was detected at Excitation: 328 nm and Emission: 393 nm (FusionR Packard plate reader). ACE2 activity was measured in presence of 10 mM lisinopril, an ACE inhibitor, to prevent any interference from ACE. Four microliter (32-40 µg) of kidney protein or 50 µl of urine samples were incubated with the substrate in a buffer (50 mM Tris, 5 mM ZnCl2, 150 mM NaCl, 10 μM lisinopril) at 37° C. The plate was read at 0, 1, 2, 3, 4 and 18 hours. The results were expressed as pmoles/hr/µg protein of cleaved substrate.

7. Immunohistochemistry

Immunohistochemistry refers to the process of localizing proteins in cells of a tissue section by using antibodies binding specifically to antigens in biological tissues. Mice were injected with ketamine/xylazine mixture (100:8 mg/kg). Mice were then perfused transcardially with ice cold PBS to flush out the blood and then with 4% paraformaldehyde (PFA) for 10 minutes. Tissues were collected and fixed in 4% PFA overnight at 4° C. Tissues were then sent to AML laboratories for microtomy and staining (Rosedale, MD). Paraffin sections (4 µm) were deparaffinized by washing the sections with xylene and subsequent hydration by 100%, 95% and 70% ethanol. Then sections were transferred to 10 mM citrate buffer (pH 8.5) for 10 minutes at 95° C. Then sections were incubated in 5% normal donkey serum (Vector, Burlingame, CA) in PBS containing 0.1% Triton-X 100, for 1 hour at R.T. Sections were then incubated with rabbit anti-AT1 primary antibody (Santa Cruz, CA) in a dilution of 1:100 at 4° C overnight. Following this, the sections were washed with PBS 3 times and incubated with donkey anti-rabbit secondary antibody conjugated with Cyanine 3 fluorescent dye
(Jackson immunoresearch, PA) for 60 minutes at R.T. Slides were then allowed to dry in air and mounted with Vectashield mounting medium (Vector, Burlingame, CA). The slides were then visualized in a fluorescence microscope (Optronics, Goleta, CA).

8. Measurement of blood glucose levels

For determination of glucose, blood samples were withdrawn from a cut made on tail vein and measured using an Accu-Check Advantage Blood Glucose Monitor (Roche Diagnostics, Indianapolis, IN). The measuring limit of this monitor was 600 mg/dL. For measurement of higher levels of blood glucose, a glucose assay kit was purchased from Sigma (St.Louis, MO). For this assay, 5 μl blood was diluted in 25 μl of PBS buffer and 300 μl of water. Ten microliter of diluted blood was incubated with 100 μl of assay reagent for 30 minutes at 37° C. Glucose is oxidized to gluconic acid and hydrogen peroxide by glucose oxidase. Hydrogen peroxide reacts with o-dianisidine in the presence of peroxidase to form a colored product. The reaction was stopped by addition of 100 μl 12N H₂SO₄. Oxidized o-dianisidine reacts with sulfuric acid to form a more stable colored product. The intensity of the pink color measured at 540 nm is proportional to the original glucose concentration. We nullify the red color of blood by measuring blank reading at 600 nm and dividing one third of individual sample reading from actual 540 nm reading.

9. Glucose tolerance test (GTT)

To study glucose handling in mice, intra-peritoneal (i.p.) GTT was performed. Mice were fasted for 16 hours. Fasting blood glucose was measured after the end of fasting period.
Mice were then given i.p. injections of 1.5 mg/kg glucose in an aqueous solution. Blood glucose was measured at 30, 60, 90, 120 minutes after glucose injection as described.

10. Urinary albumin assay

To monitor kidney function, quantitative estimation of urinary albumin was performed using a kit purchased from Bethyl Laboratories (Montgomery, TX). Urine samples were collected and stored as described above. In a 96 well plate, the wells were coated with 100 µl goat anti-mouse albumin antibody diluted 1:100 in carbonate-bicarbonate buffer overnight at 4°C. After incubation, the antibody solution was aspired from each well and washed with 150 µl TBS-T buffer 3 times. Then each well was incubated with 200 µl blocking buffer (Tris buffered saline with 1% BSA) for 30 minutes and washed 3 times with TBS-T buffer. One microliter urine samples were diluted with 1 mL of conjugate buffer. One hundred microliter of diluted urine samples were added to each well and incubated at R.T. for 1 hour. At the same time, 100 µl of standards were added to assigned wells followed by 1 hour incubation. Albumin present in samples and standards binds with the coated antibody. Wells were then washed 5 times with TBS-T buffer. Then 100 µl HRP conjugated detection antibody diluted 1:35,000 in conjugate buffer was incubated for 1 hour at R.T. for detection of bound antigen. Wells were then washed 5 times with TBS-T buffer. Equal volumes of TMB substrate A and substrate B (KPL, Gaithersburg, MD) were mixed and 100 µl of this substrate was added to each well. HRP cleaves the substrate to produce color. The intensity of the color produced is directly proportional to the amount of albumin present in the sample. Plate was finally read at 450 nm in Fusion® Packard plate reader.
11. Measurement of plasma and renal Ang II

Mice were sacrificed by decapitation and trunk blood was collected in ice chilled heparin washed tubes and kidneys were collected in dry ice. Plasma and kidney samples were processed as described above. Plasma and kidney Ang II contents were measured using a kit purchased from Alpco (Salem, NH). This technique uses double-antibody radioimmunoassay to measure Ang II. Ang II is extracted from 50 µl of plasma or 450-500 µg kidney samples treated with 1 µl EDTA using reverse phase extraction by special columns followed by vacuum evaporation of liquid to form a pellet. The pellet was reconstituted in 120 µl Tris buffer. Hundred microliter of standards and samples were incubated with 20 µl primary antibody for 16 hours at 2-8° C. After incubation, 40 µl of tracer was added to each tube and incubated at 2-8° C for 6 hours. ¹²⁵I present in the tracer competes with Ang II present in the sample and standards for the same antibody sites. After 6 hour incubation, solid phase secondary antibody (20 µl) was added to the mixture and incubated at 2-8° C for 30 minutes. The antibody bound fraction was separated from liquid by centrifugation and counted in gamma counter (Micromedic Systems, Seattle, WA). Ang II content was expressed as pg/mL.

12. Urinary creatinine assay

Urinary creatinine assays were performed using a kit purchased from Quidel (San Diego, CA). The excretion rate of creatinine 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 Jaffé reaction where alkaline picrate forms a colored solution in presence of creatinine. Urine samples were
collected and processed as described above. Samples and standards were diluted 1:40 with distilled water. Fifty microliters of diluted samples and standards were added to 96 well plate followed by addition of 150 µl of color reagent (7 mL picric acid + 1 mL 1N NaOH). This mixture was incubated for 30 minutes at R.T. and plate was read at 490 nm (Fusion R Packard plate reader). The intensity of the color produced is directly proportional to the amount of creatinine present in the sample.

**Statistical analysis**

Diabetic db/db and control mice were separated in three groups, metformin treated group (n=8), losartan treated group (n=8) and untreated group (n=19). Students unpaired two tailed t test were performed to calculate p values. For multiple comparisons between two or more groups, one-way and two-way ANOVAs were carried out followed by Fisher’s LSD test. All data obtained are presented as mean ± SEM, and the criterion for statistical significance was set at p<0.05. Data were analyzed using Statistica software (v.7).
RESULTS
Figure 1: Weight (A) and water intake (B) of db/db mice and their age matched lean controls. *p<0.05, control vs. db/db. n=8-12 per group.
<table>
<thead>
<tr>
<th>Mice</th>
<th>Age</th>
<th>Food intake (gms)</th>
<th>Blood glucose (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5 week</td>
<td>N.D.</td>
<td>145.25 ± 4.7</td>
</tr>
<tr>
<td>db/db</td>
<td></td>
<td>N.D.</td>
<td>230.38 ± 13.25*</td>
</tr>
<tr>
<td>Control</td>
<td>8 week</td>
<td>4.44 ± 0.05</td>
<td>158.8 ± 1.7</td>
</tr>
<tr>
<td>db/db</td>
<td></td>
<td>8.92 ± 0.09*</td>
<td>568 ± 32.03*</td>
</tr>
<tr>
<td>Control</td>
<td>10 week</td>
<td>3.77 ± 0.09</td>
<td>154.86 ± 7.18</td>
</tr>
<tr>
<td>db/db</td>
<td></td>
<td>7.94 ± 0.14*</td>
<td>585.39 ± 12.36*</td>
</tr>
</tbody>
</table>

**Table 1**: Time course of blood glucose and food intake in *db/db* mice and their age matched lean controls. *p*<0.05, control vs. *db/db*, n=10 per group. N.D.: not determined
<table>
<thead>
<tr>
<th>Mice</th>
<th>Age</th>
<th>Volume (mL)</th>
<th>Albumin (mg/day)</th>
<th>Protein (mg/day)</th>
<th>Creatinine (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5 week</td>
<td>0.7 ± 0.25</td>
<td>0.06 ± 0.02</td>
<td>3.41 ± 1.22</td>
<td>0.42 ± 0.04</td>
</tr>
<tr>
<td>db/db</td>
<td></td>
<td>2.7 ± 0.52</td>
<td>0.28 ± 0.05*</td>
<td>3.6 ± 0.6</td>
<td>0.19 ± 0.03*</td>
</tr>
<tr>
<td>Control</td>
<td>8 week</td>
<td>1.12 ± 0.21</td>
<td>0.09 ± 0.02</td>
<td>4.57 ± 0.78</td>
<td>0.58 ± 0.03</td>
</tr>
<tr>
<td>db/db</td>
<td></td>
<td>15.8 ± 2.2*</td>
<td>2.09 ± 0.38*</td>
<td>16.34 ± 1.82*</td>
<td>0.09 ± 0.01*</td>
</tr>
<tr>
<td>Control</td>
<td>14-15 week</td>
<td>0.61 ± 0.14</td>
<td>0.04 ± 0.01</td>
<td>4.08 ± 0.85</td>
<td>0.52 ± 0.02</td>
</tr>
<tr>
<td>db/db</td>
<td></td>
<td>25 ± 1.83*</td>
<td>8.02 ± 1.09*</td>
<td>19.46 ± 3.55*</td>
<td>0.06 ± 0.01*</td>
</tr>
<tr>
<td>Control</td>
<td>26 week</td>
<td>0.55 ± 0.07</td>
<td>0.02 ± 0.00</td>
<td>N.D.</td>
<td>0.56 ± 0.02</td>
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<tr>
<td>db/db</td>
<td></td>
<td>30.13 ± 1.23</td>
<td>4.98 ± 0.91*</td>
<td>N.D.</td>
<td>0.07 ± 0.00*</td>
</tr>
<tr>
<td>Control</td>
<td>30 week</td>
<td>1.46 ± 0.27</td>
<td>0.05 ± 0.01</td>
<td>7.61 ± 1.33</td>
<td>0.48 ± 0.02</td>
</tr>
<tr>
<td>db/db</td>
<td></td>
<td>25.29 ± 3.81†</td>
<td>2.86 ± 0.94†</td>
<td>22.12 ± 4.68†</td>
<td>0.09 ± 0.01†</td>
</tr>
</tbody>
</table>

**Table 2:** Time course of urine volume, albumin, protein and creatinine excretion in control and db/db mice. Two-way ANOVA shows main effect of strain on volume \([F_(1,64) = 488.3, \ p<0.0001]\), albumin \([F_(1,63) = 140.34, \ p<0.0001]\), protein \([F_(1,54) = 39.84, \ p<0.0001]\) and creatinine \([F_(1,65) = 870.52, \ p<0.0001]\) excretion. There was a main effect of age on volume \([F_(4,64) = 27.67, \ p<0.0001]\), albumin \([F_(4,63) = 19.75, \ p<0.0001]\), protein \([F_(4,54) = 10.4, \ p<0.0001]\) and creatinine \([F_(4,65) = 2.94, \ p<0.05]\) excretion. There is an interaction between strain and age for volume \([F_(4,64) = 27.68, \ p<0.0001]\), albumin \([F_(4,63) = 27.68, \ p<0.0001]\), protein \([F_(4,54) = 4.43, \ p<0.01]\) and creatinine \([F_(4,65) = 19.75, \ p<0.0001]\). *\(p<0.01\), control vs. db/db. †\(p<0.01\), 30 week vs. 5 week. n=5-7 per group. N.D.: not determined.
Figure 2: Plasma ACE activity in 8 week (A) and 31 week (B) db/db mice and their age matched lean controls. *$p<0.01$, control vs. db/db. n=8 per group.
**Figure 3:** Plasma renin activity in 8 week (A) and 31 week (B) db/db mice and their age matched lean controls. *p<0.05, control vs. db/db. n=8 per group.
Figure 4: Renal ACE activity in 8 week (A) and 31 week (B) db/db mice and their age matched lean controls. *p<0.05, control vs. db/db. n=7-8 per group.
Figure 5: Renal ACE2 activity in 8 week (A) and 31 week (B) db/db mice and their age matched lean controls. *p<0.05, control vs. db/db. n=8 per group.
Figure 6: Urinary ACE2 activity in 5 week (A) and 30 week (B) db/db mice and their age matched lean controls. *$p<0.05$, control vs. $db/db$. $n=6$ per group.
Figure 7: Urinary ACE2 concentration in 5 week db/db mice and their age matched lean controls. *p<0.05, control vs. db/db. n=6 per group.
Figure 8: Renal AT1R protein expression in 8 week (A) and 31 week (B) db/db mice and their age matched lean controls. *p<0.05, control vs. db/db. n=8 per group.
Figure 9: Renal ACE protein expression in 8 week (A) and 31 week (B) db/db mice and their age matched lean controls. *p<0.05, control vs. db/db. n=9 per group.
Figure 10: Renal ACE2 protein expression in 8 week (A) 31 week (B) \(db/db\) mice and their age matched lean controls. *\(p<0.05\), control vs. \(db/db\). n=8 per group.
Figure 11: Plasma Ang II content in 8 week (A) and 31 week (B) db/db mice and their age matched lean controls. *p<0.01, control vs. db/db. n=8 per group.
Figure 12: Renal Ang II content in 8 week (A) and 31 week (B) db/db mice and their age matched lean controls. n=8 per group.
Figure 13: Effect of metformin on blood glucose of control (A) and db/db (B) mice.

*p<0.05 untreated vs. metformin. n=6-8 per group.
Figure 14: Effect of losartan on blood glucose of control (A) and db/db (B) mice. n=6-8 per group.
Figure 15: Glucose tolerance test in control mice after 9 weeks of metformin or losartan treatment. One-way ANOVA showed no main effect of treatment on area under curve. n=8 per group.
Figure 16: Glucose tolerance test in db/db mice after 9 weeks of metformin treatment.

*p<0.05, untreated vs. metformin. n=8 per group.
**Figure 17:** Glucose tolerance test in *db/db* mice after 9 weeks of losartan treatment. n=8 per group.
Figure 18: Urinary albumin excretion in 8 week (A) and 14 week (B) db/db mice and their age matched lean controls. Two-way ANOVA showed a main effect of strain at 8 weeks \( F_{(1,39)} = 129.78, p<0.0001 \) and at 14 weeks \( F_{(1,37)} = 113.93, p<0.0001 \). There was also a main effect of treatment at 14 weeks \( F_{(2,37)} = 6.57, p<0.01 \) but not at 8 weeks. ANOVA also showed an interaction between strain and treatment at 14 weeks \( F_{(2,37)} = 6.56, p<0.01 \) but not at 8 weeks. * \( p<0.0001 \), control vs. db/db. # \( p<0.001 \), untreated vs. treated db/db mice. n=6-8 per group.
Figure 19: Urinary protein excretion in 8 week (A) and 14 week (B) db/db mice and their age matched lean controls. Two-way ANOVA showed a main effect of strain at 8 weeks \(F_{(1,33)} = 74.24, p<0.0001\) and at 14 weeks \(F_{(1,38)} = 38.53, p<0.0001\). There was also a main effect of treatment at 8 weeks \(F_{(2,33)} = 17.86, p<0.0001\) and at 14 weeks \(F_{(2,38)} = 12.5, p<0.0001\). ANOVA also showed an interaction between strain and treatment at 8 weeks \(F_{(2,33)} = 8.82, p<0.01\) and at 14 weeks \(F_{(2,38)} = 12.52, p<0.001\). *\(p<0.01\), control vs. db/db. #\(p<0.0001\), untreated vs. treated db/db mice. n=5-8 per group.
Figure 20: Urinary creatinine excretion in 8 week (A) and 14 week (B) db/db mice and their age matched lean controls. Two-way ANOVA showed main effect of strain at 8 weeks [$F_{(1,43)} = 228.22, p<0.0001$] and at 14 weeks [$F_{(1,43)} = 401.29, p<0.01$]. However, there was no main effect of treatments at 8 weeks and 14 weeks. ANOVA did not show a significant interaction between strain and treatment. *$p<0.0001$, control vs. $db/db$. n=7-8 per group.
Figure 21: Renal ACE activity (A) and ACE2 activity (B) of 18 week \( db/db \) mice and their age matched lean controls. Two-way ANOVA showed main effect of strain for ACE activity \( [F_{(1,25)} = 268.54, p<0.0001] \) and for ACE2 activity \( [F_{(1,27)} = 33.18, p<0.0001] \). However, there was no main effect of treatment on either ACE activity or ACE2 activity. There was no significant interaction between strain and treatment. *\( p<0.001 \), control vs. \( db/db \). \( n=4-6 \) per group.
Figure 22: Urinary ACE activity in 14 week *db/db* mice and their age matched lean controls. Two-way ANOVA shows main effect of strain \(F_{(1,30)} = 71.72, p<0.0001\) but not that of treatment. There was no significant interaction between strain and treatment. *\(p<0.01\), control vs. *db/db*. #\(p<0.05\) untreated vs. treated *db/db* mice. n=6-7 per group.
Figure 23: Urinary ACE2 activity in 8 week (A) and 14 week (B) db/db mice and their age matched lean controls. Two-way ANOVA showed main effect of strain at 8 weeks \( [F_{(1,34)} = 80.19, \ p<0.0001] \) and at 14 weeks \( [F_{(1,39)} = 117.89, \ p<0.0001] \). There was a main effect of treatment at 8 weeks \( [F_{(2,34)} = 9.3, \ p<0.001] \) and at 14 weeks \( [F_{(2,39)} = 8.8, \ p<0.001] \). There was an interaction between strain and treatment at 8 weeks \( [F_{(2,34)} = 8.58, \ p<0.01] \) and at 14 weeks \( [F_{(2,39)} = 8.56, \ p<0.001] \). *\( p<0.001 \), control vs. db/db. #\( p<0.01 \), untreated vs. treated. n=5-7 per group.
**Figure 24:** Effect of losartan on renal AT1R expression in 18 week control (A) and *db/db* (B) mice and their age matched lean controls. *p<0.05, untreated vs. treated. n=8 per group.*
Figure 25: Effect of losartan on renal ACE expression in 18 week control (A) and db/db (B) mice and their age matched lean controls. n=8 per group.
Figure 26: Effect of losartan on renal ACE2 expression in 18 week control (A) and db/db (B) mice and their age matched lean controls. *p<0.05, untreated vs. treated. n=8 per group.
Figure 27: Effect treatments on morphology of pancreatic islets. Representative trichrome staining in control mouse (A), control mouse treated with losartan (B), control mouse treated with metformin (C), db/db mouse (D), db/db mouse treated with losartan (E), db/db mouse treated with metformin (F). Metformin and losartan treatments reduce collagen and preserves integrity of β-cells.
Figure 28: Immunohistochemical staining for AT1R in renal tissue sections from 8 week old mice. Kidney sections were stained as described in materials and methods.
Figure 29: Urinary expression of ACE, ACE2, AT1 and β-actin in 8 week db/db mice.

Five week old db/db mice were treated with metformin (150 mg/kg/day) and losartan (10 mg/kg/day) in drinking water.
RESULTS

1. Anthropometric and metabolic parameters:

To evaluate the progression of diabetes, body weight, water intake, food intake and blood glucose of control and db/db mice were measured.

1.1 Body weight: Young 5 week db/db mice were obese and had significantly greater body weight compared to control mice. Diabetic db/db mice showed consistently higher body weights compared to controls throughout the 12 week study period (Figure 1A, \( p<0.05 \)).

1.2 Water intake: At 5 weeks, water intake was comparable between the two groups (control 6.15 ± 0.76 vs. db/db 7.3 ± 0.54, n=6). Starting from 6 weeks, water consumption of db/db mice increased significantly. With age db/db mice showed consistently higher water intake compared to control mice (\( p<0.05 \)).

1.3 Food intake: At 8 weeks, food intake of db/db mice was significantly higher compared to control mice (Table 1, \( p<0.05 \)). High food intake of db/db mice was also noticeable at 10 weeks (Table 1, \( p<0.05 \)).

1.4 Blood glucose: At 5 weeks, blood glucose of db/db mice was significantly high compared to controls. There was a consistent increase in the blood glucose of db/db mice (Table 1, \( p<0.05 \)). However, blood glucose of control mice remained rather constant.
2. Measurement of renal function:

Renal function was evaluated by measurement of urinary albumin, protein and creatinine. Mice were placed in metabolic cages for 24-hour collection of urine.

2.1 Urinary albumin: Albuminuria is a risk marker for diabetic nephropathy. At 5 weeks, there was a significant difference in urinary albumin excretion between control and db/db mice (Table 2, \( p<0.01 \)). With age, kidney function worsens and albumin excretion increases further. At the age of 30 weeks, db/db mice excreted almost 10 times more albumin compared to 5 week old mice (Table 2, \( p<0.01 \)).

2.2 Urinary total protein: Urinary protein was measured by using Bradford’s reagent. At 5 weeks, urinary protein excretion of db/db mice was not different compared with non-diabetic controls (Table 2). Protein excretion of db/db mice increased significantly with age. By the age of 30 weeks, urinary protein excretion from db/db mice increased 6 fold compared to that of 5 week mice (Table 2, \( p<0.01 \)).

2.3 Urinary creatinine: Urinary creatinine levels are useful in estimating the extent of impairment of kidney function. In 5 week db/db mice, urinary creatinine concentration was significantly less compared to controls (Table 2, \( p<0.01 \)). With the progression of diabetes, urinary creatinine concentration declined further. At 30 weeks also, urinary creatinine concentration of db/db mice was significantly less compared to control mice (Table 2, \( p<0.01 \)).
3. Measurement of enzyme activities:

3.1 Plasma ACE and renin activity:

3.1.1 Plasma ACE activity: Plasma ACE activity was measured using 10 µl plasma samples. At 8 weeks, db/db mice had significant increase in plasma ACE activity compared to controls (Figure 2, \( p < 0.01 \)). The increase in plasma ACE activity was also observed in 31 week db/db mice (Figure 2, \( p < 0.01 \)).

3.1.2 Plasma renin activity: Renin activity was measured in 100 µl plasma samples. There was no significant difference in renin activities between db/db and control mice at 8 weeks and at 31 weeks (Figure 3).

3.2 Renal ACE and ACE2 activity:

3.2.1 Renal ACE activity: Renal ACE activity was measured using 10 µl (80-100 µg) kidney lysate. At 8 weeks, db/db mice had significant decrease in ACE activity compared to controls (Figure 4A, \( p < 0.05 \)). ACE activity in 31 week db/db mice was also significantly reduced compared to controls (Figure 4B, \( p < 0.05 \)).

3.2.2 Renal ACE2 activity: Renal ACE2 activity was measured in 4 µl (32-40 µg) kidney lysate. Renal ACE2 activity was significantly increased in the kidneys of young 8 week db/db mice (Figure 5A, \( p < 0.05 \)). Higher ACE2 activity was also observed in 31 week old db/db mice (Figure 5B, \( p < 0.05 \)).

3.3 Urinary ACE and ACE2 activity

In this project, we propose to use urinary ACE and ACE2 as an index of intra-renal RAS status.
3.3.1 Urinary ACE activity: Urinary ACE activity was determined using ACE REA kit and 50 µl urine. Urinary ACE activity in young 5 week db/db mice was below the detectable limit (data not shown).

3.3.2 Urinary ACE2 activity: Urinary ACE2 activity was determined using fluorogenic substrate Mca-APK (Dnp) in 50 µl urine in presence of ACE inhibitor (lisinopril). Five week mice had a significant increase in urinary ACE2 activity compared to controls (Figure 6A, p<0.05). Urinary ACE2 activity increased 4-fold in db/db mice by the age 31 weeks (Figure 6B, p<0.05).

3.3.3 Urinary ACE2 content: Urinary ACE2 content was measured by ELISA in 50 µl urine. At 5 weeks, ACE2 content was increased significantly in db/db mice compared to control mice (Figure 7, p<0.05).

4. Renal protein expression of ACE, ACE2 and AT1R:

To study the effect of diabetes on renal protein expression of ACE, ACE2 and AT1R western blots were performed.

4.1 AT1R expression: Renal AT1R expression was significantly high in 8 week db/db mice (Figure 8A, p<0.05). Higher expression of renal AT1R was also observed in 31 week db/db mice (Figure 8B, p<0.05). These renal changes may explain hypertension associated with diabetes.

4.2 ACE expression: In young 8 week db/db mice, kidney ACE expression was significantly less compared to their lean controls (Figure 9A, p<0.05). Old 31 week mice
also had lower kidney ACE expression when compared to control mice (Figure 9B, \( p<0.01 \))

4.3 **ACE2 expression:** Young 8 week \( db/db \) mice exhibited significantly high renal ACE2 expression than controls (Figure 10A, \( p<0.05 \)). With progression of diabetes, kidney function declines and so does ACE2 expression. In kidneys of old 31 week mice, ACE2 expression does not differ between control and \( db/db \) mice (Figure 10B).

5. **Plasma and kidney Ang II content:**

5.1 **Plasma Ang II:** Ang II content was evaluated in 45 µl of plasma containing 1 µl EDTA. At 8 weeks, plasma Ang II content of \( db/db \) mice was increased compared with control mice (Figure 11A, \( p<0.01 \)). Plasma Ang II levels in old 31 week \( db/db \) mice were also significantly high compared to controls (Figure 11B, \( p<0.01 \)). This finding is supported by high plasma ACE activity in \( db/db \) mice.

5.2 **Kidney Ang II:** Ang II content was evaluated using 50 µl (450-500 µg) of kidney lysate. There was no significant difference in renal Ang II content between control and \( db/db \) mice at the age of 8 weeks and 31 weeks (Figure 12). This finding is supported by the fact that renal ACE expression and activity are reduced in \( db/db \) mice. Upregulation of ACE2 in kidney degrades excess Ang II, thereby keeping its deleterious effects in control.

6. **Effect of metformin:**

To study the effect of reduction in glycemia on renal and urinary outcomes, 5-8 week mice were treated with metformin (150 mg/kg/day) in drinking water for 12 weeks.
6.1 Effect of metformin on blood glucose: Metformin treatment in 8 week mice did not alter blood glucose levels (data not shown). We believe that the treatment was initiated after a significant rise in blood glucose of 8 week db/db mice. Therefore, another group of 5 week old db/db and control mice were treated with metformin for 12 weeks. Metformin treatment significantly reduced blood glucose of db/db mice during ad libitum feeding (Figure 13B, \( p < 0.05 \)). However, the treatment did not alter blood glucose of control mice (Fig 13A). To evaluate the effect of metformin on glucose handling, we performed i.p. glucose tolerance test. As expected, metformin treatment significantly improved glucose tolerance in db/db mice (Figure 16, \( p < 0.05 \)) but not in control mice (Figure 15).

6.2 Effect of metformin on renal function:

6.2.1 Albumin excretion: Three weeks of metformin treatment (150 mg/kg/day) did not alter urinary albumin excretion in db/db mice (Figure 18A). However, 9 weeks of metformin treatment significantly reduced urinary albumin excretion (Figure 18B, \( p < 0.001 \)). The albumin excretion of control mice did not change during the treatment period.

6.2.2 Total protein excretion: Treatment with metformin significantly decreased urinary total protein excretion in db/db mice (Figure 19, \( p < 0.0001 \)). However, there was no effect of the treatment on urinary total protein excretion of control mice (Figure 19).
6.2.3 Creatinine excretion: Metformin did not change creatinine concentration in urine of either db/db or control mice after 3 and 9 weeks of treatment (Figure 20A).

6.3 Effect of metformin on renal ACE and ACE2 activities: Chronic metformin treatment has no effect on renal ACE and ACE2 activities in either db/db or control mice (Figure 21).

6.4 Effect of metformin on urinary ACE and ACE2 activities: Metformin treatment has no effect on urinary ACE activity (Figure 22) or urinary ACE2 (Figure 23) activity in either db/db or control mice.

6.5 Effect of metformin on renal expression of ACE, ACE2 and AT1: Metformin treatment has no effect on the renal expression of ACE, ACE2 and AT1R in db/db and control mice (data not shown).

6.6 Effect of metformin on morphology of pancreatic islets: Pancreas sections from 18 week mice were stained with Masson’s trichrome to study the effect of metformin treatment on islet morphology. Untreated db/db mice show disarray of cellular architecture and loss of their structural integrity. Chronic metformin treatment improved islet integrity. It also reduced fibrosis by reducing collagen around islets (Figure 27).

7. Effect of losartan

To study the effect of AT1R blockade on renal and urinary outcomes 5 week mice were treated with losartan (10 mg/kg/day) in drinking water for 12 weeks.
7.1 Effect of losartan on blood glucose: Chronic losartan treatment did not alter blood glucose in control (Figure 14A) and db/db mice (Figure 14B). To evaluate the effect of losartan on glucose handling, we performed i.p. glucose tolerance test. Losartan treatment had no effect on glucose tolerance of control and db/db mice (Figure 15).

7.2 Effect losartan on renal function:

Urine samples were collected after 3 and 9 weeks of initiation of the treatments.

7.2.1 Albumin excretion: Three weeks of losartan treatment did not alter urinary albumin excretion in db/db mice (Figure 18A). However, 9 weeks of losartan treatment significantly reduced urinary albumin excretion (Figure 18B, \( p<0.001 \)). There was no effect of the treatment on urinary albumin excretion of control mice.

7.2.2 Total protein excretion: Treatment with losartan decreased urinary total protein excretion of db/db mice (Figure 19, \( p<0.0001 \)). However, there was no effect of the treatment on urinary total protein excretion of control mice.

7.2.3 Creatinine excretion: Losartan did not change creatinine concentration in urine of either db/db or control mice (Figure 20A).

7.3 Effect of losartan on renal ACE and ACE2 activities: Losartan treatment did not affect renal ACE and ACE2 activity after 12 weeks of treatment (Figure 21).

7.4 Effect of losartan on urinary ACE and ACE2 activities: Losartan increased urinary ACE2 activity in db/db mice (Figure 23, \( p<0.01 \)). However, losartan had no effect on urinary ACE2 activity of control mice. Losartan has no effect on urinary ACE activity (Figure 22).
7.5 Effect of losartan on renal expression of ACE, ACE2 and AT1R: Chronic treatment with losartan significantly increased AT1 receptor protein expression in both db/db and control mice (Figure 24, \(p<0.05\)). However, the treatment decreased ACE2 protein expression (Figure 26, \(p<0.05\)) in both db/db and control mice. Losartan treatment had no effect on renal ACE expression (Figure 25).

7.6 Effect of losartan on morphology of pancreatic islets: Untreated db/db mice show disarray of cellular architecture and loss of their structural integrity. Chronic losartan treatment improved islet integrity. It also reduced fibrosis by reducing collagen around islets (Figure 27).

8. Immunohistochemistry of kidney:

To confirm the results obtained from western blot analysis, immunohistochemistry was performed. As expected, AT1R expression was increased significantly in kidney tubules of db/db mice compared to controls (Figure 28).

9. Western blots of concentrated urine:

Urinary excretion of ACE, ACE2 and AT1R protein was studied using western blots. A total of 80 \(\mu\)g of concentrated urinary protein was added in each lane. Immunoblot of ACE revealed two immunoreactive bands at 190 kDa and \(\sim70\) kDa. The smaller band may be a degradation fragment of intact 190 kDa ACE. Immunoblot of ACE2 revealed only one band at \(\sim70\) kDa which represents degradation fragment of integral ACE2. Single immunoreactive bands of AT1R and \(\beta\)-actin were also detected at 43 kDa and 42 kDa respectively (Figure 29). The bands of \(\beta\)-actin were not consistent for each sample so as to be used as a control protein.
DISCUSSION

This study tested the hypothesis that there is upregulation of AT1 receptors and imbalance in ACE/ACE2 homeostasis in the kidneys of db/db mice. In addition we studied the effects of chronic metformin and losartan treatment on renal RAS. Metformin is widely prescribed as a blood glucose lowering drug for type 2 diabetics. It is an insulin sensitizer which is thought to act by reducing hepatic glucose output and enhancing peripheral glucose uptake (Stumvoll et al., 1995; Cusi et al., 1996). Initially a group of 8 week old db/db and control mice were treated with 150 mg/kg/day metformin in drinking water. The treatment did not affect blood glucose of either db/db or control mice. Eight week db/db mice had very high average blood glucose (more than 500 mg/dL) at the start of the treatment. Therefore, we think that metformin was ineffective in reducing blood glucose. To achieve glycemic control, young 5 week db/db and control mice were treated with metformin (150 mg/kg/day) for 12 weeks. On this occasion metformin treatment improved both glycemia and glucose tolerance in db/db mice. The time of initiation of metformin treatment is critical for lowering blood glucose.

In present study, losartan treatment had no effect on blood glucose and glucose tolerance of db/db and control mice. Some studies indicate that AT1 receptor blockers improve β-cell function and glucose tolerance and delay the onset of type 2 diabetes in humans (Lindholm et al., 2002) and in mouse (Chu et al., 2006). Some epidemiological data indicates that RAS blockade delays the onset of type 2 diabetes in patients with
hypertension (Yusuf et al., 2005). ARBs and ACEIs are thought to affect glucose metabolism by improving insulin sensitivity (Moan et al., 1996; Fogari et al., 1998). However, our previous study shows that chronic losartan treatment reduces blood pressure in \( db/db \) mice without affecting glucose tolerance (Senador et al., 2009). One reason for improved glucose tolerance by AT1R blockade could be timing of initiation of treatment. Our finding agrees with previous studies on \( db/db \) mice who initiated the treatment after glucose had started to rise (Mathew et al., 2005; Shao et al., 2006; Sugaru et al., 2007). Moreover, studies on streptozotocin induced diabetes and \( ob/ob \) mice reported failure of chronic losartan treatment to improve blood glucose (Raimondi et al., 2004; Erbe et al., 2006).

Although losartan treatment does not improve glucose tolerance, it improves morphology of pancreatic islets in \( db/db \) mice. RAS components like ACE, angiotensinogen and AT1R are reported be present in pancreatic islets (Lau et al., 2004). Activation of AT1R is believed to inhibit insulin release in response to glucose loading (Carlsson et al., 1998). Ang II also activates NAD(P)H oxidase and thus causes oxidative stress induced \( \beta \)-cell dysfunction and apoptosis (Nakayama et al., 2005). Treatment of \( db/db \) mice with candesartan improves granulation and reduces fibrosis and loss of endothelial cells in islets (Shao et al., 2006). In present study, losartan treatment improves islet morphology and integrity of \( \beta \)-cells. It should be noted that losartan treatment may increase insulin release but may not improve insulin resistance. Therefore, it may not alter glucose levels significantly.

The first sign of nephropathy in diabetics is presence of persistent albuminuria. In \( db/db \) mice, significant microalbuminuria develops as early as 8 weeks (Sharma et al., 2003).
In this study, microalbuminuria was evident at the age of 5 weeks in \( db/db \) mice. At the same time total protein excretion was not different between control and \( db/db \) mice. At this age \( db/db \) mice were hyperglycemic. Moreover, blood pressure in \( db/db \) mice starts to rise after the age of 11 weeks (Senador et al., 2009). Therefore in initial stages of kidney damage is triggered by high blood glucose levels. Hyperglycemia and RAS contribute in the development of nephropathy (Larkins & Dunlop, 1992; Andersen et al., 2000). As mice become old, kidney function declines, measured by glomerular filtration rate (Sharma et al., 2003). At the age of 31 weeks, albumin excretion of \( db/db \) mice increases 10 fold compared to 5 week mice. Hyperinsulinemia has been found to increase transcapillary escape of albumin in non-diabetic subjects, providing a link between albuminuria and insulin resistance (Niskanen & Laakso, 1993). There are conflicting reports in the literature on the effect of metformin on albuminuria. One study reports reduction in urinary albumin excretion after metformin treatment in patients with type 2 diabetes (Amador-Licona et al., 2000) while others did not find any difference between the treated and untreated groups (Imano et al., 1998; UKPDS, 1998). The fact that metformin reduces both fasting (Fujita et al., 2005) and non-fasting (Fruehwald-Schultes et al., 2002) serum insulin levels, explains why it is effective in reducing albuminuria. On the contrary, the effectiveness of losartan in reducing albuminuria in normotensive (Zandbergen et al., 2003) and hypertensive (Brenner et al., 2001; Lozano et al., 2001; Andersen et al., 2002) diabetic patients is well-known. Losartan blocks AT1R and attenuates many of the deleterious actions of Ang II in kidneys such as contraction of mesangial cells and glomerular arterioles (Manley, 2000), increase in membrane pore radius (Remuzzi et al., 1993), glomerular sclerosis and modulation of extra-cellular...
matrix (Leehey et al., 2000). In present study, metformin and losartan significantly reduced urinary total protein excretion after 3 weeks of treatment. However, reduction in urinary albumin excretion was only noticeable after 9 weeks. The duration of treatments and time-points for urine collections were selected to compare our results with the literature (Hu et al., 2009; Chu et al., 2006).

Western blot analysis of kidney shows that there is increase in ACE2 expression and decrease in ACE expression in young db/db mice. This combination attenuates Ang II accumulation and produces more Ang (1-7) in kidneys. Ang II over-activity is believed to play an important role in the pathogenesis of DN (Parving et al., 2001). Ang (1-7), produced by ACE2, is a vasodilator and anti-proliferative peptide that opposes the action of Ang II (Koitka et al., 2008). Interestingly, ACE2 expression decreases and ACE expression increases in the glomerulus of db/db mice, leading to altered glomerular permeability and albuminuria (Ye et al., 2006). Ang II can increase intraglomerular pressure by constricting both afferent and efferent arteriole, thereby stimulating urinary albumin excretion (Remuzzi & Bertani, 1998). It has also been revealed that in animals with experimental diabetes, intraglomerular pressure is increased even before systemic blood pressure rises (Hostetter et al., 1982). Therefore, kidney upregulates ACE2 to counteract pro-hypertensive processes and maintain its function. As they become old, ACE2 expression goes down and kidney function deteriorates.

One of the key findings in the present study is upregulation of AT1R in kidneys of db/db mice. AT1Rs are upregulated by conditions that increase Ang II like dehydration in rats (Barth & Gerstberger, 1999; Sanvitto et al., 1997) and sodium deficiency in mouse (Chen et al., 2003). These findings indicate that expression of AT1 receptors is affected by its
agonist Ang II. In a recent study, researchers found that systemic Ang II infusion increases AT1 receptor mRNA expression in brain tissue of rats (Wei et al., 2009). An increase in oxidative stress also leads to an increase in renal AT1R protein and mRNA expression causing stimulation of sodium transporters and hypertension (Banday & Lokhandwala, 2008). High blood glucose levels can increase reactive oxygen species and eventually oxidative stress to upregulate AT1R. AT1R mRNA and protein levels are also increased in vascular smooth muscles of type 2 diabetic patients (Hodroj et al., 2007). Another study reported upregulation of AT1 receptor together with over expression of Ang II in cavernous tissue of type 1 diabetic rats suggesting its possible role in erectile dysfunction (Yang et al., 2009). High levels of circulating insulin, as seen type 2 diabetics, can also upregulate AT1 receptors in vascular smooth muscles (Hodroj et al., 2007; Nickenig et al., 1998). Additionally, AT1R mRNA expression also increases in pancreatic islets of db/db mice (Chu et al., 2006). This study shows that db/db mice have high levels of circulating Ang II. Therefore, it is plausible that high expression of AT1 receptors in response to high circulating Ang II could lead to diabetes related hypertension in db/db diabetic mice.

Treatment with losartan increases Ang II levels in the plasma in Lewis rats (Ferrario et al., 2005). As a result, losartan also increases cardiac ACE2 activity promoting production of Ang (1-7) form Ang II. High plasma Ang II may also cause stimulation of AT2 receptors to exert its beneficial effects. There are conflicting reports on effect of ARBs on AT1R expression. Some report an increase in AT1R with ARB treatment (Wang et al., 1997; Hu et al., 2009) while others report down regulation (Wei et al., 2009). Our data show that renal AT1R protein expression increases following chronic
losartan treatment in both db/db and control mice. As mentioned earlier db/db mice already have high levels of circulating Ang II and an increase in Ang II results in an increase in AT1R. Therefore, losartan treatment is likely to up regulate AT1R in kidney, where Ang II is sequestered from plasma and is also a primary site of its action. Many reports in the literature suggest that blockade of AT1R increases ACE2 (Soler et al., 2009; Ferrario et al., 2005; Igase et al., 2005; Whaley-Connell et al., 2006). In contrast, study by Xia and others did not find any significant change in ACE2 expression after losartan treatment (Xia et al., 2009). In this study, losartan treatment significantly reduced renal ACE2 protein expression in db/db and control. A recent study shows down regulation of ACE2 mRNA in rat astrocytes after Ang II treatment (Gallagher et al., 2006). Therefore, an increase in plasma Ang II levels by losartan may cause downregulation of ACE2. Although AT1Rs are blocked by losartan, they can be constitutively active and may act as autoreceptors (Zou et al., 2004). In this case, AT1R no longer need Ang II to initiate signaling and may affect expression of ACE2. Another possible mechanism for ACE2 downregulation may be action of Ang II or its derivatives on angiotensin receptors. There was no change in expression of renal ACE protein after losartan treatment. This finding agrees with study by Gallagher and others (Gallagher et al., 2006). However, chronic metformin treatment did not affect RAS parameters in either db/db or control mice.

To confirm the data obtained from western blot, renal ACE and ACE2 activities were determined. As expected, ACE2 activity was increased and ACE activity was decreased in kidneys of 8 week db/db mice. On the contrary, plasma ACE activity and Ang II content were higher in 8 week db/db mice compared to controls and at this age mice are
not hypertensive (Senador et al., 2009). This means that kidney keeps deleterious effects of Ang II in check by upregulating ACE2. As mice become old, kidneys cannot keep up and systemic hypertension develops (Figure 5 vs. Figure 21). A ratio of high ACE2 to low ACE favors less Ang II accumulation in tissues like kidney, helping to maintain their function. Studies in the literature suggest that losartan treatment increases ACE2 activity (Ferrario et al., 2005; Xia et al., 2009; Jessup et al., 2006). In this study, losartan treatment does not change ACE activity in either db/db or control mice. Chronic losartan treatment reduces expression of ACE2 protein in the kidney, but it doesn’t change ACE2 activity. One reason for this could be sequestration of plasma Ang II by the kidney. Reduction in ACE2 protein caused by losartan in the kidney is compensated by high ACE2 activity to cleave the harmful Ang II.

It is reasonable to assume that up regulation of ACE2 in kidneys may be reflected in urine samples. A study by Wakahara et al. has shown that ACE2 mRNA expression in human renal tissue marginally correlated with the degree of proteinuria (Wakahara et al., 2007). A strong correlation between proteinuria and urinary expression of ACE and ACE2 has also been observed in humans (Wang et al., 2008). In this study, we measured urinary ACE2 activity and ACE2 content in young 5 week mice. At this age, db/db mice excreted more ACE2 in urine compared to controls. Additionally, ACE2 activity in the urine of db/db mice was also higher compared to controls. Kidney upregulates ACE2 sensing the change glycemia which is detected by urine analysis. At 5 weeks, urinary albumin excretion was also high in db/db mice but urinary total protein excretion was not different between the two groups. Furthermore, our study shows presence of ACE, ACE2 and AT1 proteins in the urine of 8 week old db/db mice. Urinary ACE and ACE2 enzyme
activities were also high in 8 week db/db mice. ACE and ACE2 are both type 1 integral membrane protein comprising a large extracellular catalytic domain. ACE has been shown to be proteolytically released from cell surface (Parkin et al., 2004). Catalytically active forms of ACE have also been found in urine (Hooper, 1991) and plasma (Senador et al., 2009). On the contrary, ACE2 activity is not detectable in plasma (Elased et al., 2006). Therefore, it likely that source of urinary ACE2 is kidney and that of urinary ACE is plasma. This study shows presence of ACE2 in urine of young db/db mice, using both sensitive catalytic assay and immunoblotting. Urinary ACE2 levels reflect intra-renal RAS status and have potential for diagnosis and prognosis of diabetic renal disease.
CONCLUSION

Cardiovascular and renal diseases are long term complications of diabetes and are leading cause of morbidity and mortality. There is evidence of activation of RAS in diabetic animals and humans. Our previous study showed effectiveness of AT1R blockade in reduction of blood pressure in db/db mice. Present study shows for the first time, that there is upregulation of AT1R protein expression in kidneys of db/db mice that may contribute in the development of diabetes related hypertension. We also show that, chronic losartan (10 mg/kg/day) treatment has no effect on blood glucose levels although it improves morphology of pancreatic islets. In addition, losartan treatment increased AT1R protein expression but decreased ACE2 protein expression in both db/db and control mice. Losartan treatment also reduces urinary albumin and protein excretion in db/db mice.

To investigate the effect of blood glucose reduction, mice were treated with metformin (150 mg/kg/day). As expected, metformin treatment improved blood glucose and glucose tolerance in db/db mice. Our study shows effectiveness of metformin in reducing urinary albumin and protein excretion. However, chronic metformin treatment did not change renal expression or activity of ACE and ACE2.

One of the aims of this study was to examine if ACE2 could be used as a predictor for early phase of diabetic nephropathy. We show that there is an increase in urinary ACE2 activity and ACE2 content in young 5 week, db/db mice. Present study also shows
presence of ACE, ACE2 and AT1R proteins in concentrated urine samples of 8 week mice. Our previous studies show that there is no detectable ACE2 activity in plasma. Therefore, kidney is the primary source of urinary ACE2. These findings suggest that urinary ACE2 could be a potential non-invasive biomarker of diabetic nephropathy.
APPENDIX A

Urinary, but Not Plasma Angiotensin-Converting Enzyme 2 (ACE2), Is Associated with Diabetic Nephropathy

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Diabetic nephropathy (DN) is a leading cause of end-stage renal disease worldwide. Alteration in the renin angiotensin system (RAS) is widely believed to contribute to kidney injury in diabetes. Currently, detection of urinary albumin is the only non-invasive technique used for diagnosis of DN. However, microalbuminuria is a poor predictor for DN, while proteinuria is only detectable in late stage nephropathy. Recent studies suggested that ACE2 is renoprotective in 8 week old murine model of type II diabetes (\(db/db\) mice). The aim of this study was to test the hypothesis that urinary ACE2 is an early marker for intra-renal RAS status and nephropathy in \(db/db\) diabetic mice. Individual urine samples were collected from 5-8 week old male \(db/db\) diabetic and control mice over 24 hours. Renal and urinary ACE2 activities were determined using the conventional fluorogenic substrate, Mca-APK (Dnp) and also using SELDI-TOF mass spectrometry (MS). Urinary ACE2 content was evaluated by ELISA. Urinary albumin, creatinine and protein were measured as an index of kidney damage. Renal ACE and ACE2 expression were determined by western blot. At an early age (5-6 weeks) \(db/db\) mice developed moderate hyperglycemia, hyperinsulinemia and mild albuminuria (p<0.01). Five week old \(db/db\) mice showed a significant increase in urinary ACE2 content (p<0.05) and activity (p<0.01) compared to controls. This finding was confirmed by SELDI-TOF MS. There was increase in renal ACE2 expression and activity of 8 week \(db/db\) mice (p<0.05) while renal ACE expression was decreased in \(db/db\) mice (p<0.01) compared to controls. Interestingly, plasma ACE activity was significantly higher in \(db/db\) mice compared to controls (p<0.01). There was no detectable ACE2 activity in plasma suggesting urinary ACE2 was derived from the kidney. In conclusion, urinary ACE2 warrants further investigation as a potential non-invasive marker for early diabetic nephropathy.

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Upregulation of Angiotensin AT1 Receptors in Hypertensive \textit{db/db} Diabetic Mice

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Hypertension is a major cause of cardiovascular and renal disease in diabetics. In our previous studies we showed that high blood pressure in obese \textit{db/db} diabetic mice can be reduced by angiotensin AT1 receptor (AT1R) blockade. The aim of this study was to assess the changes in renal and aortic AT1R expression and pressor response to chronic angiotensin II (Ang II) infusion in \textit{db/db} mice. Western blot analysis was used to measure renal and aortic protein expression in 8-18 week old male \textit{db/db} mice and their lean controls. AT1R expression was significantly increased in kidneys and aorta of \textit{db/db} mice compared to controls (p<0.01). Immunohistochemistry showed increased immunostaining for AT1R in cortical kidney tubules and glomeruli of \textit{db/db} mice compared to controls. There was also a significant increase in plasma ACE activity (p<0.01) and plasma Ang II (p<0.05) in \textit{db/db} mice compared to controls. Another group of 8 week old \textit{db/db} mice were implanted with carotid telemetric probes and 24 hr mean arterial pressure (MAP), heart rate (HR) and activity were monitored weekly. MAP began to increase in \textit{db/db} mice after the age of 11 weeks during both light and dark periods when compared to controls (p<0.01). At 15 weeks, Ang II was infused at 1000 ng/kg/h for 4 weeks to evaluate its effect on pressor response. The infusion elicited a spike in MAPs of both control and \textit{db/db} mice compared to the previous week (p<0.01). After the initial increase, MAP of control mice regressed towards baseline. However, MAP of \textit{db/db} mice remained elevated throughout the infusion period (p<0.05). This is the first report to demonstrate increases in AT1R expression in \textit{db/db} mice. The prolonged pressor response to Ang II by \textit{db/db} mice could, in part, be attributed to the upregulation of AT1R and preexisting elevation in plasma Ang II.

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