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Role of Angiotensin Converting Enzymes Ace and Ace2 in Diabetes Induced Cardiovascular Dysfunction

Keerthy Kanakamedala
Wright State University

ROLE OF ANGIOTENSIN CONVERTING ENZYMES ACE AND ACE2 IN DIABETES INDUCED CARDIOVASCULAR DYSFUNCTION

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of the requirements for the degree of
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By
Keerthy Kanakamedala
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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY **Keerthy Kanakamedala** ENTITLED "**Role of Angiotensin Converting Enzyme (ACE) and ACE2 in Diabetes Induced Cardiovascular Dysfunction"** BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF *Master of Science.*

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James Lucot, Ph.D.
Director, Graduate Program

_____
Mariana Morris, Ph.D.
Department Chair

Committee on Final Examination

_____
Dr Khalid Elased, RPh, PhD Advisor

_____
Dr James Lucot, PhD

_____
Dr David Cool, PhD

_____
Joseph F. Thomas, Jr. Ph.D.
Dean, School of Graduate Studies

ABSTRACT

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Cardiovascular disease is a long term complication of diabetes, which remains a leading cause of mortality and morbidity. There is recent evidence for activation of renin angiotensin system (RAS) in diabetic animals and humans. Emerging evidence shows that the vasoconstrictor actions of Ang II may be opposed by formation of vasodilator, Ang (1-7). There is limited data on blood pressure in murine models of type 2 diabetes. The aim is to study the role of angiotensin converting enzymes ACE and ACE2 in diabetes induced cardiovascular dysfunction using type 2 diabetic murine mouse models (*db/db* mice). Mice were implanted with carotid telemetric probes for chronic monitoring of MAP, heart rate and activity. At 8-9 weeks age, mice showed hyperinsulinemia, hyperglycemia and increase in body weight compared to their lean controls while MAP was not altered. At an older age (14-15 weeks) there was a significantly increased BP in the *db/db* mice compared to controls. In young (8 weeks) normotensive mice there was a highly significant increase in plasma ACE activity in *db/db* mice compared to controls. In contrast there was increased ACE2 activity and decreased ACE activity in kidney in 8weeks old *db/db* mice compared to controls. No significant difference between ACE and ACE2 activity was observed in lungs and brain. In addition western blot analysis for ACE/ACE2 protein expression also revealed that there was

a significant increase in kidney ACE2 and decrease in kidney ACE protein expression in 8 weeks old *db/db* mice compared to their lean controls. In addition no difference was observed between lung ACE and ACE2 protein expression. Increased plasma and kidney ACE activity and a decreased kidney ACE2 activity were observed in 24 weeks old *db/db* mice. In conclusion: 1. Despite an increase in plasma ACE activity in young (8 weeks) *db/db* mice they were normotensive 2. There was an age dependent increase of BP in *db/db* mice.

3. ACE2 may play a compensatory mechanism against development of hypertension in *db/db* mice in the early stage of type2 diabetes. 4. The up regulation of ACE coupled with a down regulation of ACE2 might be the cause for hypertension at a later stage of diabetes.

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Abbreviations

RAS …….Renin-Angiotensin system

BP ……....Blood Pressure

HR……Heart Rate

MAP…….Mean arterial pressure

SAP……...Systemic arterial pressure

ACE……..Angiotensin converting enzyme

ACE2……Angiotensin converting enzyme 2

Ang II…….....Angiotensin II

Ang (1-7)……...Angiotensin (1-7)

CVD……...Cardiovascular disease

AT1 receptor……..Angiotensin I receptor

AT2 receptor……...Angiotensin II receptor

BK……...Bradykinins

NO……...Nitric oxide

ARB…….Angiotensin receptor blocker

KO……...Knock-out

WT……...Wild type

db/db……Diabetic mice model

SEM…….Standard error of the mean

EDTA……...Ethylene Diamine Tetra acetic acid

SELDI-TOF MS…….....Surface-Enhanced Laser Desorption Ionization Time of Flight Mass Spectrometry

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INTRODUCTION

Type 2 diabetes

Diabetes is occurring at an epidemic rate in the United States and other western countries (Mokdad *et al.* 2000). The pathology of diabetes is characterized not only by hyperglycemia but also by hypertension, dyslipidemia, microalbuminaria and inflammation (Sowers and Stump 2004a). Since up to 75% of cases of cardiovascular disease (CVD) in patients with diabetes can be attributed to hypertension, aggressive management of elevated blood pressure (BP) is essential for reduction in cardiovascular morbidity (Sowers and Stump 2004a). For example, recent clinical trials demonstrated that strict glycemic control in patients with type 2 diabetes correlates with a reduction in cardiovascular complications (Horio *et al.* 2004). The patients at risk for diabetes also present higher baseline serum glucose concentration, increased body index, elevated systolic blood pressure and reduced serum high-density lipoprotein-cholesterol (Lindholm *et al.* 2002a)}.

Diabetes and hypertension

Diabetes and arterial hypertension frequently coexist (Genuth 2006), and it is well known that patients presenting with both disorders have increased antihypertensive drug requirements to achieve the recommended goals of blood pressure control and elevated cardiovascular risk (Alderman 2004). The connection between diabetes and cardiovascular disease is profound and clinically extraordinarily important. Among

people with diabetes, type 2 diabetes specifically, the major cause of death is cardiovascular disease (Chobanian *et al.* 2003). The global mortality attributable to diabetes in the year 2000 was estimated at 2.9 million deaths, representing 5.2% of all deaths globally (Jacob 1999). These figures are expected to increase, with the prevalence diabetes worldwide predicted to rise from 4 % in 1995 to 5.4% by the year 2025, by which time it is estimated that there will be around 300 million adults with diabetes globally (Horio *et al.* 2004). Diabetes and hypertension independently predispose to renal and cardiovascular complications (Campbell 1987). Despite the importance of the relationship between diabetes and cardiovascular disease, little is known about the biologic basis of this association (Chobanian *et al.* 2003).

Hypertension affects 20-60 % of the individuals with diabetes (Wingard *et al.* 1983). The prevalence of hypertension in diabetic population is 1.5 to 3 times higher than that of non-diabetic groups. In type 1 diabetes, hypertension usually occurs in association with nephropathy whereas in type 2 diabetes, it may be present at early diagnosis stage, as a part of the metabolic syndrome (Campbell 1987). Hypertension is twice as common in the type 2 diabetic, compared with the nondiabetic; it is present in 85% of subjects with nephropathy , and the coexistence of these two conditions is associated with an increase in the risk of retinopathy, renal failure, and cardiovascular disease (CVD)(Chen *et al.* 2005). An increase of 5 mm Hg in systolic or diastolic blood pressure increases the risk of CVD by 20% to 30%; a diastolic blood pressure above 70 mm Hg increases the risk of retinopathy; and end-stage renal disease is 5 to 6 times more common in the hypertensive diabetic patients (Carey *et al.* 2001).

Already in the diabetic setting, agents that interrupt the renin-angiotensin system and increasingly also the aldosterone pathway appear to be particularly useful in diabetic renal and cardiovascular disease (Pitt *et al.* 2001). Hypertension in diabetic patients has to be controlled to reduce the occurrence of macro vascular complications of diabetes. Observational studies show that people with both diabetes and hypertension have approximately twice the risk of cardiovascular disease as non-diabetic people with hypertension. In addition to its role in macro vascular complications, hypertension greatly increases the risk of renal insufficiency and diabetic nephropathy (Wingard *et al.* 1983).

People with diabetes are two to four times more likely to suffer strokes and once having had a stroke, are two to four times as likely to have a recurrence (Mokdad *et al.* 2000). Deaths from heart disease in women with diabetes have increased 23 percent over the past 30 years compared to a 27 percent decrease in women without diabetes. Deaths from heart disease in men with diabetes have decreased by only 13 percent compared to 36 percent decrease in men without diabetes (Mokdad *et al.* 2000). Diabetes is a leading cause of death in the United States of America (USA). Based on the statistics in the year 2007, diabetes contributed to approximately 224,092 deaths (Brenner *et al.* 2001). In the year 2007, the total estimated economic costs spent on type2 diabetes were approximately $132 billion. There are 20.8 million children and adults in the United States, or 7% of the population, who have diabetes (Mokdad *et al.* 2001). While an estimated 14.6 million have been diagnosed, unfortunately, 6.2 million people (or nearly one-third) are unaware that they have the disease (Mokdad *et al.* 2001).

Renin-Angiotensin system (RAS)

The implication of the renin-angiotensin system (RAS) in the regulation of the cardiovascular system has been well known for many years (Brosnihan *et al.* 2003;Carey *et al.* 2000). Accordingly, many medications have been developed to treat several pathologies, e.g. diabetes related hypertension, hypertension and heart failure and RAS is emerging as one of the major target in the treatment of cardiovascular diseases (Danilczyk *et al.* 2003).Therefore, the whole dynamic of RAS has to be evaluated. In this project the main emphasis will be on RAS and its enzyme components ACE (Angiotensin converting enzyme) and ACE2 (Angiotensin converting enzyme 2) and their specific effect in diabetes related hypertension offering a new perspective on this potential target for the treatment of cardiovascular diseases and hypertension. A better understanding of the mechanisms of RAS will open new horizons for novel drug therapy by RAS inhibition in treating type 2 diabetes worldwide (Crackower *et al.* 2002). The importance of the Renin-Angiotensin system (RAS) and the pivotal role of angiotensin II (Ang II) in the pathogenesis of hypertension and other cardiovascular diseases is widely investigated. (Ferrario and Chappell 2004). The RAS has long been recognized to play a crucial role in the regulation of blood pressure and electrolyte balance. It is an enzymatic cascade reaction. The processing scheme begins with the conversion of angiotensinogen (AGT) to angiotensin I (Ang I) via renin (de *et al.* 2000;Peach 1977). Ang I has little or no biological activity but is converted across vascular beds, particularly in the lungs, to the octapeptide Ang II (Carey *et al.* 2001). Angiotensin converting enzyme (ACE) plays a central role in the scheme; it catalyzes the cleavage of the C-terminal dipeptide (L-histidyl-L-leucine) from the inactive decapeptide Ang I, to produce potent

vasoconstrictor Ang II. The traditional view that Ang II is the key product of RAS has been questioned with the discovery of angiotensin converting enzyme 2 (ACE2), as well as growing evidence for a physiological role for Ang (1-7)) (Donoghue *et al.* 2000;Tikellis *et al.* 2003). ACE2 converts Ang I to Ang 1-9, which can be further hydrolyzed by ACE to form Ang 1-7(Ferrario and Chappell 2004). ACE2 also catalyses the generation of Ang 1-7 by removal of a COOH-terminal amino acid from Ang II (Tipnis *et al.* 2000;Vickers *et al.* 2002b). The putative product of ACE2, Ang 1-7, is a potent vasodilator and mediates effects opposite to those of Ang II in several tissues (Ferrario and Chappell 2004).

Angiotensin Converting Enzyme (ACE)

ACE was discovered in plasma in 1956 by Leonard T. Skeggs (Skeggs, Jr. 1993). ACE is also present in other organs such as kidney, heart, pancreas, and brain. ACE is a monomeric, membrane bound, zinc and chloride dependent di-peptidyl carboxypeptidase. ACE is an important therapeutic target for the management of hypertension during diabetes (Lieberman *et al.* 1986). Inhibitors of ACE that reduce the formation of Ang II have been highly successful in the management of hypertension in type 2 diabetes. Pathological activation of tissue ACE with resulting increase in local Ang II produces deleterious effects on the kidney and the heart during organ remodeling,

ischemic injury and restenosis (Ferrario and Chappell 2004). The kidney, under the regulation of Ang II (produced by ACE) and aldosterone, maintains the electrolyte balance in the body. In rats, there was dramatic increase in ACE associated with development of hypertension (Crespo *et al.* 2003). The present study of increased ACE

expression revealed that higher ACE was associated with an increased diabetic pathology, in terms of renal function and blood pressure (Fernandez *et al.* 2003).

Angiotensin converting enzyme 2 (ACE2)

ACE2, a recently identified homologue of ACE, represents a new potentially important target in cardiovascular disease in type2 diabetes. ACE2 was identified and cloned recently by Donoghue *et al* and Tipnis *et al* and was described in kidney, heart and the testis. ACE2 also expresses in the brain, pancreas, lungs and other tissues (Brosnihan *et al.* 2003;Ferrario and Chappell 2004). There is no evidence for the presence of ACE2 in the plasma or circulating ACE2. Previous studies have shown that ACE2 knock-out mice had an elevation of plasma Ang II levels, supporting the hypothesis that ACE2 provides an alternative pathway for Ang I and Ang II degradation (Carey *et al.* 2001). The vasoconstrictor actions of Ang II may be opposed by the formation of Ang (1-7). ACE2 converts Ang II into Ang (1-7) with a higher catalytic efficiency than any other known Ang (1-7) forming enzyme. The reported potential involvement of ACE2 in both Ang II degradation and Ang (1-7) production add another arm of complexity to the RAS. Ang (1-7) binds to a non-Ang II type 1/Ang II type 2 receptor originally defined as the Mas oncogene and mediates functions that oppose the actions of Ang II (Santos *et al.* 2003). ACE2 displays 42% amino acid identity to ACE and, like ACE, is a type I integral membrane protein that can be proteolytically shed from the plasma membrane. More recently, ACE2 has gained considerable attention as being a receptor for the corona virus that causes severe acute respiratory syndrome (SARS) and as being protective against severe acute lung failure (Muller-Wieland *et al.* 1998). ACE2 is not inhibited in any way

by the ACE inhibitors, captopril and lisinopril {Wysocki, 2006 378 /id. Importantly, ACE2 not only controls angiotensin II levels but functions as a protease on additional molecular targets that could contribute to the observed in vivo phenotypes of ACE2 mutant mice(Danilczyk and Penninger 2006).

Renin Angiotensin System Enzyme Cascade

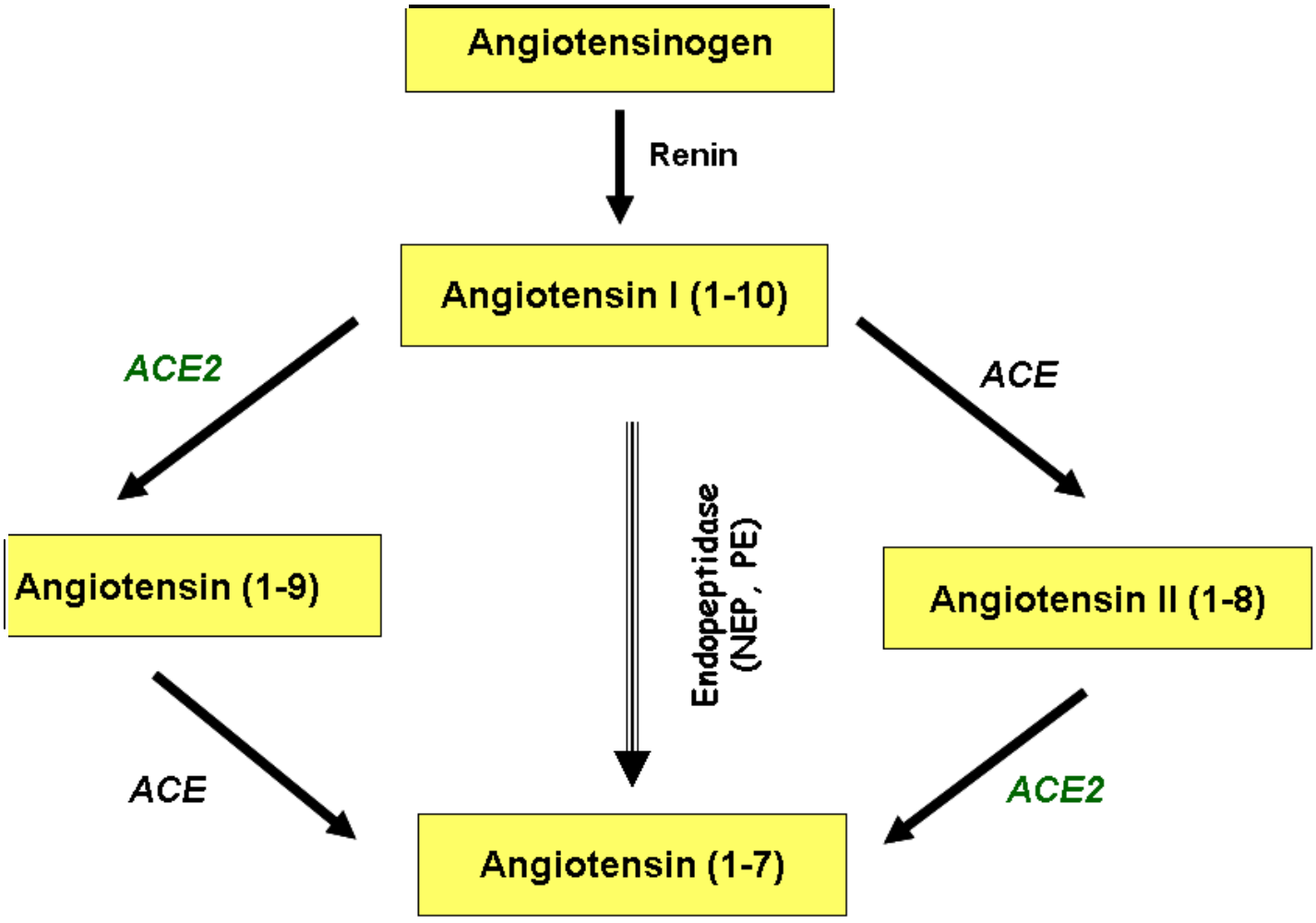


Diagram 1: A schematic representation of the Renin-Angiotensin system cascade

Crackower *et al*. reported that several hypertensive animal models exhibit reduced ACE2 expression in the kidney and that the ACE2 knockout mice exhibit severe glomerulosclerosis (Crackower *et al.* 2002). Despite the growing evidence that ACE2 is a component of the RAS, the influence of ACE2 on angiotensin metabolism within the kidney of other tissues is not well known, particularly in animal models. In the human kidney, ACE2 was described in the proximal tubule epithelial cells. Additionally, conflicting results have been reported in renal pathologies, such as diabetic nephropathy in humans and rodents, suggesting both an increase and decrease in ACE2 protein (Ye *et al.* 2004). *Tikellis et al* were the first to report that ACE2 expression were reduced in the kidneys of rats with longstanding diabetes mellitus (Tikellis *et al.* 2003), whereas more recently, *Ye et al,* and *Wyoscki et al,* reported that there was an early increase in ACE2 expression and activity in *db/db* diabetic mice (Wysocki *et al.* 2006;Ye *et al.* 2004).

There is no evidence for the presence of ACE2 in the plasma (*Elased*, 2005 1 /id). In a previous human study it has been shown that circulating ACE2 was ~ 100- fold lower than ACE and was detectable in only 40 subjects (Rice *et al.* 2006). In this project we planned to investigate for detectable levels of ACE2 in plasma. There is no data related to blood pressure values in these mice at an early and later stage of type 2 diabetes. The rationale for the present study was twofold: first, activation of RAS plays an important role in the development of experimental and clinical diabetes induced hypertension, and second, the recent discovery of ACE2, has revised our understanding of Ang peptide processing.

Local Ang II constricts the efferent glomelular arterioles thereby maintaining the glomelular filtration rate, and preserves the filtration function of the kidney during changes in the blood pressure (BP) (Bruemmer *et al.* 2003;Li and Ren 2006). An increased ACE2 activity increases Ang (1-9), which in turn may compete with Ang I for ACE thereby having an additional effect on the decrease of Ang II formation (Turner *et al.* 2002).

The primary function of RAS is to maintain homeostatic blood perfusion particularly during conditions of blood volume depletion or in the presence of hypotensive stimuli. This is accomplished by AngII's regulation of arterial pressure by stimulating sympathetic tone, vasoconstriction and secretion of aldosterone that leads to sodium and water retention by the kidney (Hummel *et al.* 1966). However, the loss of RAS's homeostatic balance induces hypertension that is associated with cardiovascular and renal diseases such as heart failure, hypertrophic remodeling, myocardial infarction (MI), atherosclerosis and nephrosclerosis (Hummel *et al.* 1966). Some of the most compelling evidence for RAS involvement in hypertension comes from genetic manipulation of its constituents. Mice lacking key components of this system such as renin, ACE, AGT or the AT1 receptors have greatly advanced our knowledge of the RAS and confirmed its role in BP control since these mutants all show reduced BP (Jacob 1999;Shiuchi *et al.* 2004).

The balance between Ang II and Ang (1-7), reflecting ACE and ACE2 activities, respectively, is to be considered as physiologically significant ratio (Huentelman *et al.*

2004). Over reactivity of the renin-angiotensin system (RAS) has been identified as an important determinant that is implicated in the etiology of diabetes, heart disease and therefore represents a major target for therapy (Parving *et al.* 2001).

Angiotensin II is the main effector hormone of the renin-angiotensin system (RAS), which regulates blood volume, arterial pressure, and cardiac and vascular function. Ang II binds to two distinct receptors, type I (AT1) and type II (AT2) (Ferrario *et al.* 2005b). The Ang AT1 receptor mediates most of the known physiological and pathophysiological actions of Ang II in renal, cardiovascular and neuronal systems (Campbell 1987). Ang II also binds to AT1 and AT2 receptors, inducing a counter-regulatory vasodilatation that is largely mediated by bradykinins (BK) and nitric oxide (NO) (Carey *et al.* 2001).

There is clinical data to document the effectiveness of using ACE inhibitors and Angiotensin receptor blockers (ARB) in diabetics (Shinozaki *et al.* 2004). There is evidence that ACE levels are increased in diabetic animal models and that ACE inhibitors and ARBs (AT1 receptor blockers) lower blood pressure and improves the kidney function (Guo *et al.* 2005a). Recent comparative trials in patients with type2 diabetes and hypertension have suggested that AT1 receptor antagonists may be superior to all other alternative antihypertensive agents (Chu *et al.* 2006a).

Losartan (AT1 antagonist) treatment in some animal models has improved the sensitivity to insulin. However, there is no information on the specific role of Ang AT1a receptors in controlling the blood pressure (Shinozaki *et al.* 2004). There is very little information as

to the mechanisms by which AT1 receptor blockers like losartan produce reduction in blood pressure and have control on the chronic cardiovascular changes in mice during type2 diabetes mellitus. There is clinical data to document the effectiveness of ACE inhibitors and Ang receptor blockers (ARB) in diabetics (Gilbert *et al.* 2003). In addition, long term treatment with losartan lowered the risk of developing diabetes (Lindholm *et al.* 2002b). A general role for the RAS in diabetes-induced hypertension is supported by the depressor effects of ACE inhibitors and ARBs in rats (Stubbs *et al.* 1994). In a recent clinical trial candesartan appears to prevent diabetes in heart failure patients, suggesting that the RAS is implicated in glucose regulation (Yusuf *et al.* 2000).

A better understanding of the protective effects of RAS inhibition on type 2 diabetes is of profound importance to human health, given the massive rise in the incidence of type2 diabetes and related cardiovascular problems worldwide (Chu *et al.* 2006b). It is for this reason that we used animal models to study the evolution of hypertension in diabetic mice in relation to changes in blood glucose, insulin and indices of the RAS

Animal models of type2 diabetes (*db/db* mice)

In the present study we used the *db/db* genetic mouse model that has a point mutation in the diabetes (*db*) gene encoding the leptin receptor gene (Coleman 1983). At early stages, they serve as a good model for type 2 diabetes, characterized by hyperinsulinemia, and progressive hyperglycemia (Coleman and Hummel 1974). Although there are several reports of vascular hyperreactivity in *db/db* mice(Sowers and Stump 2004b), there is limited and conflicting data on the in vivo cardiovascular changes in these models.

Recently there are reports of increased (Bagi *et al.* 2005), decreased (Kosugi *et al.* 2006) or no change in BP in *db/db* mice (Moriyama *et al.* 2004). However, cardiovascular studies in these genetic models are limited with little information on data on indices of the RAS. We conducted comprehensive cardiovascular studies to establish the role of diabetes in triggering an increase in BP and changes in other cardiovascular parameters in *db/db* diabetic mice.

A major role for the local RAS in the development and progression of diabetic nephropathy has been clearly demonstrated (Brenner *et al.* 2001). Although ACE2 expression seems to be down regulated in diabetes induced kidney disease, there is evidence suggesting that ACE2 expression is increased in early phases of diabetes in the absence of renal injury (Ye *et al.* 2004). It has been shown previously that the significantly increased ACE2 activity and expression in young (6-8 weeks) *db/db* mice might be the cause for renal protection at that early stage (Ye *et al.* 2004). But there is limited data on the blood pressure and heart rate in these young mice. Thus, it would now be of interest to specifically examine the ACE2 levels in young and old mice in relation to the changes in cardiovascular parameters. Previous data suggests that in mice, early development of diabetes paralleled increased ACE2 protein expression in the renal cortical tubules while ACE expression is decreased ACE2 might be renoprotective in early stages of type 2 diabetes (Ye *et al.* 2004).

There is much information to show that the RAS is critical in regulation of blood pressure, water balance and endocrine secretion. Nevertheless, there are questions as to

exact mechanisms by which RAS functions, particularly as to the relationship between ACE and ACE2 in the regulation of cardiovascular parameters in murine models of type2 diabetes. To close this knowledge gap we studied the role of ACE and ACE2 in diabetes induced hypertension with a particular focus on the newly discovered component of RAS, ACE2.

Objective:

The objective of this project is to study the changes of blood pressure in relation to changes in ACE/ACE2 balance in murine models of type 2 diabetes. For this project we have used (*db/db)* mice as type 2 diabetic murine models. Another goal is to investigate the role of Ang AT1 receptors in the development of cardiovascular dysfunction in diabetic (*db/db*) mice. Radio telemetric probes were inserted in 8-9 weeks old *db/db* mice and blood pressure was monitored 14-15 weeks. To investigate the role AT1 receptor in diabetes induced hypertension, mice were treated with losartan (10 mg/kg/day for 12 weeks) and cardiovascular parameters were measured. In addition, ACE and ACE2 enzyme activities and protein expression were measured in young (8 weeks) and old (24 weeks) *db/db* mice. The concurrent measurement of ACE and ACE2 activity should be helpful in the evaluation of tissue specific alterations of these two enzymes, involved in local Ang II formation and degradation.

Hypothesis and Specific aims

Hypothesis

The hypothesis of this proposal is that cardiovascular complication of diabetes such as increased blood pressure is related to an upregulation of ACE and downregulation of ACE2.

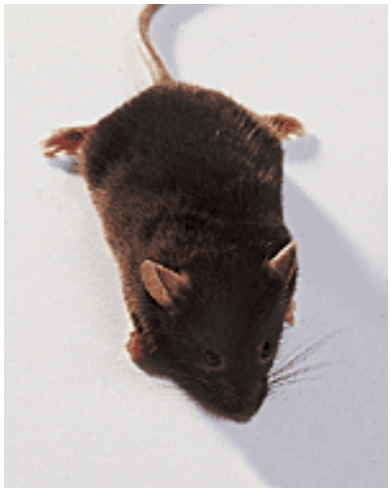
Specific Aims

1. To test the hypothesis that there is an increase in blood pressure (BP) during the course of diabetes in *db/db* mice. (Cardiovascular parameters were measured in young and old *db/db* mice by radio telemetry).
2. To test the hypothesis that there is up regulation of ACE and down regulation of ACE2 in *db/db* mice which contributes to the high blood pressure at a later stage of diabetes (ACE1 and ACE2 enzyme activity and protein expression were measured in normotensive and hypertensive *db/db* mice and their lean controls).
3. To investigate the role of AT1 receptor on blood pressure in *db/db* mice. Eight weeks old *db/db* mice were treated with losartan and cardiovascular parameters were measured.

Materials and methods

Animals:

Genetically diabetic male 7-8 weeks old BKS.Cg-*m* +/+ *Lepr* $^{db/J}$ (*db*/*db*) mice and their age matched non-diabetic littermates were used for the experiments. The animals were obtained from the Jackson Immunoresearch Laboratory (West grove, PA). Animals were housed individually at 22°C at room temperature with a 12:12-h light/dark cycle (0600 h – 1700 h, lights on) with *ad-libitum* access to water and standard mouse chow. All experimental protocols were approved by the WSU Animal care and Use Committee.



Lean Control mice

Obese diabetic mice

Diagram 2: Lean control mice and their age matching obese diabetic mice.

1. Measurement of cardiovascular parameters:

Radio telemetry is a direct method of blood pressure. It is the method preferable for long-term recording of blood pressure and heart rate. Radio telemetric measurement of mouse blood pressure, while initially invasive, has been shown to accurately monitor systolic pressure, diastolic pressure, heart rate, and loco motor activity. For the surgical procedure, mice were anesthetized with ketamine/xylazine (120:20 mg/kg, im). Radio telemetric catheters (model TA11PA-C10, Data Sciences International, St. Paul, MN) were inserted into the left common carotid artery, with the transmitter body positioned subcutaneously on the right flank. Recordings were made after the mice had fully recovered from surgery (6-7 days). Continuous 24 hours recordings of the blood pressure (BP) and heart rate (HR) were made every week after the mice had fully recovered from surgery (5-7 days). Locomotor activity was also recorded by radio telemetry which detects animal movement.

2. Chronic treatment with losartan:

Eight week old obese *db/db* mice were randomly assigned to losartan dose groups. Each group consisted of 6–8 mice. The mice received 10 $mg \cdot kg^{-1} \cdot day^{-1}$ of losartan dissolved in their drinking water for 12 weeks. An additional two groups of age-matched control mice received the same treatment as the experimental groups.

3. Measurement of blood glucose levels:

Blood glucose samples were determined using an Accu – Check Advantage Blood Glucose Monitor (Roche Diagnostic Corporation, Indianapolis, IN). Blood glucose samples were taken from a cut made on the tail vein. Blood glucose is expressed in mg/dl.

4. Collection of tissue and plasma samples:

Mice were decapitated and trunk blood was collected in ice-chilled heparinized tubes. Blood was centrifuged at 10,000 x g for 10 mins. The supernatant clear plasma was immediately separated and stored frozen at -80°C for future use. Kidney, brain, liver, pancreas, and lungs were quickly removed, frozen in liquid nitrogen and stored at -80°C for enzyme activity assays. Tissues were homogenized on ice in 1:9 (w/v) of Tris Hcl (50 mM, pH 7.4) containing 2 mM PMSF. The homogenate was centrifuged at 10,000 x g for 10 min to remove cellular debris. Total protein content was determined in the supernatant using the Bradford protein assay with BSA as a standard (BioRad Protein Assay Reagent, BioRad Labs, Hercules, CA).

5. Measurement of plasma insulin levels:

Insulin levels were measured in 10µl aliquots of plasma in *db/db* and control mice using Insulin ELISA Immunoassay kit from Linco Research Ltd, CA. The absorption at 450 nm and 590 nm was read in a Fusion^{R} Packard plate Reader. The final concentration of plasma insulin was expressed in ng/ml of plasma.

6. Measurement of ACE activity:

ACE activity assays were performed using an assay kit purchased from ALPCO Diagnostics (Windham, NH, USA). In minin tubes 10 µl plasma or tissue extracts (50-100 µg) were incubated with 100 µl of HEPES buffer (pH 8) containing the synthetic substrate H^3 hippuryl glycine glycine (H^3 Hip-Gly-Gly) at 37º C in a constant shaking water bath . After 60 min incubation the reaction was terminated by adding 50 µl of 1 N hydrochloric acid solution. Liberated H^3-Hippuric acid due to ACE activity in samples was separated from unreacted substrate by addition of 1.5 ml of scintillant cocktail (from the kit) and measured in a beta counter (Packard 1800TR Liquid Scintillation Analyzer). ACE activity was expressed in units/µg protein used as previously described. (Neels *et al.* 1982) One unit (U) of angiotensin converting enzyme activity is defined as the amount of enzyme required to release 1 µmol of hippuric acid per minute per litre of plasma at 37º C under the assay conditions described (i.e. U/ml = kU/L = µmol/litre per hour).

7. Measurement of ACE2 activity

ACE2 activity was measured using two flouregenic substrates, 7-Mca-*YVADAPK* [Dnp] purchased from (R&D sys Inc, Minneapolis, MN) and 7-Mca-*APK* [Dnp] purchased from (BIOMOL systems, Plymouth meeting, PA), as described before (Vickers *et al.* 2002a;Wysocki *et al.* 2006) with some modifications. Cleavage of this substrate by ACE2 removes the 2, 4-dinitrophenyl moieties that quench the fluorescence of the 7-methoxy-coumarin moiety, thus resulting in increased fluorescence (Vickers *et al.* 2002a). The specificity of the ACE2 enzyme activity assay was determined using kidney extract from ACE2 Knockout (KO) mice. ACE2 flouregenic activity assays were

performed with final concentration of 100µM flouregenic substrate in total volume of 100 µl of the ACE2 assay buffer (75 mM Tris buffer, 300mM NaCl, 10 µM of Zinc Chloride, pH-7.4). Reactions were initiated by the addition of tissue extract at room temperature and carried out for 2 or 18 hours. The liberated fluorescence was read at an excitation wavelength = 328 nm and emission wavelength = 393 nm (Fusion^{R} Packard plate reader). The reaction products (fluorescence units) were quantified by using a standard solution of Mca and ACE2 activity was expressed as pmoles of Mca / hr/ microgram of protein as previously described(Vickers *et al.* 2002a).

8. Western Blot Analysis of ACE and ACE2:

Kidney, brain and lungs from control and diabetic mice were quickly removed and homogenized on ice in lysis buffer containing protease inhibitors and EDTA (Complete, Minin, EDTA Tablet, Roche Diagnostics, Mannheim, Germany). Tissue homogenate was centrifuged at 10,000 x g for 10 min to remove cellular debris. Total protein content was determined in the supernatant using the Bradford protein assay with BSA as a standard (BioRad Protein Assay Reagent, BioRad Labs, Hercules, CA). Samples were mixed with 25 µl Laemli loading buffer (4% SDS, 100 mmol Tris-Cl, pH 6.8, 20% glycerol, 0.2% bromophenol blue, and 200 mmol/L dithiothreitol) and boiled for 10 minutes. Twenty miclolitre (30-50 µg protein per lane) were be loaded onto a 10% sodium dodecylsulfate (SDS) –tris-glycine polyacrylamide gel and separated by electrophoresis. Subsequently, protein were electro transferred onto a PVDF membrane in transfer buffer (20Mm Tris, 150mM Glycine, 400 ml Methanol) for 2 hr. The PVDF membranes were blocked for 1 hour in 10% non-fat dry milk solution in 2 % Tween-PBS. For analysis of ACE the

membranes were probed with primary monoclonal mouse anti- ACE (1:200, Chemicon International ltd, Temecula, CA) for 3 days at 4ºC. The membrane was then washed in Tween-PBS, and incubated with secondary antibody HRP-conjugated donkey Anti-Mouse IgG (1:40000; Jackson immunoresearch Laboratories, PA). For analysis of ACE2, the membranes were probed with primary polyclonal rabbit anti-ACE2 (1:200, Santa Cruz biotechnology Ltd, Santa Cruz,) for 3 days at 4ºC. The membrane was then washed in 2% Tween-PBS, and incubated with secondary antibody HRP-conjugated donkey Anti-rabbit IgG (1:40000; Jackson immunoresearch Laboratories, PA) for 2 hours and washed (3*15 minutes TBST). Blots were detected using an enhanced chemiluminescence and visualized using Fuji film image analyzer (LAS 3000 image Quant, Sunnyvale, *CA*). The ACE and ACE2 bands were visualized at an apparent molecular weight of 170 kDa and 90 kDa respectively.

Statistical Analysis

Results are expressed as means ±SEM for all groups. When only two groups were compared in this project, the probalities of chance differences between the experimental groups were calculated with Student's unpaired two tailed *t* test. When two same groups of animals are considered then a Student's paired two tailed *t* test was done. For all the comparisons, $P<0.05$ was considered to be statistically significant. For multiple comparisons between two or more groups ANOVA was done to check whether the results are significant or not.

RESULTS

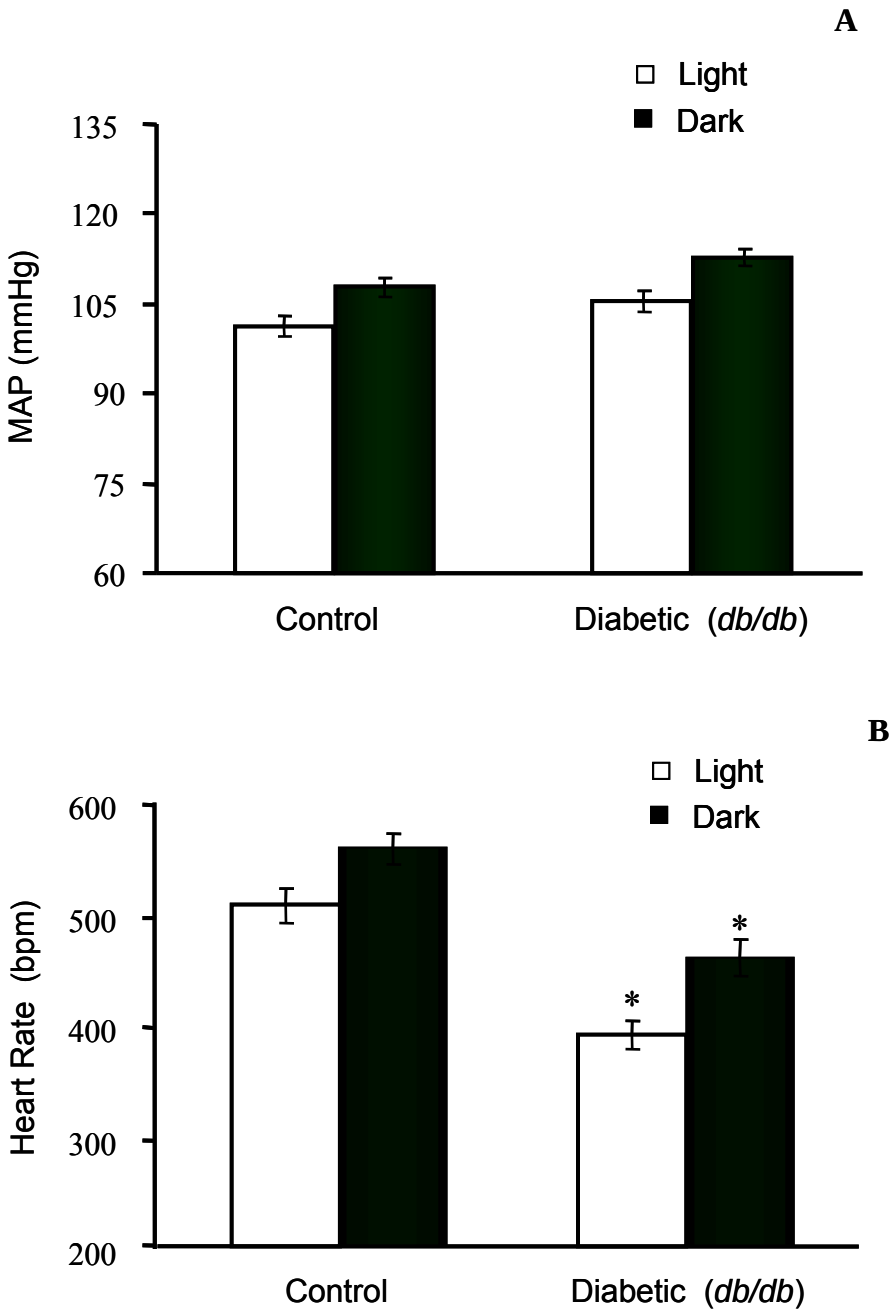


Figure 1: Mean arterial pressure (MAP) and heart rate (HR) in young (8 weeks) *db/db* diabetic mice. MAP and HR were recorded in young *db/db* mice and their age matching control mice in both light and dark periods (**P*<0.01 control vs. diabetic, n=6). Values are expressed as ± SEM.

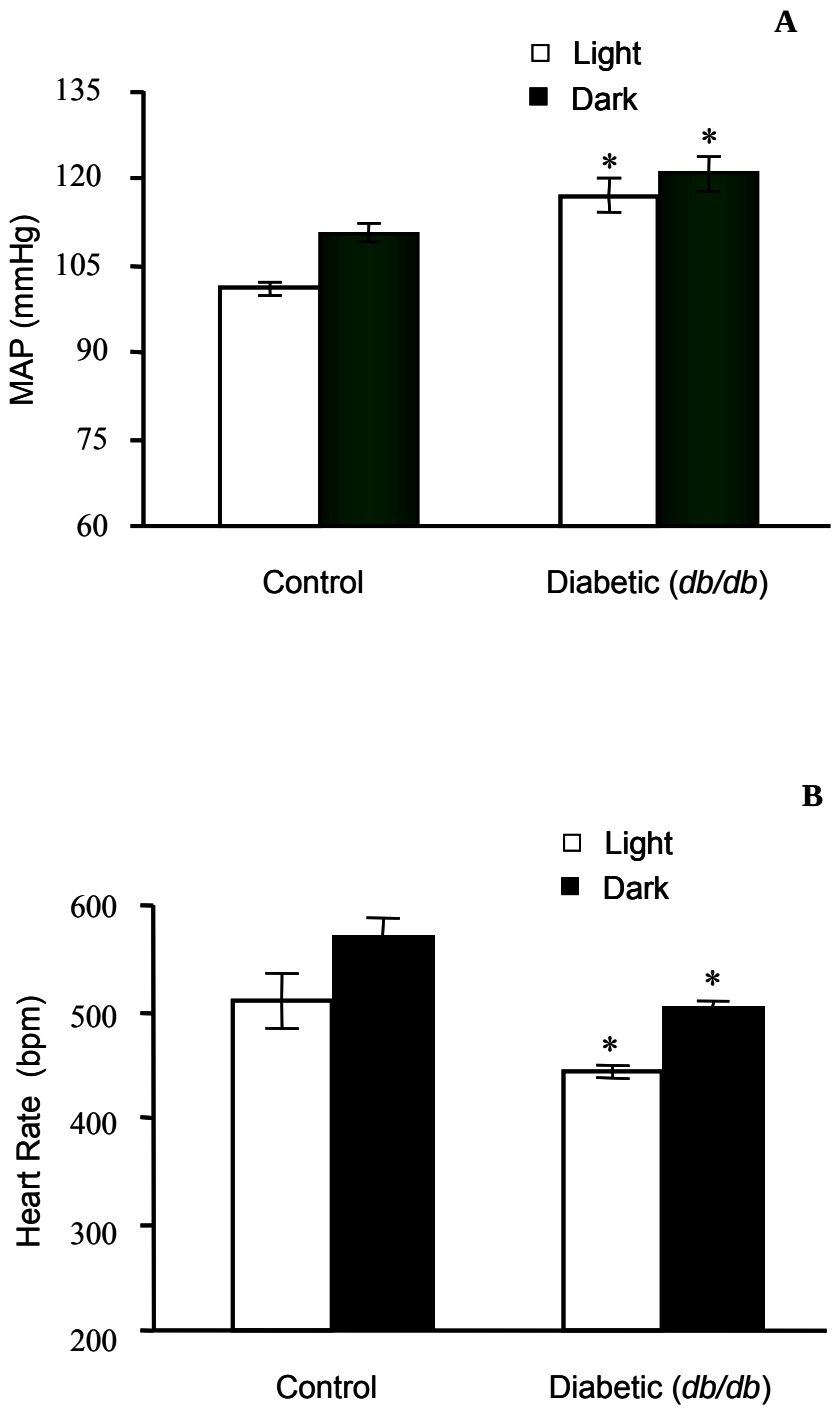


Figure 2: Mean arterial pressure (MAP) and heart rate (HR) in (12-13 weeks old) *db/db* mice. MAP and HR were recorded in 12-13 weeks old *db/db* mice and their age matching controls in both light and dark periods (*P< 0.01 control vs. diabetic, n=6). Values are expressed as ± SEM.

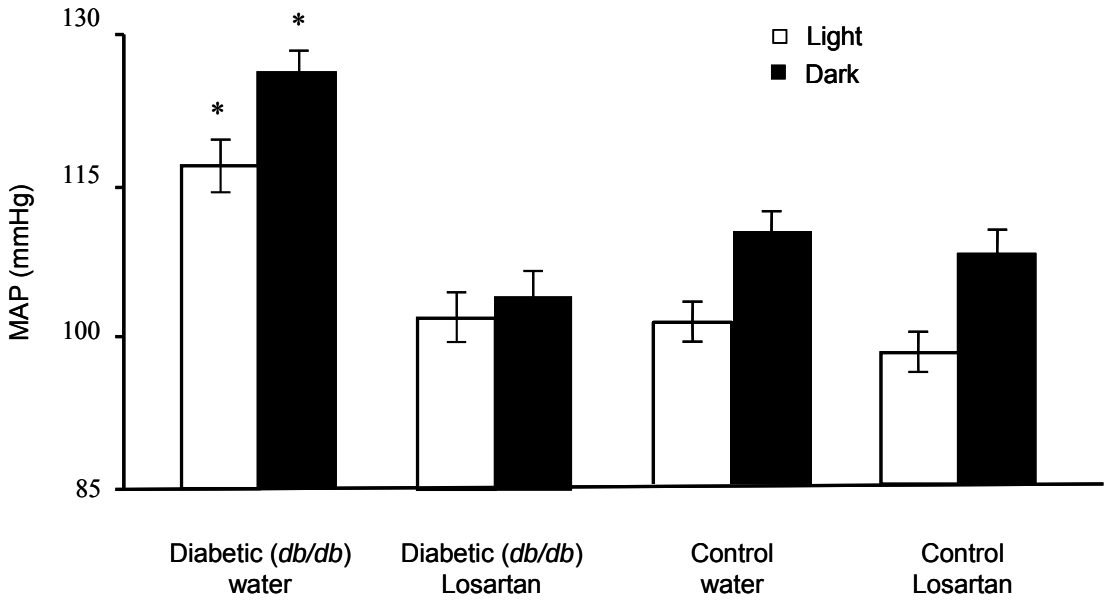


Figure 3: Effect of chronic treatment with losartan on blood pressure in old (20 weeks) *db/db* diabetic mice. 8 weeks old *db/db* diabetic mice and their lean controls were treated with AT1 antagonist, losartan (10 mg/kg/day) for 12 weeks (**P*<0.01 water vs. losartan, n=6). Values are expressed as ± SEM.

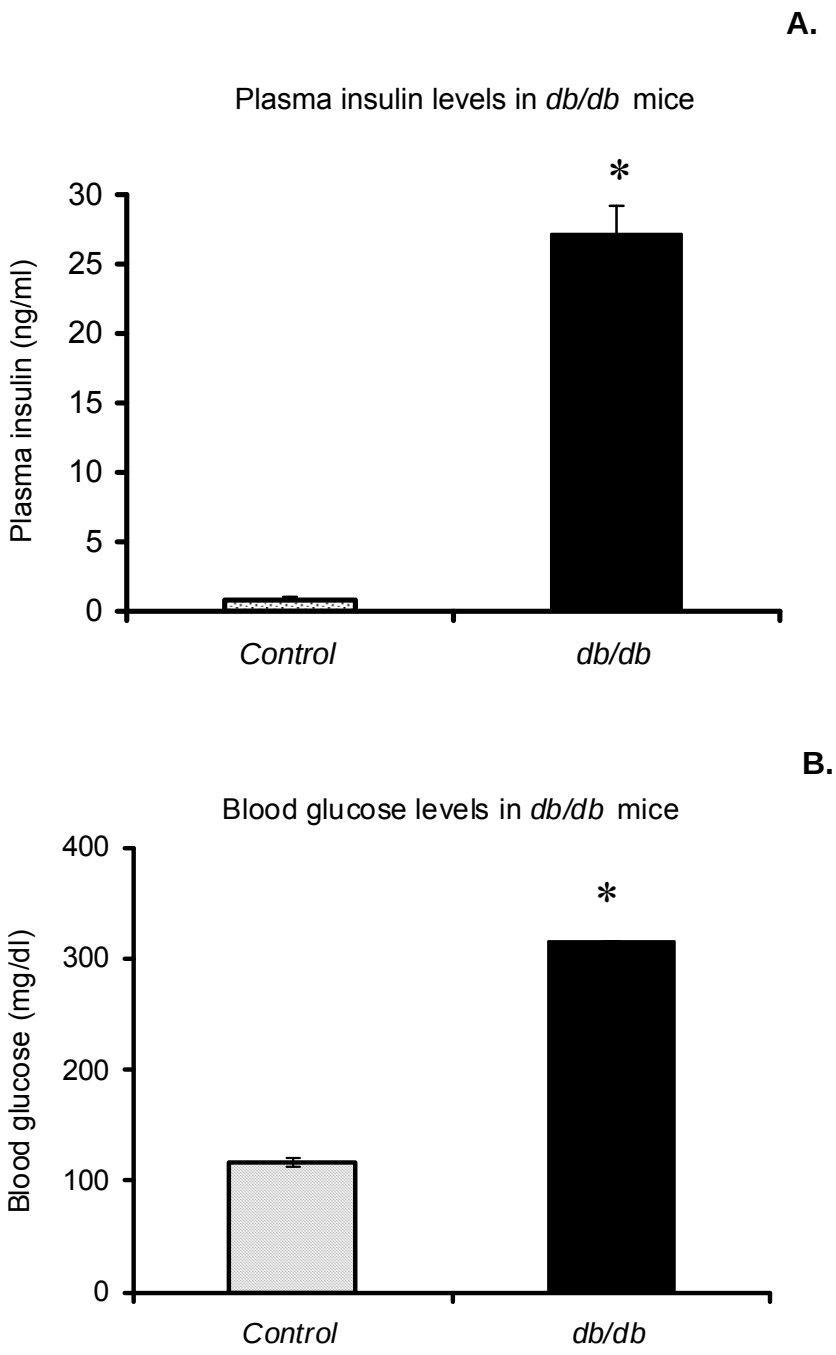


Figure 4: Plasma insulin level and blood glucose levels were measured in the *db/db* mice and their lean controls at 8-9 weeks of age (*P<0.01 control vs. diabetic, n=6). Values are expressed as ± SEM.

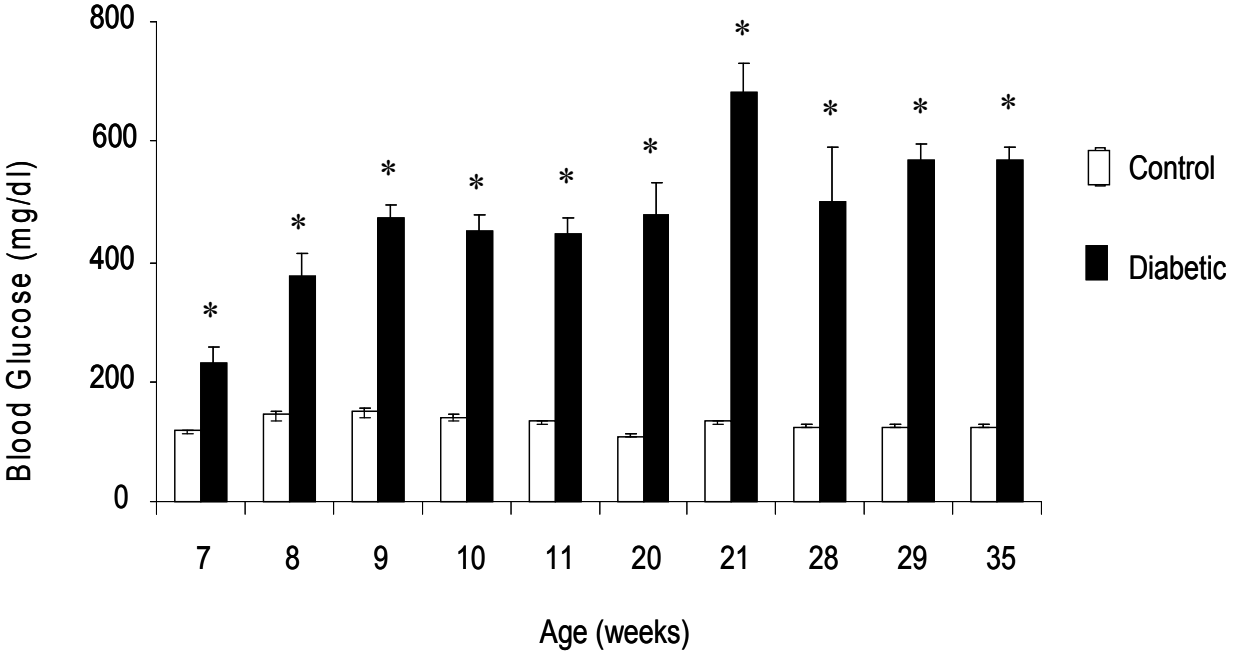


Figure 5: Age dependent increase in blood glucose in *db/db* diabetic mice. Blood glucose was measured in *db/db* mice and their lean controls from 7-35 week (**P*<0.01 control vs. diabetic, n=6). Values are expressed as ±SEM.

Mice	Age (week)	Weight (gram)	Blood Glucose (mg/dL)	Insulin (ng/ml)
Lean Control	8	23.13 ± 0.32	116.17 ± 0.3	0.99 ± 0.23
Diabetic *db/db*	8	33.5 ± 1.1 *	315 ± 27.9 *	27.17 ± 2.02 *
Lean Control	14	25.4 ± 0.3	117.1 ± 0.5	1.2 ± 0.25
Diabetic *db/db*	14	35.5 ± 1.2 *	550 ± 25 *	4.5 ± 0.8 *

Table 1: Age dependent changes in body weight, blood glucose and plasma insulin levels. Body weight, blood glucose and plasma insulin was measured in 8 weeks and 14 weeks old *db/db* mice and their lean controls. (*P<0.01 control vs. diabetic, n=6). All the values are reported as mean ±SEM.

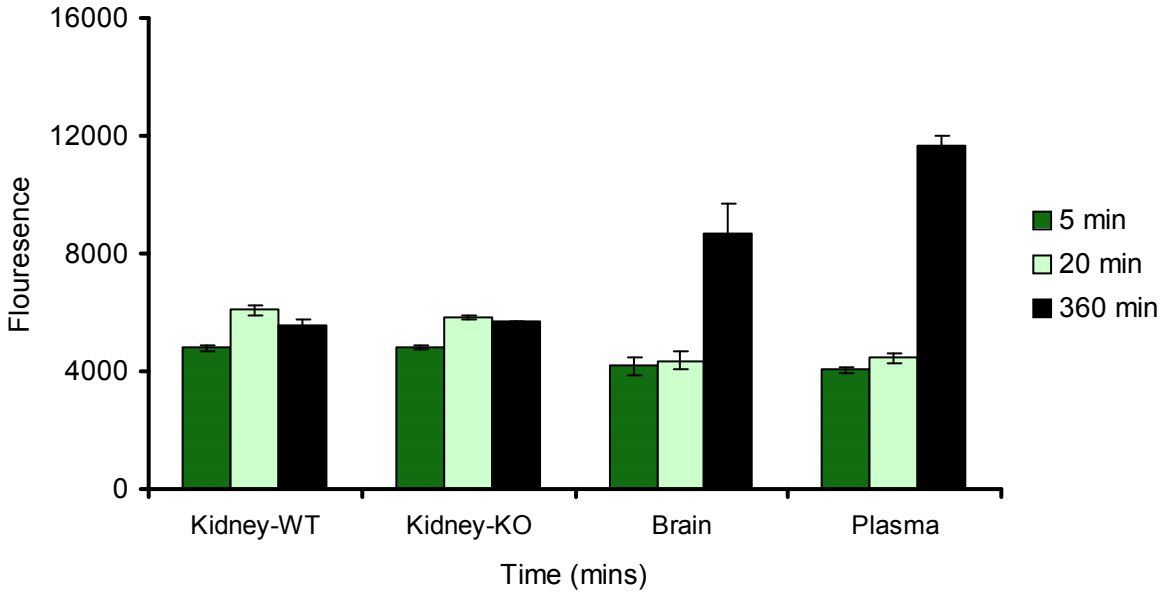


Figure 6: ACE2 activity assay using Mca-*YVADAPK* [Dnp] substrate. Plasma (10 µl), and tissue extracts (50 µg protein) from ACE2-WT and ACE2- KO were incubated with Mca-*YVADAPK* [Dnp] substrate and ACE2 activity was measured at different time points.

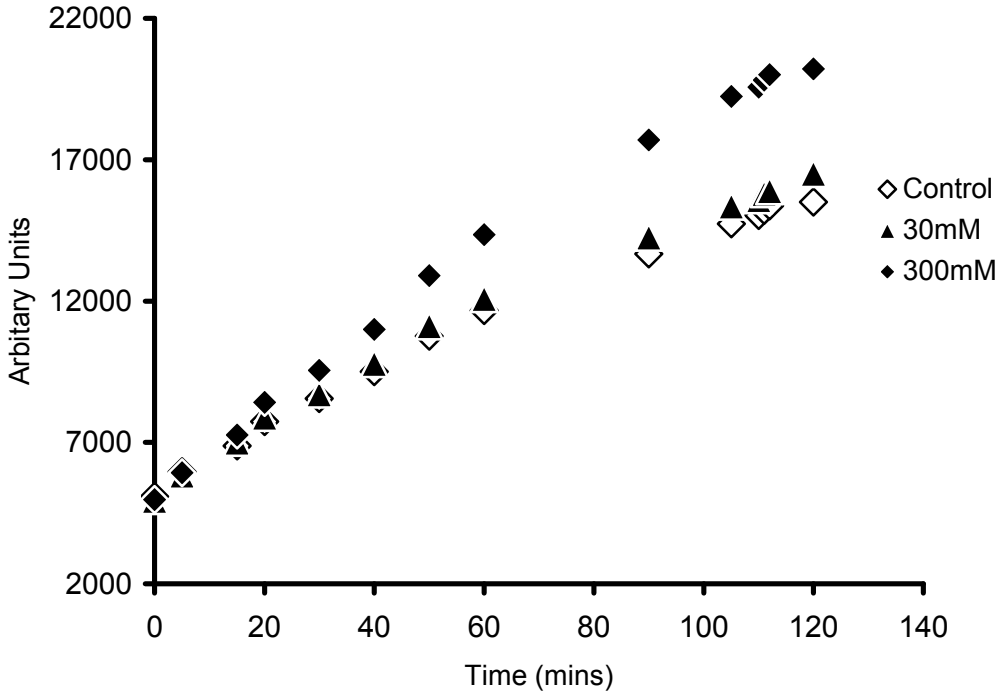


Figure 7: Effect of NaCl on ACE2 activity using ACE2 flouregenic substrate Mca-*APK*-[Dnp]. ACE2 activity was measured in kidney extract (50 μg protein) with different concentrations of NaCl.

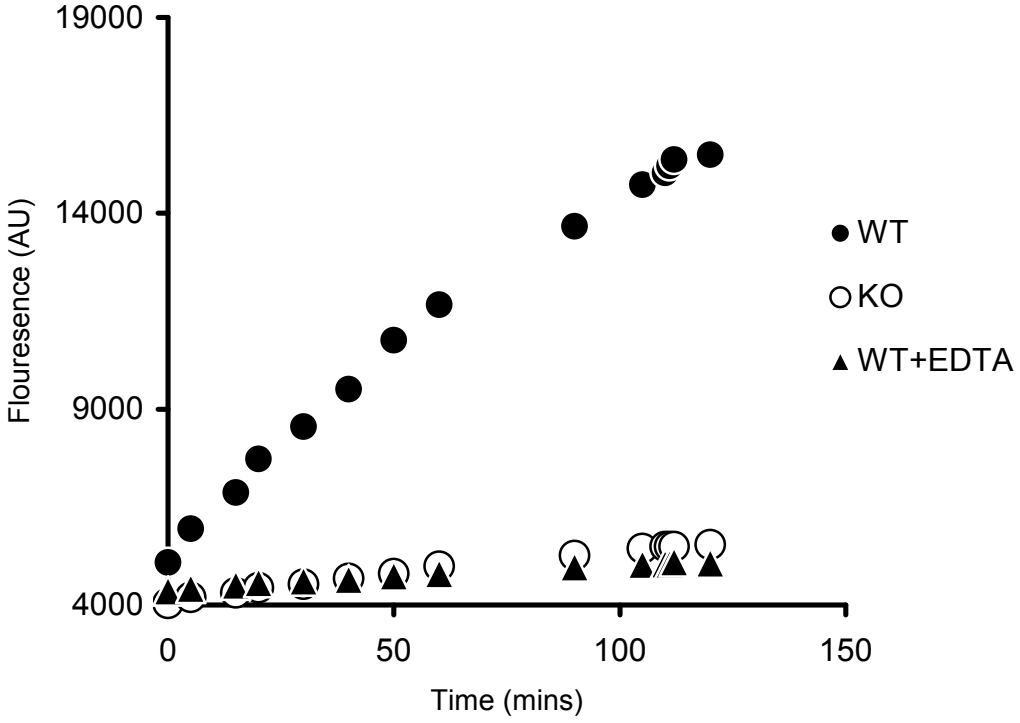


Figure 8: Kidney ACE2 activity using Mca-*APK* [Dnp] substrate. Kidney extracts (50 μg protein) from was incubated with Mca-*APK* [Dnp] substrate in presence and absence of 100 mM EDTA. To validate the assay kidney lysate from ACE2 KO was incubated in Mca-*APK* [Dnp] substrate.

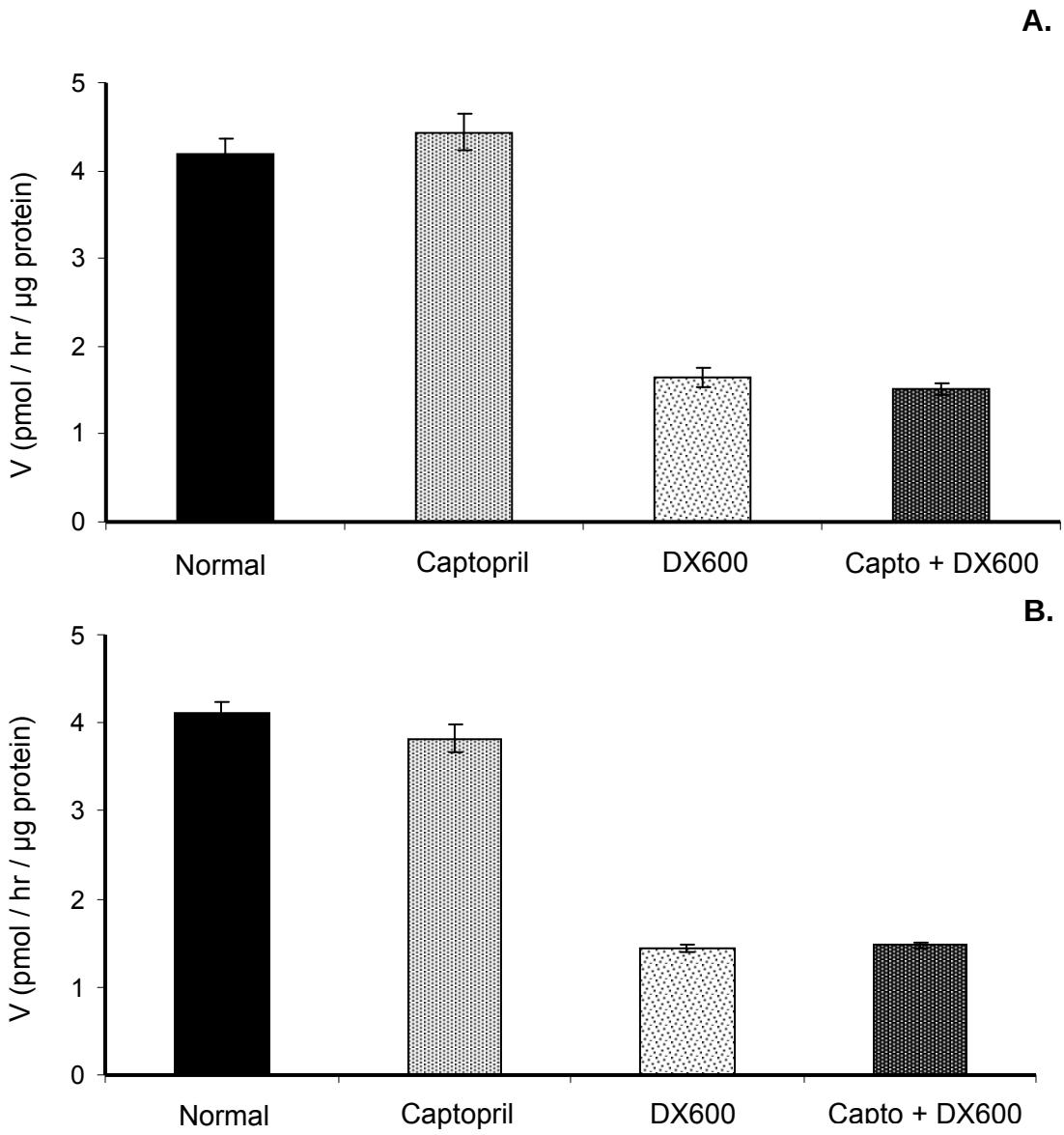


Figure 9: Brain ACE2 activity in *db/db* diabetic mice (B) and their lean controls (A) using 7-Mca-*APK* [Dnp] as substrate in the presence and absence of selective ACE inhibitor (captopril, 100 µM) and selective ACE2 inhibitor, (DX-600, 10 µM). Each bar represents ±SEM.

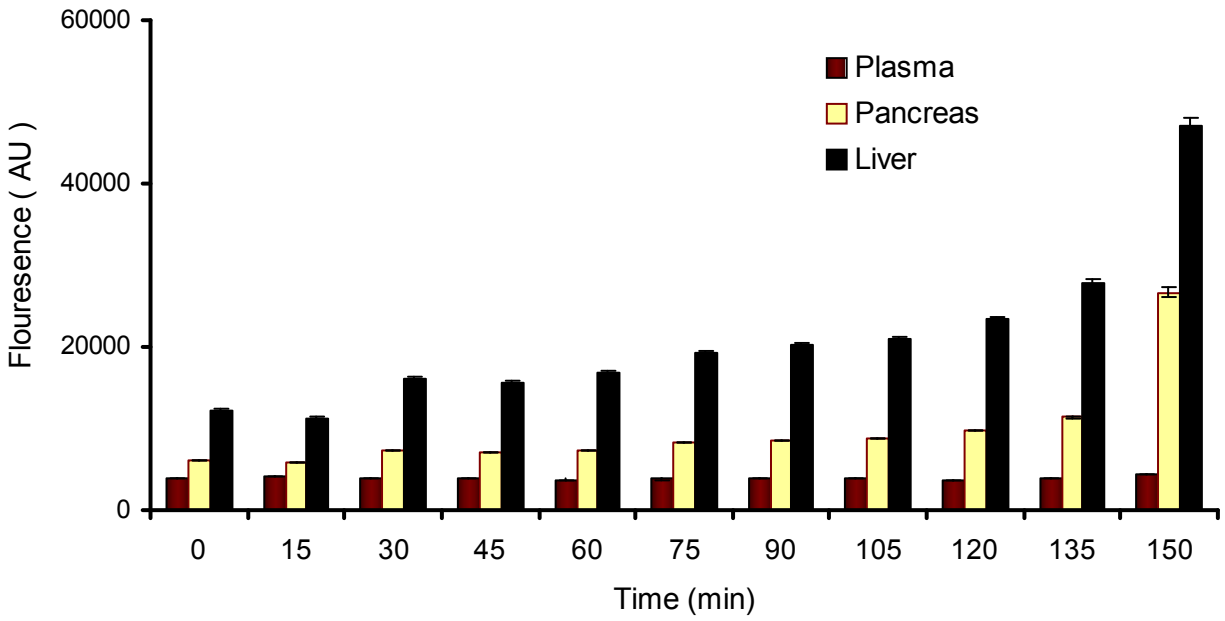


Figure 10: Effect of time on ACE2 activity using Mca-*APK* [Dnp] as substrate in plasma, liver and pancreas. Values are expressed as ±SEM.

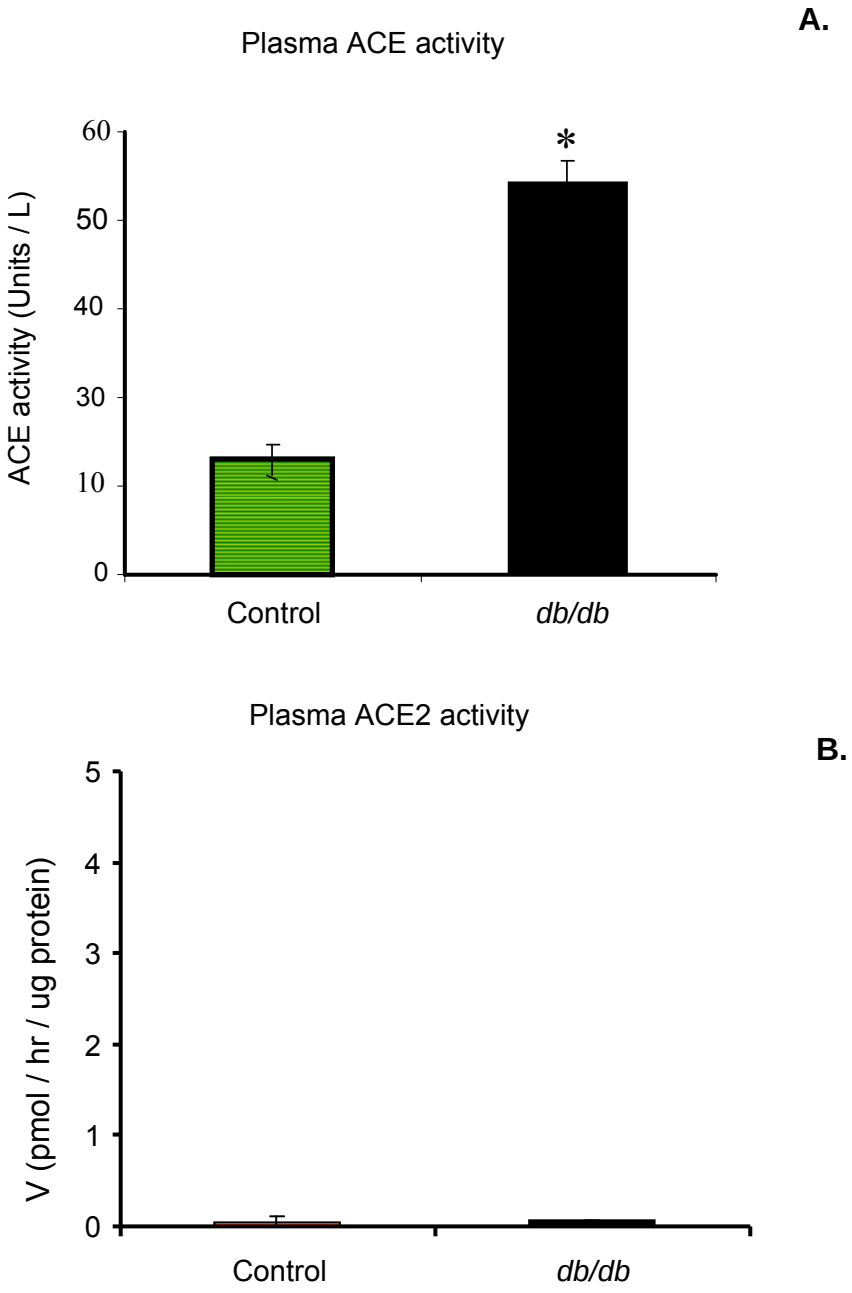


Figure 11: Plasma ACE and ACE2 activities were determined in 8 weeks old *db/db* mice and their controls. (**P*<0.01 control vs. diabetic, n=6). The minimum detectable limits being 1000 FU (fluorescence units). Values are expressed as ±SEM.

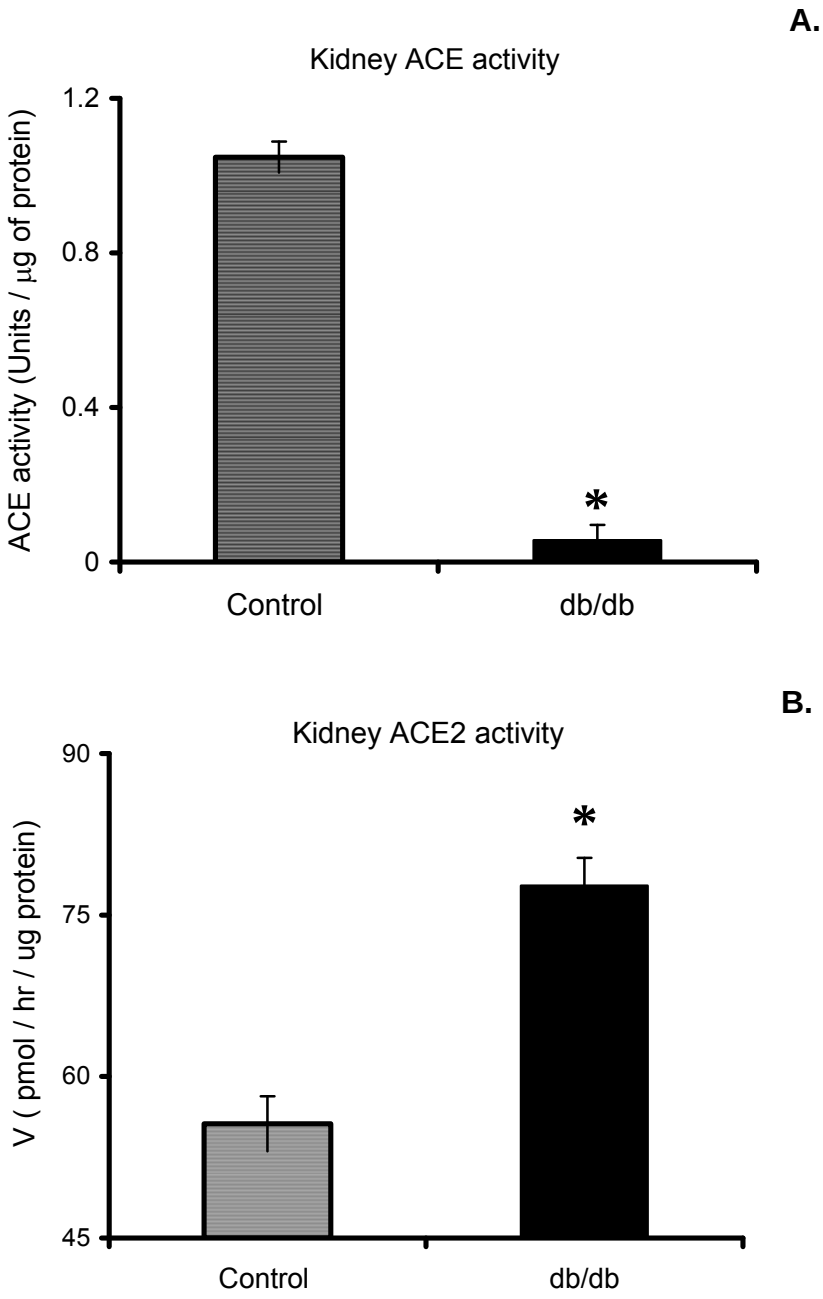


Figure 12: Kidney ACE activity and ACE2 enzyme activities were measured in kidney extract (50 µg protein) from 8-9 weeks old *db/db* diabetic mice and their age matched controls (**P*<0.01, control vs. diabetic; n=6). Values are expressed as ± SEM.

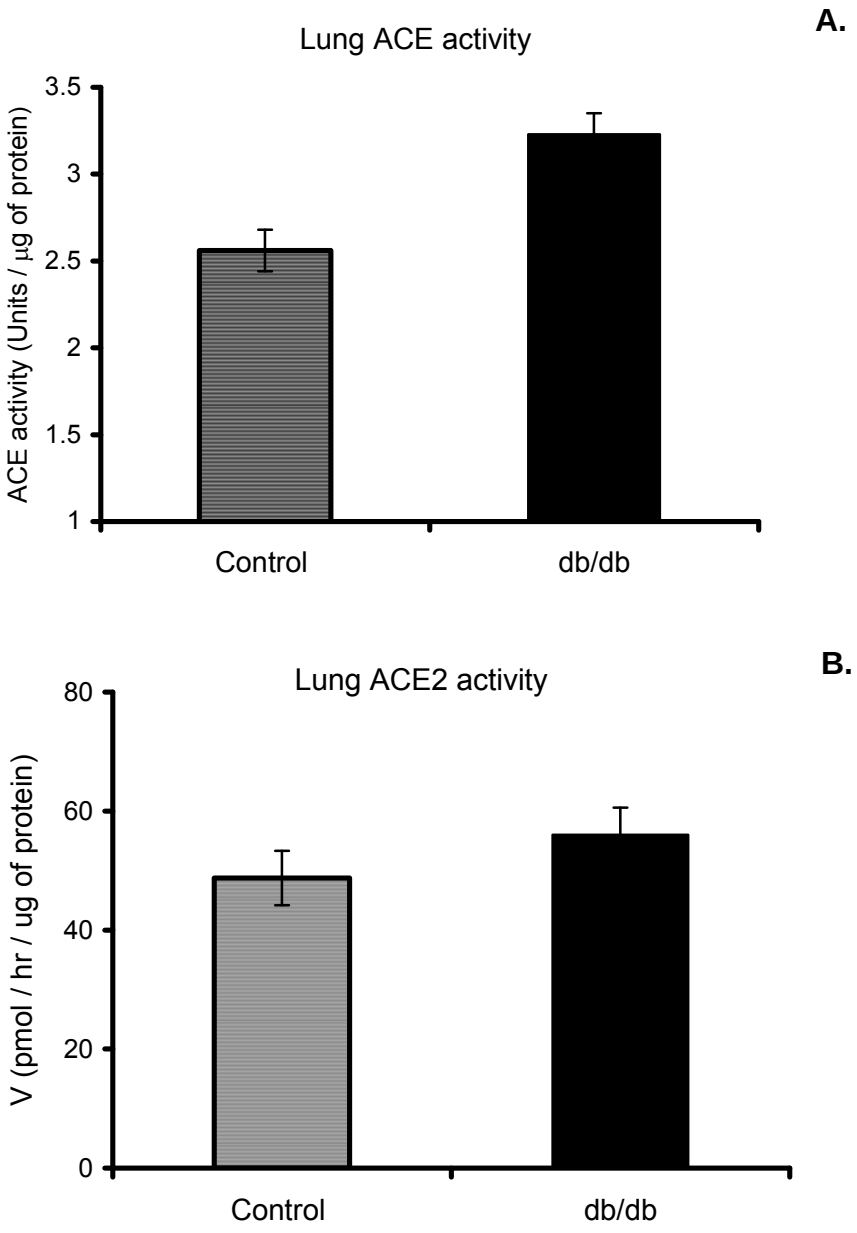


Figure 13: Lung ACE and ACE2 enzyme activities were measured in lungs extract (50 µg protein) in 8-9 weeks old *db/db* diabetic and their age matched lean controls. Values are expressed as ± SEM; n=6.

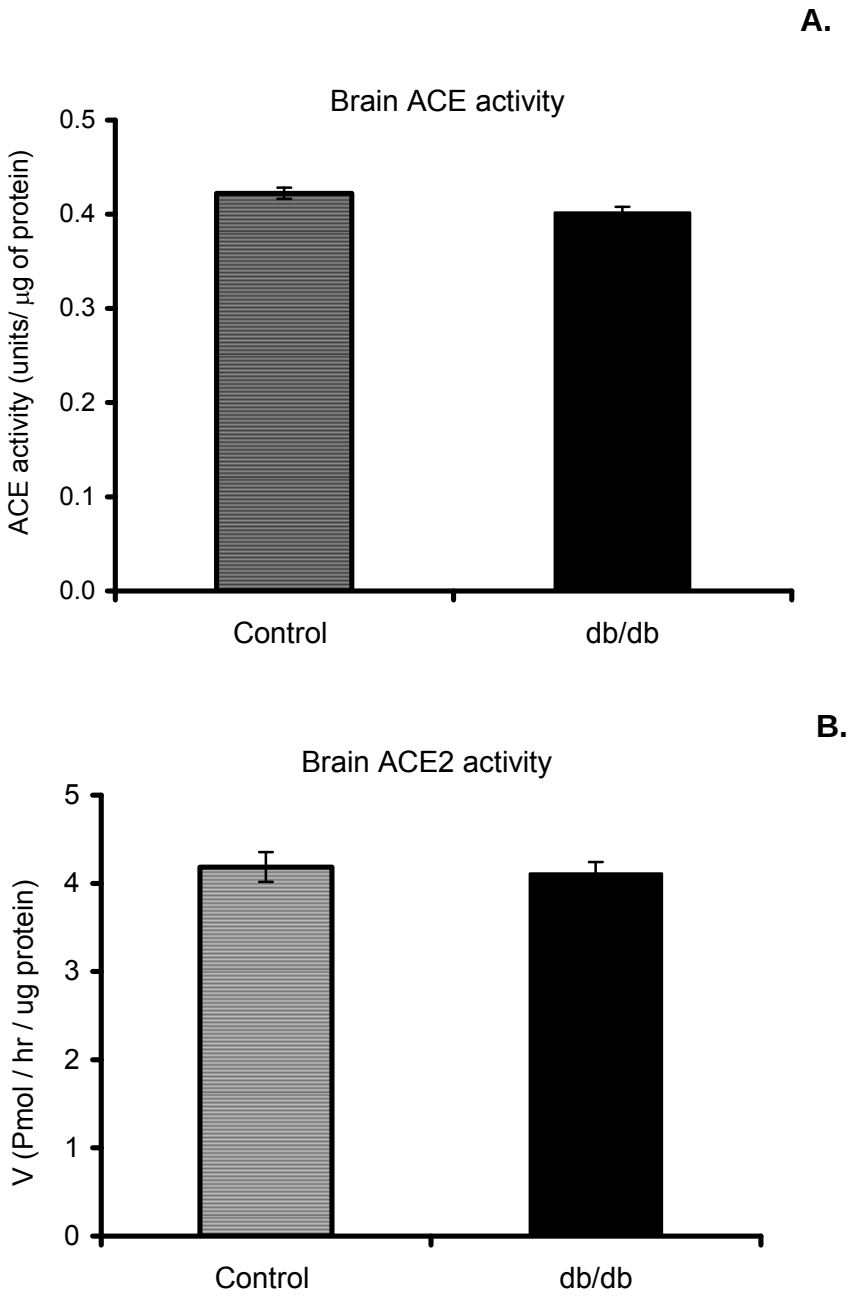


Figure 14: Brain ACE and ACE2 enzyme activities were measured in brain extract (85.5 µg protein) in 8-9 weeks old *db/db* diabetic and their lean controls. Values are expressed as ± SEM.

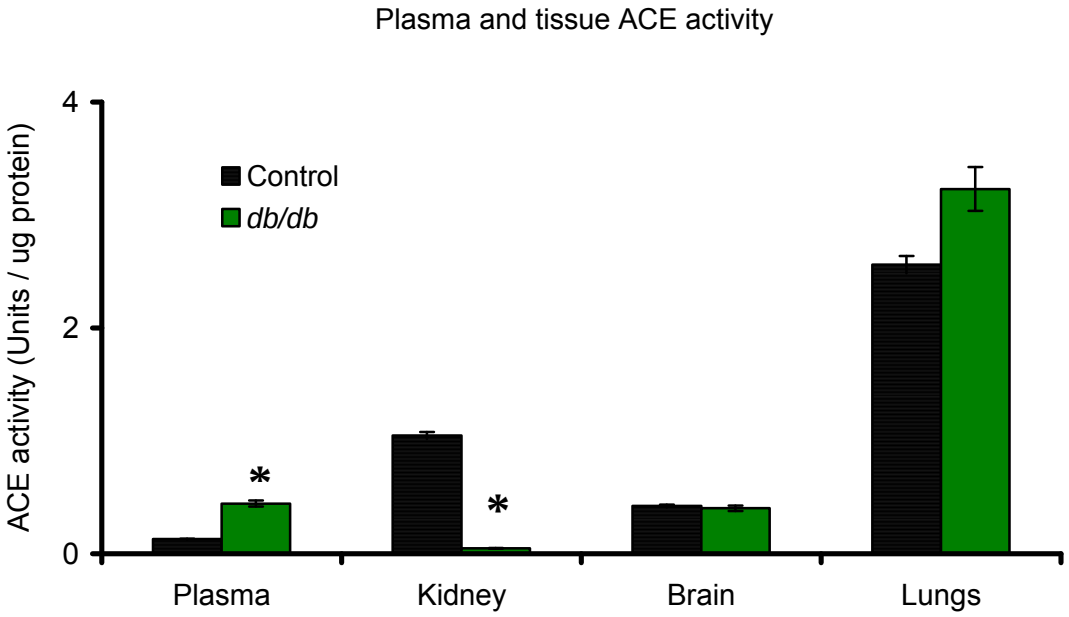


Figure 15: Summary of ACE activity in plasma, kidney, brain and lung tissue extracts from 8 weeks old *db/db* diabetic mice and their lean controls (**P*<0.01, control vs. diabetic; n=6). Values are expressed as ± SEM.

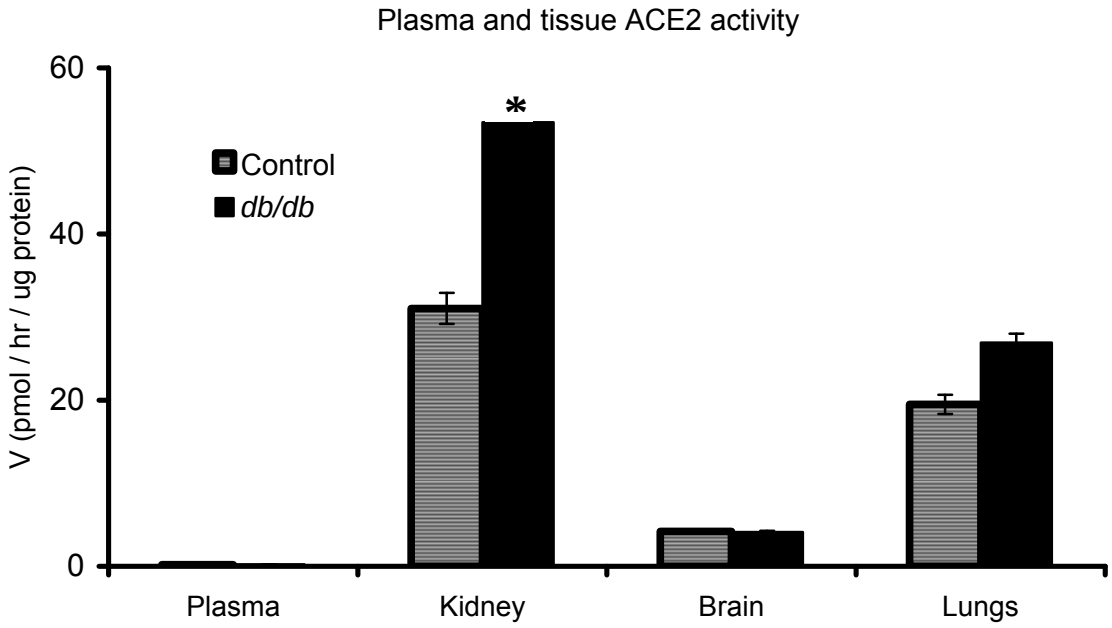


Figure 16: Summary of ACE2 activity in plasma, kidney, brain and lung tissue extracts from 8 weeks old *db/db* diabetic mice and their lean controls (**P*<0.01, control vs. diabetic; n=6). Values are expressed as ± SEM.

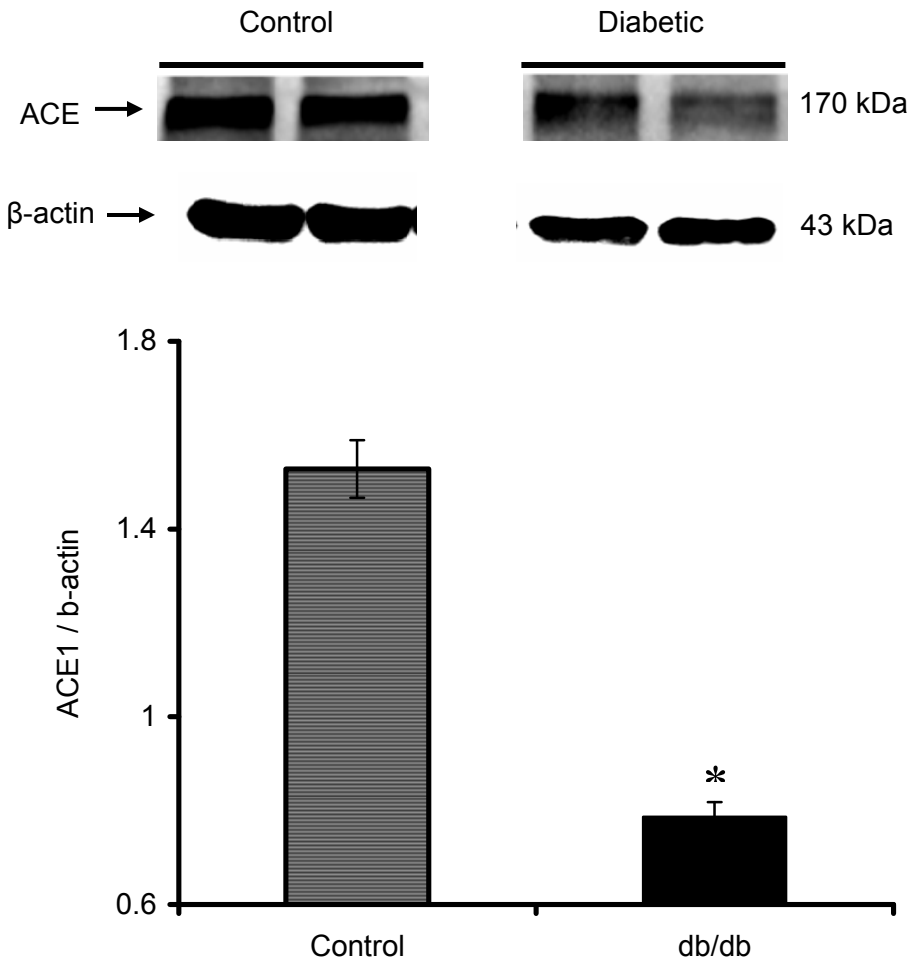


Figure 17: Western blot analysis of kidney ACE protein expression in 8 weeks old *db/db* mice and their lean controls. Kidney ACE band (top panel) at 170 kDa band was detected by ACE antibody using β-actin (bottom panel) as an internal standard (*P<0.01 control vs. diabetic, n=6). Values are expressed as ± SEM.

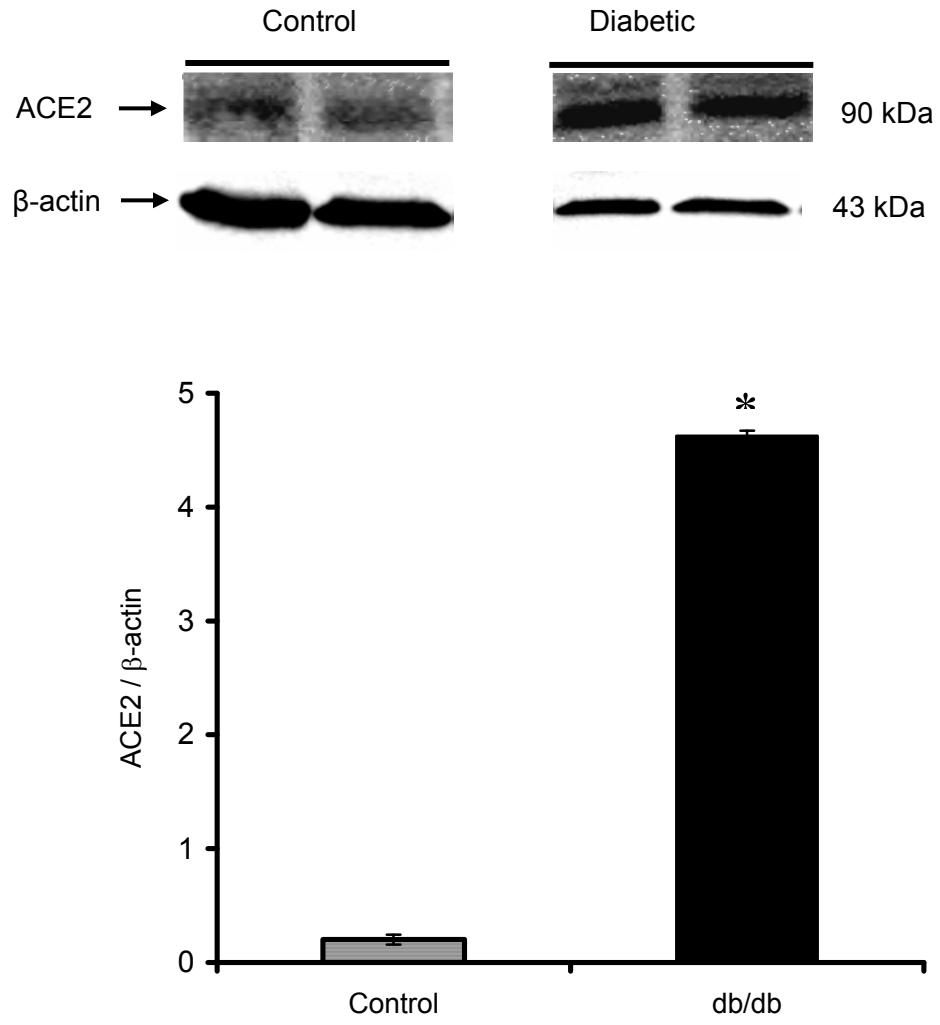


Figure 18: Western blot analysis of kidney ACE2 protein expression in 8 weeks old *db/db* mice and their lean controls. Kidney ACE2 band (top panel) at 90 kDa band was detected by ACE2 antibody using β-actin (bottom panel) as an internal standard (**P*<0.01 control vs. diabetic, n=6). Values are expressed as ± SEM.

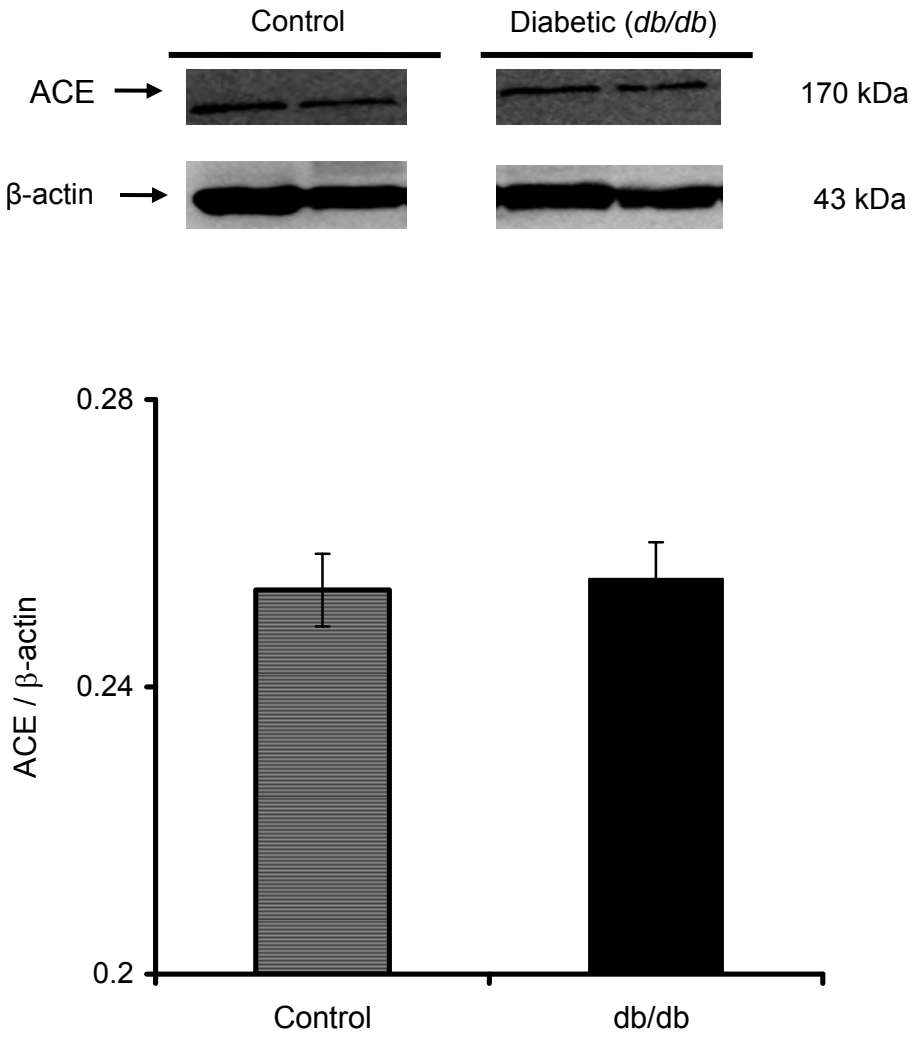


Figure 19: Western blot analysis of lung ACE protein expression in 8 weeks old *db/db* mice and their lean controls. Lung ACE band (top panel) at 170 kDa band was detected by ACE antibody using β-actin (bottom panel) as an internal standard. Values are expressed as ± SEM.

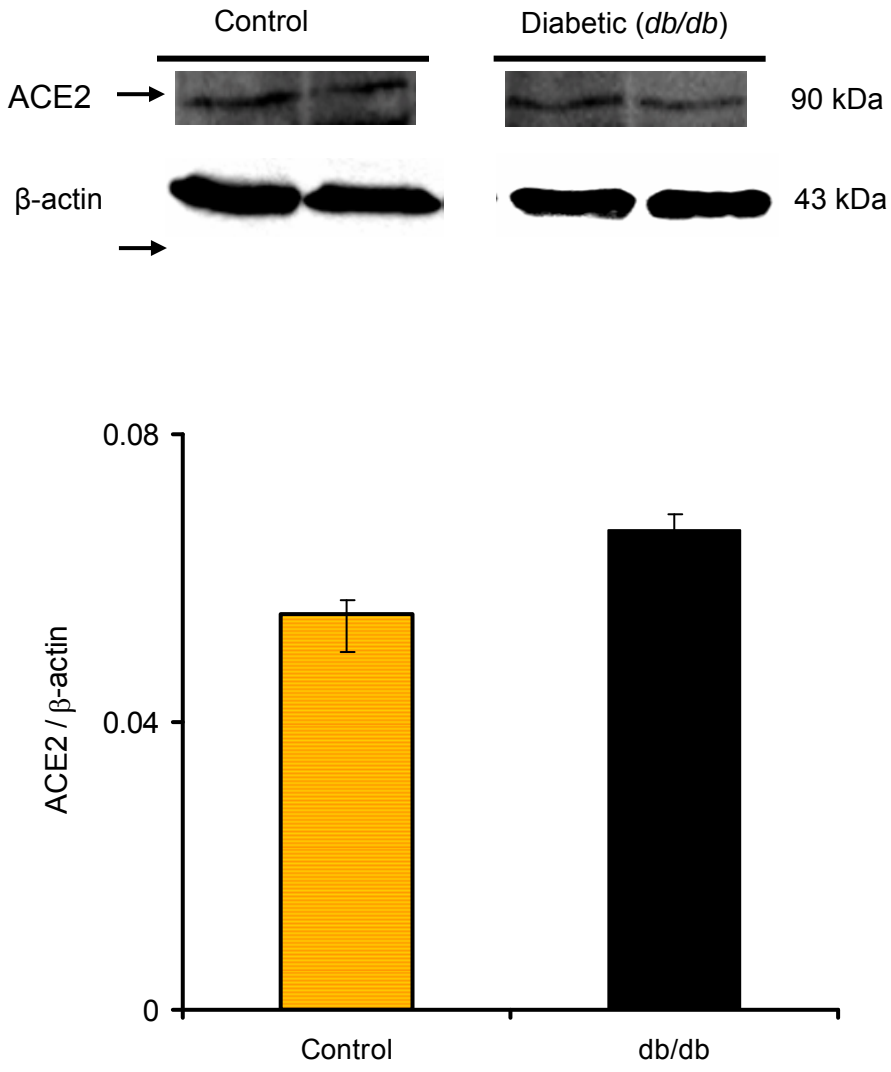


Figure 20: Western blot analysis of lung ACE2 protein expression in 8 weeks old *db/db* mice and their lean controls. Lung ACE2 band (top panel) at 90 kDa band was detected by ACE2 antibody using β-actin (bottom panel) as an internal standard. Values are expressed as ± SEM.

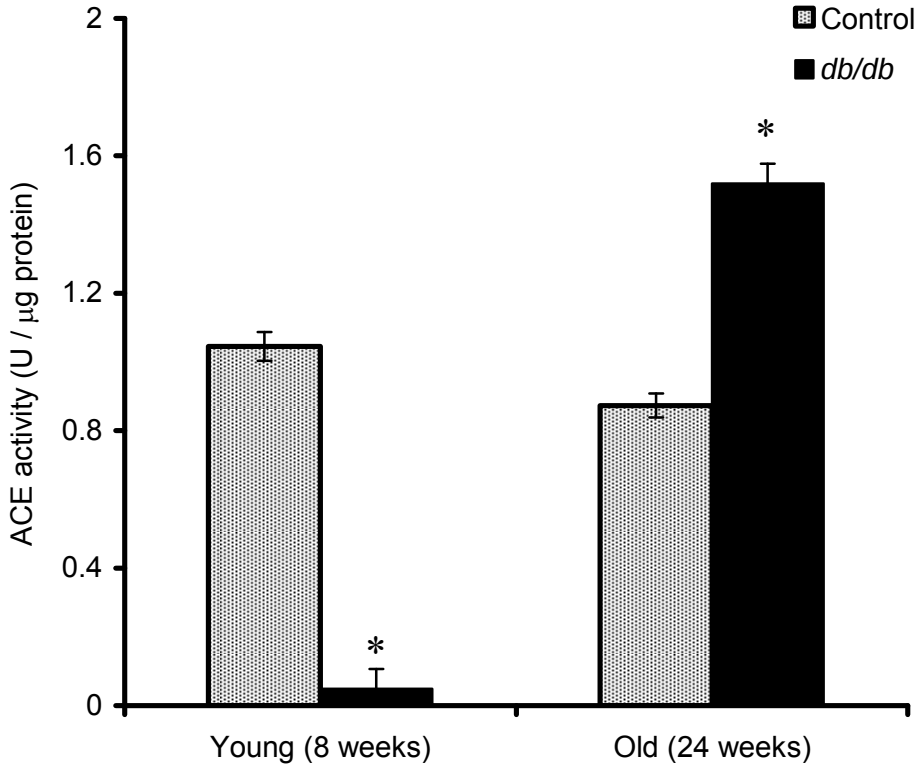


Figure 21: Increased kidney ACE activity in the old (24 weeks) hypertensive *db/db* mice. ACE activity was determined in the kidney by using radioactive $1H^3$ tritiated hippuryl glycine in young *db/db* mice. There was a significant increase in kidney ACE activity with age. (**P*<0.01, Control vs. diabetic; n=6). Each data point represents mean ±SEM.

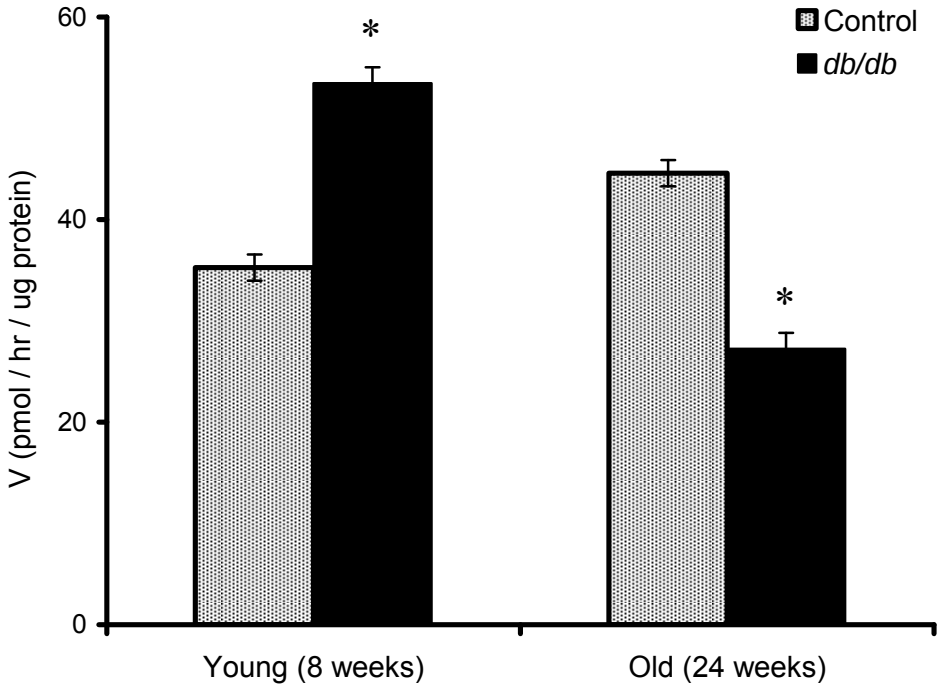


Figure 22: Decreased kidney ACE2 activity in the old (24 weeks) hypertensive *db/db* mice. ACE2 activity was determined in the kidney by using flouregenic substrate 7-Mca-*APK*-[Dnp] in young (8 weeks) and old *db/db* mice (26 weeks) (**P*<0.01, Control vs. diabetic; n=6). Each data point represents mean ± SEM.

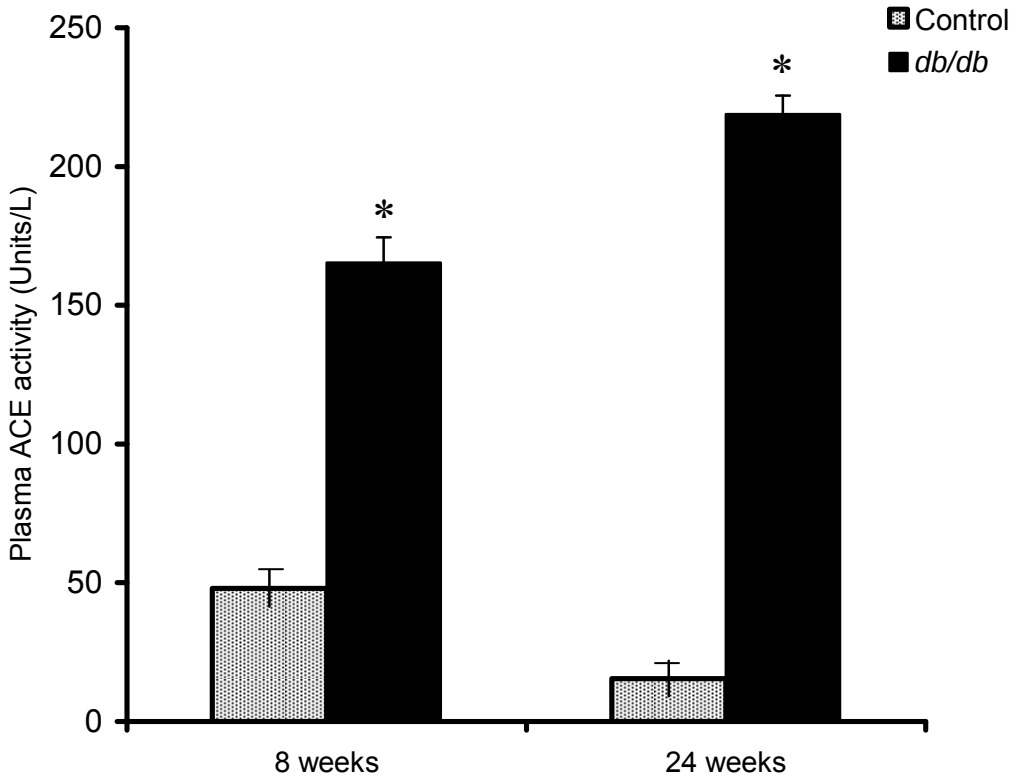


Figure 23: Increased plasma ACE activity in the old (24 weeks) hypertensive *db/db* mice. ACE activity was determined in plasma by using radioactive H^3-hippuryl glycine glycine in young (8 weeks) and old (24 weeks) *db/db* mice and their controls (**P*<0.01 control vs. diabetic, n=6). Each data point represents mean ±SEM.

RESULTS

1. ***Measurement of Cardiovascular Parameters:***

Mean arterial pressure (MAP) and heart rate (HR) were monitored for 24 hours every week for 20 weeks. At early age (8-9 weeks) there was no difference in MAP between diabetic and control mice (*Figure 1A),* but showed a significant decrease in HR (*Figure 1B*, $P<0.01$; n=6. MAP gradually increased with age in *db/db* diabetic mice compared to the controls (*Figure 2*). By 14-15 weeks MAP in diabetics increased compared to their lean controls both during day (101 ± 1 vs. 117 ± 2 mmHg) and night (110 ± 1.7 vs. 3.1 mmHg) (*Figure 2A*; $P<0.01$, n=6). To investigate the role of AT1 receptor in mediating the increased BP in *db/db* mice, mice (7-8 weeks) were treated with AT1 blocker, losartan (10 mg/kg/day in drinking water for 20 weeks. Chronic treatment with losartan had no effect on the MAP in control animals (*Figure 3, *P*<0.01; n=6). However losartan significantly blocked the progressive increase in MAP in diabetic mice during day (102 ± 4 vs. 117 ± 2.5 mmHg) and night (104 ± 4 vs. 126 ± 2.2 mmHg) (*Figure 3, P*<0.01; n=6).

2. Measurement of blood glucose and plasma insulin:

The basic animal characteristics are as shown in the Table 1. At 8 weeks age *db/db* diabetic mice gained more weight than their lean controls (33.5 ± 1.1 vs. 23.13 ± 0.32

gm, diabetic vs. control, $P<0.01$; n=6). At early age *db/db* mice showed the signs of diabetes and insulin resistance. *Figure 4* shows that at a young age (8 weeks) the *db/db* mice had already demonstrated high blood glucose levels (315 ± 28 vs. 117 ± 0.3 mg/dl, diabetic vs. lean control, $P<0.01$; n=6) and high insulin levels (27 ± 2 vs. 1 ± 0.2 ng/dL, diabetic vs. lean control, $P<0.01$; n=6) (Table 1) when compared to their lean controls. By 14 weeks of age diabetic mice exhibited marked obesity, and pronounced hyperglycemia (550 ± 25 vs. 117.1 ± 0.5 ng/dL, diabetic vs. lean control, $P<0.01$; n=6) and hyperinsulinemia (4.5 ± 0.8 vs. 1.2 ± 0.25 ng/dL, diabetic vs. lean control, $P<0.01$; n=6) compared to their lean controls. The decrease in insulin levels from 8 weeks to 14 weeks might be due to the insufficiency of the β cells of the pancreas to secrete more insulin. As shown in *figure 5* there was an age dependent increase in blood glucose levels of db/db mice from 7 weeks to 35 weeks. The *db/db* mice survive more than 40 weeks of age and later die due to diabetic acute nephropathy or other cardiovascular complications of type2 diabetes.

3. Optimization of ACE2 flouregenic assays:

ACE2 flouregenic assays were optimized using two flouregenic substrates Mca-*YVADAPK* [Dnp] and Mca-*APK* [Dnp]. Plasma (10 µl) and tissue extracts (50 mg protein) from ACE2 wild type (WT) and ACE2 knockout (KO) mice were incubated with Mca-*YVADAPK* [Dnp] and ACE2 activity was measured at different time points (*Figure* 6). There was no difference between WT and KO kidney ACE2 activity after 20 min and 6 hours incubation. In addition there was no detectable plasma and brain activity after 20 min incubation. Unexpectedly, there was a significant plasma ACE2 activity after 6 hours

(*Figure 7*). This indicates that the substrate Mca-*YVADAPK* [Dnp] might not be a good substrate for measurement of ACE2 activity. To determine whether Mca-*APK* [Dnp] substrate was specific to ACE2 kidney lysate (50 μg protein) from ACE2 WT and ACE2 KO was incubated in presence and absence of 100 Mm EDTA. No ACE2 activity was observed in ACE2 KO indicating the specificity and selectivity of Mca –*APK* [Dnp] to ACE2 (*Figure 8*). To study the effects of NaCl on ACE2 activity kidney lysate (50 μg protein) was incubated with Mca-*APK* [Dnp] in the presence of different concentrations of NaCl (0, 30, 300 Mm) (*Figure 8*) (Guy *et al*. 2003). Kidney ACE2 activity increased with increased concentrations of NaCl. (*Figure 7)* (Guy *et al.* 2003). Brain ACE2 activity in *db/db* diabetic mice and their lean controls was determined using the substrate Mca-*APK* [Dnp] in the presence and absence of captopril (selective ACE inhibitor) and DX-600 (selective ACE2 inhibitor). DX-600 significantly inhibited ACE2 activity indicating the specificity of the assay (*Figure 9*). Mca-*APK* [Dnp] substrate was used for ACE2 activity assays. Effect of time on ACE2 activity was determined using Mca- *APK* Dnp] in plasma, liver and pancreas from 0-2.5 hours. There was a gradual increase in ACE2 activity in the liver and pancreas with time and there was no detectable plasma ACE2 activity (*Figure 10*).

4. Measurement of ACE activity in young (8 weeks) mice:

ACE activity was determined in plasma, kidney cortex, lung, and brain in young (8 weeks) *db/db* diabetic mice and their age matched controls. At this age the *db/db* mice were hyperglycemic, hyperinsulinemic and normotensive at this age. Plasma ACE activity was markedly increased in *db/db* diabetic mice when compared to their lean

controls (*Figure 11*; (*db/db* 0.44 ± 0.11 U/μg protein vs. control 0.13 ± 0.09 U/μg protein, $P<0.001$; n=6). By contrast kidney ACE activity was significantly decreased in *db/db* diabetic mice compared to their lean littermates (*Figure12; db/db* 1.045 ± 0.09 U/μg protein vs. control 0.048 ± 0.065 U/μg protein, $P<0.01$; n=6). Lung ACE activity was observed to be high but there is no significant difference observed between *db/db* mice and their controls (*Figure 13; db/db* 2.56 ± 0.83 U/μg protein vs. control 3.23 ± 0.4 U/μg protein, n=6). There was no significant difference observed in brain ACE activity in *db/db* diabetic mice compared to their controls (Figure 14; *db/db* 0.42 ± 0.057 U/μg protein versus control 0.40 ± 0.042 U/μg protein, n=6). Significantly decreased ACE activity in young (8 weeks) *db/db* diabetic mice seems to be organ specific to the kidney.

5. Measurement of ACE2 activity in young (8 weeks) db/db mice:

Plasma, kidney, lung and brain ACE2 activity was determined in young (8 weeks) *db/db* diabetic mice and their lean littermates. Young *db/db* diabetic mice were normotensive, hyperglycemic and hyperinsulinemic. There was no detectable levels of plasma ACE2 activity in *db/db* mice and their controls (*Figure 11*; n=6). Kidney ACE2 activity was significantly increased in the *db/db* diabetic mice when compared to the controls (*Figure 12; db/db* 66.22 ± 0.9 pmol/hr/μg protein vs. control 43.68 ± 0.8 pmol/hr/μg protein, $P<0.01$; n=6). In lungs by contrast, there was no significant difference in ACE2 activity in both *db/db* diabetic mice and their lean controls (*Figure 13*; *db/db* 56.004 ± 0.16 pmol/hr/μg protein vs. control 46.73 ± 0.14 pmol/hr/μg protein, n=6). There was no significant difference in brain ACE2 activity between *db/db* mice and their lean controls (*Figure 14*; *db/db* 4.11 ± 0.17 pmol/hr/μg protein vs. control 4.19 ± 0.21 pmol/hr/μg

protein, n=6). A highly significant increase in ACE2 activity in *db/db* diabetic mice appears to be organ specific to the kidney at a young age (8 weeks).

6. Summary of ACE and ACE2 activities in young mice (8 weeks)

To summarize the plasma and tissue ACE activities in the young (8 weeks) *db/db* mice, there was a significantly increased plasma ACE activity and significantly decreased kidney ACE activity in *db/db* mice compared to the controls. However there was no significant difference in ACE activities in lung and brain between *db/db* mice and their controls (*Figure 15*). There were no detectable levels of plasma ACE2 in db/db mice and their controls (*Figure 16*). But there was a significantly increased kidney ACE2 in db/db mice compared to controls. In the lungs and brain there was no significant difference between *db/db* mice and their lean controls (*Figure 16*).

7. Western Blot Analysis (young db/db mice):

ACE and ACE2 protein expression levels in young mice (8 weeks) were quantified by western blot analysis. In kidney and lungs, a single band of protein was detected at 170 kDa for ACE (*Figures 17 and 18*) and 90 kDa (*Figures 19 and 20*) for ACE2 when membranes were probed with respective antibodies. This result is consistent with the molecular weights of the enzymes ACE and ACE2, respectively as reported by others (Vickers *et al.* 2002b) (Eriksson *et al.* 2002).

Kidney ACE protein expression was markedly reduced in *db/db* diabetic mice compared to their lean controls (*Figure 17*; *db/db* 0.83 ± 0.12 n=6 vs. control 1.52 ± 0.15 **P*<0.01; n=6). Kidney ACE2 protein expression, by contrast, was increased significantly, in *db/db*

diabetic mice than in the lean controls (*Figure 18*; *db/db* 4.46 ± 0.11 n=6 vs. control 0.43 ± 0.16, **P*<0.01 n=6). In lungs there was no significant difference between *db/db* diabetic mice and their controls in either ACE (*Figure 19*; *db/db* 0.25 ± 0.09 n=6 vs. control 0.27 ± 0.06, n=6) or ACE2 protein abundance (*Figure 20*; *db/db* 0.56 ± 0.17 n=6 vs. control 0.78 ± 0.14, n=6).

8. Measurement of ACE activity in old (24 weeks) db/db mice:

To investigate the progressive changes in ACE activity in *db/db* mice at a later stage of type2 diabetes, plasma and kidney ACE activity were measured in 24 weeks old *db/db* diabetic mice and their lean controls. There was a significant increase in the kidney ACE activity in the old *db/db* mice when compared to the young mice (Figure 21, *db/db* old 1.76 ± 0.147 U/ µg protein vs. *db/db* young 0.048 ± 0.047 U/ µg protein, **P*<0.01, n=6). In the plasma there was a significant increase in ACE activity in the old *db/db* mice when compared to the young mice (*Figure 22*; *db/db* old 218.90 ± 6.7 U/L protein vs. *db/db* young 165.4 ± 9.1 U/L protein, **P*<0.01, n=6).

9. Measurement of ACE2 activity in old (24 weeks) db/db mice:

ACE2 activity was measured in the kidney old mice (24 weeks) to study the changes in ACE2 levels at a later stage of type2 diabetes. There was a significant decrease in kidney ACE2 activity in the old *db/db* mice when compared to the young mice (*Figure 22*; *db/db* old 53.463 ± 1.57 U/ µg protein vs. *db/db* young 27.24 ± 1.30 U/ µg protein) **P*<0.01, n=6. Increased ACE activity and decreased ACE2 activity was observed in *db/db* diabetic mice at a later stage of type2 diabetes.

DISCUSSION

The RAS is a coordinated hormonal cascade and a central regulator of cardiovascular, renal and adrenal functions that regulate fluid and electrolyte balance and arterial pressure (Hackenthal *et al.* 1990). In addition, RAS plays a key role in the pathophysiology of various diseases of the cardiovascular and renal systems. The RAS is an attractive target for medications designed for management of cardiovascular dysfunction (Vickers *et al.* 2002a). ACE and ACE2 are the two primary components of RAS. The traditional view that Ang II is the key product of the RAS has been challenged with the recent discovery of a new carboxypeptidase, ACE2, and growing evidence for a physiological role of its peptide product, Ang (1-7)(Ferrario *et al.* 2005a). A recent report suggested that the deletion of the ACE2 gene leads to the development of Ang II dependent cardiovascular and renal damage (Oudit *et al.* 2006). It is the ability of ACE2 to inactivate the vasoconstrictor, Ang II, and generate the putative cardio protective metabolite, Ang (1-7), that implicates ACE2 as an important participant in cardiovascular homeostasis (Guy *et al.* 2003). However no quantitative assessment has been made for the enzyme activity and protein expression of ACE and ACE2 and hence, it is of interest to characterize circulating and localized RAS especially in the kidney, brain and lungs.

The aim of the present study is to examine the changes in blood pressure in relation to changes in ACE/ACE2 balance in murine models of type 2 diabetes mellitus. Murine

models of type 2 diabetes (*db/db*) mice and their lean controls aged 8-20 weeks were used as a bioassay.

One of the features of the present study was the application of radio telemetry to measure the blood pressure and day/night rhythms in cardiovascular parameters in *db/db* and control mice. This method accurately monitors BP, heart rate, and locomotor activity in conscious, untethered, freely moving mice with minimum human interference. Diabetic (*db/db)* mice have been extensively studied as diabetic murine models but there is little information about blood pressure in measurements in these mice. Besides the scarcity of data, most of the available data was collected using the conventional tail- cuff method (Kosugi *et al.* 2006) (Guo *et al.* 2006) and also presents conflicting results, such as increased (Bagi *et al.* 2005;Guo *et al.* 2005b), decreased (Kosugi *et al.* 2006) or no changes in BP in *db/db* mice(Moriyama *et al.* 2004). This divergence in the results may be due to the differences in the animal age and also the methodology used to acquire blood pressure measurements in the previous data. The focal result of the present project is that diabetic *db/db* mice develop hypertension with age that is associated with changes in the balance of ACE/ACE2 activity and expression.

Our data show that the BP is significantly increased in *db/db* mice with age (*Figure 1 and 2.* Our data also show differences in BP, HR and locomotor activity in day/night pattern. The key issue is that the blood pressure in the night time is higher than in the day. There is clinical evidence that verifies a relationship between diabetes and BP rhythm perturbations {Carey, 2001 383 /id}. Diabetic patients present hypertension and reduced

gradient between light and dark BP levels (Jermendy *et al.* 1996). Although lean control mice were not hypertensive, this group showed, as expected, an increase in the blood pressure during the dark phase(Farah *et al.* 2000;Farah *et al.* 2004). In the present study diabetic *db/db* mice also presented differences in the BP levels between the light and dark phases.

The *db/db* diabetic mice have insulin resistance from an early age (8 weeks) serving as good models for type 2 diabetes mellitus. One of the goals of this project was to investigate BP control and the role of angiotensin AT1 receptors in the *db/db* model of type2 diabetes. Chronic treatment with losartan (10 mg/kg/day) significantly blocked the progression of the MAP changes in diabetic mice during the day and night (day 102 ± 4 vs 117 ± 2.5 mmHg vs. night (104 ± 4 vs 126 ± 2.2 mmHg, $P<0.01$, n=6) (*Figure 3*). Our results document the increase in BP in *db*/*db* diabetic mice and the importance of Ang AT1 receptors in mediating the BP changes in this murine model of type 2 diabetes (*Figure 3*). There is a previous study showing the increase in blood glucose level after losartan was administered (Chu *et al.* 2006a). These findings provide a novel and at least partial explanation for the reduced incidence of type 2 diabetes related hypertension that has been observed in a number of clinical trials applying AT1R inhibition to individuals at high risk of cardiovascular problems during type 2 diabetes.

Our hypothesis is that there is a change in balance of ACE and ACE2 during the course of diabetes in *db/db* diabetic mice. In the present study, we investigated ACE and ACE2

enzyme activity and protein expression in plasma, kidney and lungs from young (8 weeks) and old (24 weeks) *db/db* diabetic mice and age matched lean controls.

There is previous data that shows that chloride ions affect the enzyme activity of ACE2 (Oudit *et al.* 2003). Therefore we optimized the ACE2 activity assays and decided to perform the assays under identical conditions that closely mimic the salt concentration and pH of the extra cellular environment (i.e. 300mM NaCl and pH 7.4) {Wysocki *et al.*, 2006). In a recent study using *db/db* and streptozocin diabetic mice an alteration in ACE and ACE2 activities were observed compared to non-diabetic controls (Wysocki *et al.* 2006). Assays to measure ACE activity use spectrophotometric, flouremetric, high-performance liquid chromatography, or radiometric approaches and are quite reliable, but there is limited information on ACE2 assays (Danilczyk *et al.* 2003). There is a recent study that used SELDI-TOF MS approach to measure plasma ACE and Ang II levels. (Elased *et al.* 2005). Previously ACE2 activity has been previously measured using two flouregenic substrate, Mca-*YVADAPK* [Dnp] (Wysocki *et al.* 2006) and Mca-*APK*[Dnp] (Herath *et al.* 2007). ACE2 activity assays were optimized using both of these substrates. With Mca-*YVADAPK* [Dnp] substrate no difference in ACE2 activity between kidney WT and KO mice was observed indicating that the substrate is not specific to ACE2 (*Figure 8*) . There is no evidence for the presence of ACE2 in the plasma. Using Mca-*YVADAPK* [Dnp] substrate we found high ACE2 activity in the plasma (*Figure 8*). We have shown that Mca-*YVADAPK* [Dnp] has less specificity and suitability for ACE2. We developed an assay to measure tissue ACE2 activity specifically using 7-Mca-*APK* [Dnp]. The selective cleavage of this substrate by ACE2 allowed us to measure ACE2 activity. The specificity and selectivity of this substrate to ACE2 was confirmed by our

experiments with ACE2 KO mice and by using a metalloprotease inhibitor, EDTA In ACE2 KO mice there was no ACE2 activity observed and in ACE2 WT a significant increase in ACE2 activity was observed indicating that the substrate 7-Mca-*APK* [Dnp] is highly selective to ACE2 (*Figure 9*). Brain ACE2 activity in *db/db* diabetic mice and their lean controls was determined using the substrate Mca-*APK* [Dnp] in the presence and absence of captopril (selective ACE inhibitor) and DX-600 (selective ACE2 inhibitor). DX-600 significantly inhibited ACE2 activity indicating the specificity of the assay (*Figure 10*). Mca-*APK* [Dnp] substrate was used for ACE2 activity assays. Hence ACE2 assays were conducted using Mca-*APK* [Dnp] substrate for measuring plasma and tissue ACE2 activity. Measurement of circulating forms of ACE2 has proved problematic. In normal plasma, there was no detectable ACE2 activity (*Figure 12*). A recent clinical trial showed that plasma ACE2 was only detectable in 10% of individuals, a total study population of 500 (Rice *et al.* 2006).

In a recent study it was shown that increased ACE2 and decreased ACE protein in young *db/db* mice acted as renoprotective combination (Ye *et al.* 2004). We have shown that there is a significant increase in kidney ACE2 activity and protein expression in *db/db mice* compared to their controls (*Figure 13, 19; P<0.01*). Kidney ACE protein expression, by contrast, was significantly decreased in young (8-9 weeks) normotensive *db/db* diabetic mice compared to their non-diabetic controls. The reduction of ACE protein activity and expression coupled with augmentation of ACE2 protein activity and expression in young *db/db* diabetic mice was tissue specific. In lungs there was no significant difference between *db/db* mice and their lean controls. In plasma by contrast

we observed significantly increased ACE activity and practically no ACE2 activity; *Figure 12 (A) and (B)*. This agrees with previous studies performed by using SELDI-TOF-MS technique (Elased *et al.* 2006). This is the first report to show that there is significant increase in plasma ACE activity in *db/db* diabetic mice.

Western blot analysis was performed to investigate ACE and ACE2 protein expression in the kidney, lungs and the brain from young *db/db* mice (8 weeks) old. We have shown that that in 8-9 weeks *db/db* diabetic mice there was a significant increased kidney ACE2 protein compared to the controls (*Figure 22*,$P<0.01$). ACE protein expression, by contrast, was significantly decreased in kidney from *db/db* mice compared with their non-diabetic controls (*Figure 21*, $P<0.01$). The reduction of tissue ACE protein expression and increase in ACE2 protein expression was tissue specific. In the lungs there was no significant difference in ACE and ACE2 protein expression. There was a good correlation between ACE and ACE2 enzymatic activity and the protein expression in the kidney, lungs and brain. The down regulation of ACE and the up regulation of ACE2 in the kidney is an intrigue finding. The mechanism for tissue specific changes in ACE2 activity is unknown, but it seems reasonable to speculate that it could be, directly or indirectly, a consequence of hyperglycemia and insulin resistance. The fact that the up regulation of ACE2 is organ specific (renal tissue but not the lungs) argues however against a generalized effect of hyperglycemia on ACE2.

In the old (24 weeks) *db/db* mice ACE2 enzyme activity has significantly decreased in the kidney (*Figure 23*; $P<0.01$). ACE enzyme activity by contrast has increased significantly when compared to the young *db/db* mice (*Figure 22* $P<0.01$). On the basis of these findings we can infer that the increase in ACE may result in increased local

levels of Ang II leading to hypertension. In the plasma, ACE enzyme activity was even higher compared to young *db/db* mice (*Figure 24*; $P<0.01$). Hence by this we can infer that the increase in circulating ACE can be the primary reason for increase in blood pressure at a late stage of diabetes. We speculate that this pattern of decreased ACE2 activity coupled with increased ACE may be the primary cause of hypertension and might lead to various cardiovascular dysfunctions. A high level of ACE2 in the young *db/db* mice prevents Ang II accumulation while favoring Ang (1-7) formation. ACE2 has vasodilatory, natriuretic and antiproliferative properties.

CONCLUSION

The counterbalancing role of ACE and ACE2 in the RAS and their involvement in controlling the blood pressure and electrolyte balance is gaining credence. ACE2 has been implicated in a number of disease conditions including hypertension, diabetes and cardiac dysfunction. Most recently structural solutions of both ACE and ACE2 should facilitate further design of potent and selective ACE inhibitors. The findings in the present study have answered some of the questions as to how cardiovascular parameters are affected by changes in ACE/ACE2 balance in different stages of type2 diabetes mellitus.

Results show that the *db/db* diabetic mice are hyperglycemic and hyperinsulinemic at a young age (8 weeks). *db/db* diabetic mice are good animal models for type 2 diabetes. The data from the project implicates that there is a progressive increase in the blood pressure in *db/db* mice showing that these mice become hypertensive as type 2 diabetes progresses from 14-15 weeks of age. The reduction of the blood pressure in losartan treated mice helps us to understand the critical role of Angiotensin ATI receptors in mediating BP changes in diabetic *db/db* mice. Antihypertensive agents such as Angiotensin receptor blockers (ARBs) might be a valuable therapeutic approach for the

treatment of diabetes induced cardiovascular complications as well as diabetic nephropathy.

We have also shown that in the young *db/db* mice, there is a profound reduction in the ACE protein expression and enzyme activity in the kidney, whereas ACE2 protein expression is increased at early stage of diabetes. It seems reasonable to propose that such a combination would attenuate angiotensin II formation and could exert a protective effect against the development of hypertension controlling increase in BP at early stages of type2 diabetes and probably against the development of nephropathy. This kind of pattern of ACE/ACE2 was observed to be tissue specific to the kidney during the course of type2 diabetes in 8-20 weeks old *db/db* mice. Decreased ACE2 activity and protein expression and increased ACE activity might be the sole reason for Ang II accumulation, increase in blood pressure and end stage renal disease at a later stage of diabetes mellitus. We have also shown that there is a significant increase in circulating ACE in db*/db* diabetic mice. ACE2, on the contrast is completely localized in the tissues. Our project has given a novel and a partial explanation as to how the different components of RAS are regulated in different stages of type2 diabetes. As the complexity of this crucial pathway is unraveled, there is growing interest in the therapeutic potential of agents that modulate the activity of ACE and ACE2.

APPENDIX A

Role of angiotensin AT1 receptors in the development of cardiovascular dysfunction in type 2 diabetic (*db*/*db*) mice

Keerthy Kanakamedala, Danielle Senador, Yanfang Chen, Mariana Morris, Khalid. M. Elased
Wright State University Boonshoft School of Medicine, Dayton, OH

Cardiovascular disease is a long term complication of diabetes, which remains a leading cause of mortality and morbidity. There is evidence for activation of the renin angiotensin system (RAS) in diabetic animals and humans. We have shown previously that a high fructose diet in mice produces glucose tolerance and increased blood pressure (BP). This goal of this study was to investigated BP control and the role of the angiotensin AT1 receptors in the *db*/*db* model of type 2 diabetes. Mice (8-9 weeks) were implanted with carotid telemetric probes and 24 hr mean arterial pressure (MAP), heart rate (HR) and activity was monitored every week for up to 20 weeks. At early age (9-10 weeks), *db*/*db* mice developed significant hyperglycemia and hyperinsulinemia which was not associated with changes in MAP. HR and activity were decreased. MAP gradually increased with age in *db*/*db* diabetic mice. By 12 weeks MAP in diabetics was higher as compared to their lean controls both during day (101 ± 1 vs 117 ± 2 mmHg, $p<0.01$) and night (110 ± 1.7 vs 121 ± 3.1 mmHg, $p<0.01$). Chronic treatment with losartan (10 mg/kg/day in drinking water) for 12 weeks significantly blocked the progression of the MAP changes in diabetic mice during day (102 ± 4 vs 117 ± 2.5 mmHg, $p<0.01$) and night (104 ± 4 vs 126 ± 2.2 mmHg, $p<0.01$). Results document the increase in BP in *db*/*db* diabetic mice and the importance of Ang AT1 receptors in mediating the BP changes in this murine model of type 2 diabetes.

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APPENDIX B

Increased ACE activity in young normotensive diabetic (*db/db*) mice

Keerthy Kanakamedala, Danielle Senador, Wenfeng Zhang, Mariana Morris,
Khalid. M. Elased
Wright State University Boonshoft School of Medicine, Dayton, OH

There is evidence for activation of the renin angiotensin system (RAS) in some diabetic animals and humans**.** Emerging evidence shows that the vasoconstrictor actions of Ang II may be opposed by formation of the vasodilator, Ang- (1-7), partly generated by action of angiotensin converting enzyme 2 (ACE2). Recent studies showed increased ACE2 and decreased ACE protein in 8 weeks *db*/*db* mice. However, the role of ACE2 in diabetes, hypertension and renal disease is currently in the early phases of investigation. The aim of this study was to investigate the role of diabetes in triggering an increase in blood pressure (BP) and changes in ACE and ACE2 indices in *db/db* diabetic mice. Mice (7-8 weeks) were implanted with carotid telemetric probes for measurement of BP. Conventional and Surface Enhanced Laser Desorption/Ionization Mass Spectrometry (SELDI-TOF-MS) were used to evaluate plasma, tissue and urine ACE and ACE2 activity. At young age (8-9 weeks), *db*/*db* mice developed hyperglycemia and hyperinsulinemia which was not associated with changes in BP. Unexpectedly in young normotensive mice there was a highly significant increase in plasma ACE activity in *db/db* mice compared to controls ($P<0.001$, n=6). In contrast kidney ACE and ACE2 activities were significantly decreased in young normotensive mice ($P<0.05$, n=6). SELDI-TOF-MS data in plasma demonstrated the formation of a peak corresponding to Ang II (1045, *M/Z*) indicating ACE activity. In addition there was no peak corresponding to Ang- (1-7) (899, *M/Z*), indicating no detectable plasma ACE2 using SELDI-TOF-MS analysis. There was a significant increase in ACE2 activity in *db*/*db* urinary spot samples compared to controls. Western blot analysis show decreased kidney ACE1 and increased ACE2 in hypertensive *db*/*db* mice ($P<0.05$, n=6). Immunofluorescence study of ACE1 and ACE2 in kidney glomeruli showed increased ACE1 and decreased ACE2 staining in hypertensive diabetic mice compared to normal controls. Conclusion: 1) this is the first report of increased plasma ACE activity in *db*/*db* mice, 2) loss of ACE2 in urine and reduced kidney ACE2 protein could contribute to diabetes-induced hypertension, 3) ACE2 may play a compensatory mechanism against development of hypertension in *db*/*db* mice.

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APPENDIX C

Activation of Renin Angiotensin System Caused High Blood Pressure in (*db*/*db*) Diabetic Mice.

Danielle Senador, Keerthy Kanakamedala, Wenfeng Zhang, Yanfang Chen, Mariana Morris, Khalid. M. Elased

Wright State University Boonshoft School of Medicine, Dayton, OH

Cardiovascular disease is a long term complication of diabetes, which remains a leading cause of mortality and morbidity. The goal of this study was to unravel the role of renin angiotensin system (RAS) in the control of cardiovascular parameters in *db*/*db* model of type 2 diabetes. Mice (8-9 weeks) were implanted with carotid telemetric probes and 24 hr mean arterial pressure (MAP), heart rate (HR) and activity was monitored every week for up to 15 weeks. At early age (8-10 weeks), *db*/*db* mice developed significant hyperglycemia and hyperinsulinemia which was not associated with changes in MAP. By 12 weeks MAP in diabetics was higher as compared to their lean controls both during day (101 ± 1 vs 117 ± 2 mmHg, $p<0.01$) and night (110 ± 1.7 vs 121 ± 3.1 mmHg, $p<0.01$). Chronic treatment with losartan (10 mg/kg/day in drinking water) for 12 weeks significantly blocked the progression of the MAP changes in diabetic mice during day (102 ± 4 vs 117 ± 2.5 mmHg, $p<0.01$) and night (104 ± 4 vs 126 ± 2.2 mmHg, $p<0.01$). Surface Enhanced Laser Desorption/Ionization Mass Spectrometry (SELDI-TOF-MS) was used to evaluate RAS enzyme activity. Plasma aliquots (1 µl) from normal and 14 weeks old *db*/*db*-diabetic mice were added to 50 µl MES buffer (50 mM, pH 6.5) containing bestatin and PMSF, spiked with Ang I (1296, *M/Z*) and incubated for 2 hours at 37^{o}C. Protein Chips (CM10) were spotted with 1µl of the reaction mixture and analyzed. Results demonstrated the formation of peak corresponding to Ang II (1045, *M/Z*) indicating angiotensin converting enzyme (ACE1) activity. However there was no peak corresponding to Ang 1-7 (899, *M/Z*) indicating no detectable plasma ACE2 in both groups. There was a significant increase in plasma ACE activity in *db*/*db*-diabetics compared to controls ($p<0.05$, $n=6$). Results document: 1) increase of BP in *db*/*db* diabetic mice, 2) importance of Ang AT1 receptors and plasma ACE1 in mediating the BP changes in this model of type 2 diabetes, 3) potential for using SELDI-TOF-MS to study the processing of angiotensin peptides.

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Reference List

Alderman M H 2004 Dietary sodium and cardiovascular health in hypertensive patients: the case against universal sodium restriction; *J. Am. Soc. Nephrol.* **15 Suppl 1** S47-S50

Bagi Z, Erdei N, Toth A, Li W, Hintze T H, Koller A, and Kaley G 2005 Type 2 diabetic mice have increased arteriolar tone and blood pressure: enhanced release of COX-2-derived constrictor prostaglandins; *Arterioscler. Thromb. Vasc. Biol.* **25** 1610-1616

Brenner B M, Cooper M E, de Z D, Keane W F, Mitch W E, Parving H H, Remuzzi G, Snapinn S M, Zhang Z, and Shahinfar S 2001 Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy; *N. Engl. J. Med.* **345** 861-869

Brosnihan K B, Neves L A, Joyner J, Averill D B, Chappell M C, Sarao R, Penninger J, and Ferrario C M 2003 Enhanced renal immunocytochemical expression of ANG-(1-7) and ACE2 during pregnancy; *Hypertension* **42** 749-753

Bruemmer D, Yin F, Liu J, Berger J P, Kiyono T, Chen J, Fleck E, Van Herle A J, Forman B M, and Law R E 2003 Peroxisome proliferator-activated receptor gamma inhibits expression of minichromosome maintenance proteins in vascular smooth muscle cells; *Mol. Endocrinol.* **17** 1005-1018

Campbell D J 1987 Circulating and tissue angiotensin systems; *J. Clin. Invest* **79** 1-6

Carey R M, Howell N L, Jin X H, and Siragy H M 2001 Angiotensin type 2 receptor-mediated hypotension in angiotensin type-1 receptor-blocked rats; *Hypertension* **38** 1272-1277

Carey R M, Wang Z Q, and Siragy H M 2000 Update: role of the angiotensin type-2 (AT(2)) receptor in blood pressure regulation; *Curr. Hypertens. Rep.* **2** 198-201

Chen Y, Joaquim L F, Farah V M, Wichi R B, Fazan R, Jr., Salgado H C, and Morris M 2005 Cardiovascular autonomic control in mice lacking angiotensin AT1a receptors; *Am. J. Physiol Regul. Integr. Comp Physiol* **288** R1071-R1077

Chobanian A V, Bakris G L, Black H R, Cushman W C, Green L A, Izzo J L, Jr., Jones D W, Materson B J, Oparil S, Wright J T, Jr., and Roccella E J 2003 The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: the JNC 7 report; *JAMA* **289** 2560-2572

Chu K Y, Lau T, Carlsson P O, and Leung P S 2006b Angiotensin II type 1 receptor blockade improves beta-cell function and glucose tolerance in a mouse model of type 2 diabetes; *Diabetes* **55** 367-374

Chu K Y, Lau T, Carlsson P O, and Leung P S 2006a Angiotensin II type 1 receptor blockade improves beta-cell function and glucose tolerance in a mouse model of type 2 diabetes; *Diabetes* **55** 367-374

Coleman D L 1983 Lessons from studies with genetic forms of diabetes in the mouse; *Metabolism* **32** 162-164

Coleman D L and Hummel K P 1974 Hyperinsulinemia in pre-weaning diabetes (db) mice; *Diabetologia* **10 Suppl** 607-610

Crackower M A, Sarao R, Oudit G Y, Yagil C, Kozieradzki I, Scanga S E, Oliveira-dos-Santos A J, da C J, Zhang L, Pei Y, Scholey J, Ferrario C M, Manoukian A S, Chappell M C, Backx P H, Yagil Y, and Penninger J M 2002 Angiotensin-converting enzyme 2 is an essential regulator of heart function; *Nature* **417** 822-828

Crespo M J, Moreta S, and Gonzalez J 2003 Cardiovascular deterioration in STZ-diabetic rats: possible role of vascular RAS; *Pharmacology* **68** 1-8

Danilczyk U, Eriksson U, Crackower M A, and Penninger J M 2003 A story of two ACEs; *J. Mol. Med.* **81** 227-234

Danilczyk U and Penninger J M 2006 Angiotensin-converting enzyme II in the heart and the kidney; *Circ. Res.* **98** 463-471

de G M, Catt K J, Inagami T, Wright J W, and Unger T 2000 International union of pharmacology. XXIII. The angiotensin II receptors; *Pharmacol. Rev.* **52** 415-472

Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart R E, and Acton S 2000 A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9; *Circ. Res.* **87** E1-E9

Elased K M, Cool D R, and Morris M 2005 Novel mass spectrometric methods for evaluation of plasma angiotensin converting enzyme 1 and renin activity; *Hypertension* **46** 953-959

Elased K M, Cunha T S, Gurley S B, Coffman T M, and Morris M 2006 New Mass Spectrometric Assay for Angiotensin-Converting Enzyme 2 Activity; *Hypertension*

Eriksson U, Danilczyk U, and Penninger J M 2002 Just the beginning: novel functions for angiotensin-converting enzymes; *Curr. Biol.* **12** R745-R752

Farah V M, Joaquim L F, Bernatova I, and Morris M 2004 Acute and chronic stress influence blood pressure variability in mice; *Physiol Behav.* **83** 135-142

Farah V M, Moreira E D, Ushizima M, Cestari I A, Irigoyen M C, and Krieger E M 2000 Acute AT1 receptor blockade does not improve the depressed baroreflex in rats with chronic renal hypertension; *Braz. J. Med. Biol. Res.* **33** 1491-1496

Fernandez S F, Huang M H, Davidson B A, Knight P R, III, and Izzo J L, Jr. 2003 Modulation of angiotensin II responses in sympathetic neurons by cytosolic calcium; *Hypertension* **41** 56-63

Ferrario C M and Chappell M C 2004 Novel angiotensin peptides; *Cell Mol. Life Sci.* **61** 2720-2727

Ferrario C M, Jessup J, Chappell M C, Averill D B, Brosnihan K B, Tallant E A, Diz D I, and Gallagher P E 2005a Effect of angiotensin-converting enzyme inhibition and angiotensin II receptor blockers on cardiac angiotensin-converting enzyme 2; *Circulation* **111** 2605-2610

Ferrario C M, Jessup J, Gallagher P E, Averill D B, Brosnihan K B, Ann T E, Smith R D, and Chappell M C 2005b Effects of renin-angiotensin system blockade on renal angiotensin-(1-7) forming enzymes and receptors; *Kidney Int.* **68** 2189-2196

Genuth S 2006 Insights from the diabetes control and complications trial/epidemiology of diabetes interventions and complications study on the use of intensive glycemic treatment to reduce the risk of complications of type 1 diabetes; *Endocr. Pract.* **12 Suppl 1** 34-41

Gilbert R E, Krum H, Wilkinson-Berka J, and Kelly D J 2003 The renin-angiotensin system and the long-term complications of diabetes: pathophysiological and therapeutic considerations; *Diabet. Med.* **20** 607-621

Guo C, Martinez-Vasquez D, Mendez G P, Toniolo M F, Yao T M, Oestreicher E M, Kikuchi T, Lapointe N, Pojoga L, Williams G H, Ricchiuti V, and Adler G K 2006 Mineralocorticoid receptor antagonist reduces renal injury in rodent models of types 1 and 2 diabetes mellitus; *Endocrinology* **147** 5363-5373

Guo M, Ricardo S D, Deane J A, Shi M, Cullen-McEwen L, and Bertram J F 2005a A stereological study of the renal glomerular vasculature in the db/db mouse model of diabetic nephropathy; *J. Anat.* **207** 813-821

Guo Z, Su W, Allen S, Pang H, Daugherty A, Smart E, and Gong M C 2005b COX-2 up-regulation and vascular smooth muscle contractile hyperreactivity in spontaneous diabetic db/db mice; *Cardiovasc. Res.* **67** 723-735

Guy J L, Jackson R M, Acharya K R, Sturrock E D, Hooper N M, and Turner A J 2003 Angiotensin-converting enzyme-2 (ACE2): comparative modeling of the active site, specificity requirements, and chloride dependence; *Biochemistry* **42** 13185-13192

Hackenthal E, Paul M, Ganten D, and Taugner R 1990 Morphology, physiology, and molecular biology of renin secretion; *Physiol Rev.* **70** 1067-1116

Herath C B, Warner F J, Lubel J S, Dean R G, Jia Z, Lew R A, Smith A I, Burrell L M, and Angus P W 2007 Upregulation of hepatic angiotensin-converting enzyme 2 (ACE2) and angiotensin-(1-7) levels in experimental biliary fibrosis; *J. Hepatol.*

Horio N, Clermont A C, Abiko A, Abiko T, Shoelson B D, Bursell S E, and Feener E P 2004 Angiotensin AT(1) receptor antagonism normalizes retinal blood flow and acetylcholine-induced vasodilatation in normotensive diabetic rats; *Diabetologia* **47** 113-123

Huentelman M J, Zubcevic J, Katovich M J, and Raizada M K 2004 Cloning and characterization of a secreted form of angiotensin-converting enzyme 2; *Regul. Pept.* **122** 61-67

Hummel K P, Dickie M M, and Coleman D L 1966 Diabetes, a new mutation in the mouse; *Science* **153** 1127-1128

Jacob H J 1999 Physiological genetics: application to hypertension research; *Clin. Exp. Pharmacol. Physiol* **26** 530-535

Jermendy G, Ferenczi J, Hernandez E, Farkas K, and Nadas J 1996 Day-night blood pressure variation in normotensive and hypertensive NIDDM patients with asymptomatic autonomic neuropathy; *Diabetes Res. Clin. Pract.* **34** 107-114

Kosugi R, Shioi T, Watanabe-Maeda K, Yoshida Y, Takahashi K, Machida Y, and Izumi T 2006 Angiotensin II receptor antagonist attenuates expression of aging markers in diabetic mouse heart; *Circ. J.* **70** 482-488

Li Q and Ren J 2006 CARDIAC OVEREXPRESSION OF METALLOTHIONEIN RESCUES CHRONIC ALCOHOL INTAKE-INDUCED CARDIOMYOCYTE DYSFUNCTION: ROLE OF AKT, MAMMALIAN TARGET OF RAPAMYCIN AND RIBOSOMAL P70S6 KINASE; *Alcohol Alcohol*

Lieberman J, Krauthammer M, and Sastre A 1986 Serum angiotensin-converting-enzyme in rabbits with and without pulmonary granulomatosis. Granulomatosis induced with complete-Freund's-adjuvant or BCG; *Sarcoidosis.* **3** 60-66

Lindholm L H, Ibsen H, Borch-Johnsen K, Olsen M H, Wachtell K, Dahlof B, Devereux R B, Beevers G, de F U, Fyhrquist F, Julius S, Kjeldsen S E, Kristianson K, Lederballe-Pedersen O, Nieminen M S, Omvik P, Oparil S, Wedel H, Aurup P, Edelman J M, and Snapinn S 2002b Risk of new-onset diabetes in the Losartan Intervention For Endpoint reduction in hypertension study; *J. Hypertens.* **20** 1879-1886

Lindholm L H, Ibsen H, Borch-Johnsen K, Olsen M H, Wachtell K, Dahlof B, Devereux R B, Beevers G, de F U, Fyhrquist F, Julius S, Kjeldsen S E, Kristianson K, Lederballe-Pedersen O, Nieminen M S, Omvik P, Oparil S, Wedel H, Aurup P, Edelman J M, and Snapinn S 2002a Risk of new-onset diabetes in the Losartan Intervention For Endpoint reduction in hypertension study; *J. Hypertens.* **20** 1879-1886

Mokdad A H, Bowman B A, Ford E S, Vinicor F, Marks J S, and Koplan J P 2001 The continuing epidemics of obesity and diabetes in the United States; *JAMA* **286** 1195-1200

Mokdad A H, Serdula M K, Dietz W H, Bowman B A, Marks J S, and Koplan J P 2000 The continuing epidemic of obesity in the United States; *JAMA* **284** 1650-1651

Moriyama T, Oka K, Ueda H, and Imai E 2004 Nilvadipine attenuates mesangial expansion and glomerular hypertrophy in diabetic db/db mice, a model for type 2 diabetes; *Clin. Exp. Nephrol.* **8** 230-236

Muller-Wieland D, Kotzka J, Knebel B, and Krone W 1998 Metabolic syndrome and hypertension: pathophysiology and molecular basis of insulin resistance; *Basic Res. Cardiol.* **93 Suppl 2** 131-134

Neels H M, Scharpe S L, van Sande M E, Verkerk R M, and Van Acker K J 1982 Improved micromethod for assay of serum angiotensin converting enzyme; *Clin. Chem.* **28** 1352-1355

Oudit G Y, Crackower M A, Backx P H, and Penninger J M 2003 The role of ACE2 in cardiovascular physiology; *Trends Cardiovasc. Med.* **13** 93-101

Oudit G Y, Herzenberg A M, Kassiri Z, Wong D, Reich H, Khokha R, Crackower M A, Backx P H, Penninger J M, and Scholey J W 2006 Loss of angiotensin-converting enzyme-2 leads to the late development of angiotensin II-dependent glomerulosclerosis; *Am. J. Pathol.* **168** 1808-1820

Parving H H, Lehnert H, Brochner-Mortensen J, Gomis R, Andersen S, and Arner P 2001 The effect of irbesartan on the development of diabetic nephropathy in patients with type 2 diabetes; *N. Engl. J. Med.* **345** 870-878

Peach M J 1977 Renin-angiotensin system: biochemistry and mechanisms of action; *Physiol Rev.* **57** 313-370

Pitt B, Williams G, Remme W, Martinez F, Lopez-Sendon J, Zannad F, Neaton J, Roniker B, Hurley S, Burns D, Bittman R, and Kleiman J 2001 The EPHESUS trial: eplerenone in patients with heart failure due to systolic dysfunction complicating acute myocardial infarction. Eplerenone Post-AMI Heart Failure Efficacy and Survival Study; *Cardiovasc. Drugs Ther.* **15** 79-87

Rice G I, Jones A L, Grant P J, Carter A M, Turner A J, and Hooper N M 2006 Circulating activities of angiotensin-converting enzyme, its homolog, angiotensin-converting enzyme 2, and neprilysin in a family study; *Hypertension* **48** 914-920

Santos R A, Simoes e Silva AC, Maric C, Silva D M, Machado R P, de B, I, Heringer-Walther S, Pinheiro S V, Lopes M T, Bader M, Mendes E P, Lemos V S, Campagnole-Santos M J, Schultheiss H P, Speth R, and Walther T 2003 Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas; *Proc. Natl. Acad. Sci. U. S. A* **100** 8258-8263

Shinozaki K, Ayajiki K, Nishio Y, Sugaya T, Kashiwagi A, and Okamura T 2004 Evidence for a causal role of the renin-angiotensin system in vascular dysfunction associated with insulin resistance; *Hypertension* **43** 255-262

Shiuchi T, Iwai M, Li H S, Wu L, Min L J, Li J M, Okumura M, Cui T X, and Horiuchi M 2004 Angiotensin II type-1 receptor blocker valsartan enhances insulin sensitivity in skeletal muscles of diabetic mice; *Hypertension* **43** 1003-1010

Skeggs L T, Jr. 1993 Discovery of the two angiotensin peptides and the angiotensin converting enzyme; *Hypertension* **21** 259-260

Sowers J R and Stump C S 2004b Insights into the biology of diabetic vascular disease: what's new?; *Am. J. Hypertens.* **17** 2S-6S

Sowers J R and Stump C S 2004a Insights into the biology of diabetic vascular disease: what's new?; *Am. J. Hypertens.* **17** 2S-6S

Stubbs M, Guberski D L, and Like A A 1994 Preservation of GLUT 2 expression in islet beta cells of Kilham rat virus (KRV)-infected diabetes-resistant BB/Wor rats; *Diabetologia* **37** 1186-1194

Tikellis C, Johnston C I, Forbes J M, Burns W C, Burrell L M, Risvanis J, and Cooper M E 2003 Characterization of renal angiotensin-converting enzyme 2 in diabetic nephropathy; *Hypertension* **41** 392-397

Tipnis S R, Hooper N M, Hyde R, Karran E, Christie G, and Turner A J 2000 A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase; *J. Biol. Chem.* **275** 33238-33243

Turner A J, Tipnis S R, Guy J L, Rice G, and Hooper N M 2002 ACEH/ACE2 is a novel mammalian metallocarboxypeptidase and a homologue of angiotensin-converting enzyme insensitive to ACE inhibitors; *Can. J. Physiol Pharmacol.* **80** 346-353

Vickers C, Hales P, Kaushik V, Dick L, Gavin J, Tang J, Godbout K, Parsons T, Baronas E, Hsieh F, Acton S, Patane M, Nichols A, and Tummino P 2002a Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase; *J. Biol. Chem.* **277** 14838-14843

Vickers C, Hales P, Kaushik V, Dick L, Gavin J, Tang J, Godbout K, Parsons T, Baronas E, Hsieh F, Acton S, Patane M, Nichols A, and Tummino P 2002b Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase; *J. Biol. Chem.* **277** 14838-14843

Wingard D L, Barrett-Connor E, Criqui M H, and Suarez L 1983 Clustering of heart disease risk factors in diabetic compared to nondiabetic adults; *Am. J. Epidemiol.* **117** 19-26

Wysocki J, Ye M, Soler M J, Gurley S B, Xiao H D, Bernstein K E, Coffman T M, Chen S, and Batlle D 2006 ACE and ACE2 activity in diabetic mice; *Diabetes* **55** 2132-2139

Ye M, Wysocki J, Naaz P, Salabat M R, LaPointe M S, and Batlle D 2004 Increased ACE 2 and decreased ACE protein in renal tubules from diabetic mice: a renoprotective combination?; *Hypertension* **43** 1120-1125

Yusuf S, Sleight P, Pogue J, Bosch J, Davies R, and Dagenais G 2000 Effects of an angiotensin-converting-enzyme inhibitor, ramipril, on cardiovascular events in high-risk patients. The Heart Outcomes Prevention Evaluation Study Investigators; *N. Engl. J. Med.* **342** 145-153