Insulin Treatment Attenuates Renal ADAM17 and ACE2 Shedding in Akita Diabetic Mice

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Insulin Treatment Attenuates Renal ADAM17 and ACE2 Shedding in Akita Diabetic Mice

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

By

ESAM SAID BUSAH SALEM
MD, Zawia University, Medical College, Zawia, Libya 2007

2013
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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY ESAM S.B. SALEM ENTITLED “INSULIN TREATMENT ATTENUATES RENAL ADAM17 AND ACE2 SHEDDING IN AKITA DIABETIC MICE” BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT

Salem, Esam. M.S., Department of Pharmacology and Toxicology, Wright State University, 2013. Insulin treatment attenuates renal ADAM17 and ACE2 shedding in Akita diabetic mice.

Diabetic patients have a 40%-50% lifetime chance of developing chronic kidney disease, which remains one of the leading causes of morbidity and mortality. Alterations within renin angiotensin system balance contribute to the pathogenesis of diabetic kidney disease. Angiotensin converting enzyme 2 (ACE2) has an endogenous renoprotective role due to its ability to form angiotensin (1-7) (Ang 1-7) by degrading angiotensin II (Ang II). We have shown previously that hyperglycemia increases urinary ACE2 and albumin excretion in db/db diabetic mice. The protease, disintegrin and metalloprotease (ADAM) 17, is involved in the shedding of several transmembrane proteins, including ACE2 in vitro. Tissue inhibitor metalloproteinase-3 (TIMP3) is known to be an endogenous inhibitor of ADAM17. We tested the hypothesis that normalizing hyperglycemia in Akita mice with insulin decreases renal ADAM17, increases TIMP3 protein expression and reduces urinary ACE2 and albumin excretion. Metabolic parameters were monitored weekly. Urine was collected over 24 hours period to measure urinary albumin, creatinine and ACE2 activity. Akita mice demonstrated hyperglycemia and a significant increase in urinary ACE2 and albumin excretion. Treatment of Akita mice with insulin implants for 20 weeks normalized hyperglycemia, decreased urinary ACE2 and albumin excretion. Western blotting demonstrated increased renal ACE2 and ADAM17 protein expression. Immunostaining revealed colocalization of ACE2 with ADAM17 in renal tubules. However, renal TIMP3 expression was not altered in Akita diabetic mice. Normalizing hyperglycemia with insulin also decreased renal ACE2, ADAM17, but had no effect on TIMP3 expression. There was a positive linear correlation between urinary ACE2 levels and albuminuria, blood glucose, and plasma levels of creatinine, glucagon and triglycerides. In conclusion, Akita diabetic mice exhibit increased expression of renal ACE2 and urinary ACE2 excretion, which in turn correlates with a significant increase in renal ADAM17 protein expression. This is the first report showing an association between hyperglycemia and increased shedding of urinary ACE2 in Akita diabetic mice. Therefore, urinary ACE2 may be used as a biomarker for early prediction of diabetic nephropathy as well as for monitoring patients to define effective therapeutic strategies.
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1. INTRODUCTION

Diabetes and its classifications

Diabetes mellitus (DM) is not merely a disease, but rather a heterogeneous multisystemic syndrome with variable degrees of obesity, insulin secretion, and intrinsic resistance to insulin (1). DM has been identified as the most serious, costly and fast-growing chronic epidemic disease worldwide (2). Affected individuals with diabetes will double from 171 million to 366 million in 2050 (3). Over time, diabetes causes serious multiple organ complications mostly related to microangiopathic syndromes such as those associated with diabetic nephropathy, neuropathy, retinopathy, and diabetic cardiomyopathy (4). In fact, diabetic nephropathy and cardiomyopathy are the main causes of morbidity and mortality among diabetic patients. Approximately 70% of deaths in patients with diabetes are caused by cardiovascular disease, yet diabetic kidney disease is considered to be an independent risk factor for cardiovascular mortality (5). DM increases 2-4 fold the risk of premature atherosclerosis, 2-fold renal diseases and 3-fold cerebrovascular disease (6). Recent studies have reported that DM is associated with shortened life span because of its contribution to development of multiple types of cancers, especially colorectal cancer (7).

Most cases of diabetes can be classified into two groups, type 1 diabetes (insulin-dependent diabetes mellitus) and type 2 diabetes with annual estimation of 150,000 newly diagnosed patients with type 1 and 1.3 million with type 2 in the United States (8). Patients with type 1 diabetes constitute 5-10% of the total cases of diabetes worldwide and its prevalence is approximately 1 in 300 adults in the USA (9). The disease is a chronic autoimmune disorder and
characterized by an absolute deficiency of insulin caused by selective autoimmune destruction of β-cells (insulin-secreting cells) of the pancreas. The devastation of β-cells can be caused by external stimuli, such as viral infection or by a genetic predisposition that causes the immune system to identify the β-cells as foreign. Activated T-lymphocytes infiltrate the pancreatic tissue resulting in a pathological condition known as insulitis. As time progresses, pancreatic β-cells are depleted. Nevertheless, manifestations of the disease (β-cells failure) appear when 90% of the β-cells have been destroyed. Frequent hypoglycemic episodes, coma and ketoacidosis are more likely in type 1 compared with type 2 diabetes (8;10). Conversely, type 2 diabetes is the common form of the disease representing 90% of the patients with DM in USA. Also, the prevalence and incidence of the disease is annually rising because of extending life-span as well as increasing prevalence of obesity and sedentary lifestyles in United States (11). Type 2 diabetic patients have a combination of relative insulin resistance mainly observed in liver, adipose and muscle tissues and impaired insulin secretion due to pancreatic β-cells dysfunction. Insulin resistance by itself does not result in type 2 diabetes but rather the combination of tissue resistance to the action of insulin and β-cells hormone deficiency. Obese people overcome the insulin resistance by elevating levels of blood insulin to control fasting blood glucose concentration below 126mg/dl as well as HbA1c below 7% of total hemoglobin (12). The pathogenesis of disease develops gradually with common symptoms such as polyuria, polydipsia and polyphagia. Type 2 diabetes’ pathogenesis, unlike type 1, does not involve autoimmune reactions, but rather associated with family history of diabetes and obesity, for instance; body mass index (BMI) more than 30kg/m² or waist/hip ratio (WHR) more than 0.9 for men, and 0.8 for women.

Adipose tissue is not simply a storage tissue but also an endocrine one, it secretes leptin and adiponectin hormones that contribute to insulin resistance (13). The most common metabolic
alteration of type 2 DM is hyperglycemia which may lead to nonketotic hyperosmolar coma especially in the elderly. Due to the toxic effects of sustained hyperglycemia and elevated levels of free fatty acids (FFA), these patients are also at higher risk for developing premature coronary artery disease which is the main cause of death in both type 1 and 2 diabetes (9;14). Gestational diabetes, a third type, occurs during pregnancy and has been linked to an increased risk of developing type 2 diabetes after labor (15).

**Renin angiotensin system (RAS)**

RAS is recognized as a key contributor of cardiorenal function and dysfunction (16). It regulates blood pressure, blood volume and electrolyte concentration (17;18). Therefore, ACE has been a promising target to control blood pressure (19). Accumulating evidence emphasizes the role of local RAS in the regulation of regional blood pressure in specific tissues, such as the kidney, particularly in the nephron where all components of the RAS are expressed (18;20).

The RAS cascade (as shown in the diagram) is initiated with hydrolytic conversion of hepatic-derived protein, angiotensinogen, to the inactive decapeptide, angiotensin I (Ang I) by an aspartic protease known as renin (21). In turn, Ang I is converted to an active octapeptide, angiotensin II (Ang II), by the membrane-bound dipeptidyl carboxyl zinc metalloprotease, angiotensin converting enzyme (ACE) (22). In fact, the vasoconstrictor peptide Ang II is inactivated by removal of the carboxy-terminal residue through the action of the angiotensin converting enzyme 2 (ACE2) to produce a vasodilator peptide known as angiotensin (1-7) [Ang (1-7)]. Ang (1-7) is proposed to bind and activate its own specific G-protein coupled receptor MAS. Ang (1-7) does not bind the predominant Ang II type 1 receptor (AT1R) (23). Although it is known that additional angiotensin peptides formed during activation of the RAS cascade, such as Ang (1-9), Ang III [Ang (2-8)] and Ang IV [Ang (3-8)] may have potential renoprotective
roles, it is Ang (1-7) the most promising therapeutic target due to its opposing effects to Ang II (24). Conceptually, the balance between ACE and ACE2 activity is proposed to be the main contributor to the tissue and circulating levels of Ang II. Therefore, any imbalance of the ACE/ACE2 activity ratio may contribute to the development and progression of kidney disease (25).

**RAS and diabetes**

The renin angiotensin system is involved in the pathogenesis of diabetic nephropathy. Plasma Ang II is increased in diabetic patients (26). Indeed, some reports have determined Ang II levels 1000 times higher in the diabetic population relative to controls (27).

Ang II effects are mediated by binding to angiotensin type 1 receptor (AT1R), the predominant receptors, or to angiotensin type 2 receptor (AT2R). Like AT1R, AT2R is a G-protein coupled receptor (28). Ang II, a pressor peptide of the RAS, damages the renal structural compartments and declines kidney functions by influencing several pathways: first; its vasoconstrictor properties effect the efferent arteriole, influencing hydrostatic pressure of the glomerular filtration, second; Ang II induces cellular dedifferentiation and proliferation, generates reactive oxygen species and produces inflammation, apoptosis and tubuloglomerular fibrosis that can alter the permeability and selectivity of glomeruli, and third; Ang II causes sodium retention by acting directly on renal tubules or through stimulation aldosterone secretion (29-31).

In contrast to AT1R, AT2R promotes vasodilatation by suppressing the synthesis and release of renin from juxtaglomerular apparatus (32) and inhibits cellular proliferation, inflammation, apoptosis and fibrosis by binding interaction transduced with Ang II (33-36). In this regard, a study has demonstrated that hyperglycemia decreases renal AT2R expression in streptozotocin
(STZ)-induced diabetic rats, which can be reversed by insulin treatment (35). Another study has reported that STZ diabetic AT$_2$R KO mice developed diabetic nephropathy (DN) much faster and worse than observed in diabetic control mice (32). Therefore, targeting the RAS by specific inhibitors, including the angiotensin converting enzyme inhibitors (ACEIs) or angiotensin receptor blockers (ARBs), attenuates urinary albumin excretion and retards the progression of established DN in type 1 and 2 diabetic patients (37). Further, blockage the RAS by dual treatment with the AT$_1$R blocker losartan (AT$_1$RB) and perindopril (ACEI), prevents albuminuria, tubulointerstitial sclerosis and progression of nephropathy in Akita mice (38). On the other hand, a study has reported that upregulation of renal angiotensinogen is involved in local RAS activation and DN development in Zucker diabetic fatty rats (39). Therefore, scientific efforts have been made to produce aliskiren, a pharmacological direct renin inhibitor (DRI), which blocks the rate-limiting reaction of the RAS by preventing the creation of Ang I from angiotensinogen. Aliskiren has shown serious adverse effects in ALTITUDE clinical trial when used in combination with ordinary RAS inhibitors such as ACEIs or ARBs. However, aliskiren had a significant value for treatment of DN. Therefore, according to European Medicines Agency recommendation, aliskiren must not be given with ACEIs or ARBs to diabetic patients (40).
Renin Angiotensin System (RAS) enzyme cascade

- Vasoconstriction
- Cell proliferation
- Inflammation
- Fibrosis

Angiotensinogen (Alpha-2-globulin, 453 a.a.)

Renin

Angiotensin I
Ang (1-10)

ACE2
Angiotensin (1-9)

ACE
Angiotensin II
Ang (1-8)

ACE2

Angiotensin (1-7)

ACE
Angiotensin (1-5)

AT1-R

AT2-R

MAS-R

- Vasodilatation
- Anti-proliferation
- Anti-inflammation
- Anti-fibrosis
**Angiotensin (1-7) (Ang 1-7) forming enzymes**

Angiotensin (1-7), a heptapeptide, plays an important role in RAS because it counteracts the effects of Ang II (such as vasoconstriction, cell proliferation and fibrosis) in many pathological conditions including kidney disease (41). Ang (1-7) can be produced directly from Ang II by removing of the carboxy-terminal amino acid phenylalanine via ACE2 (42). Ang (1-7) can also be generated indirectly from Ang I by removing of the carboxy-terminal amino acid leucine to form the biologically inactive peptide Ang (1-9) via ACE2 (43;44), and then cleaved to produce Ang (1-7) via ACE or neprilysin (NEP) (45). In addition to the classic pathway of Ang (1-7) generation described above, there are other enzymatic pathways that can generate Ang (1-7) from Ang I or Ang II including prolylcarboxypeptidase (PCP), prolylendopeptidase (PEP) and thimet oligopeptidase (46-48). Finally, Ang (1-7) is degraded by removing two amino acids from its carboxy-terminal via ACE resulting in Ang (1-5) which undergoes further degradation producing either Ang (3-5) or Ang (1-4) (27).

**Angiotensin converting enzyme 2 (ACE2)**

ACE2 is a type 1 integral membrane glycoprotein (49). ACE2 is a recently identified component of the RAS with 60% similarity to ACE. ACE2 function is inhibited by the Ca$^{2+}$/Mg$^{2+}$ chelating agent ethylenediaminetetraacetic acid (EDTA), but contrary to ACE, it is not inhibited by lisinopril, captopril or enalapril, traditional ACE inhibitors (50). The protein sequence of ACE2 consists of 805 amino acids with a short C-terminal cytoplasmic domain (22 a.a), a transmembrane domain and a large N-terminal ectodomain anchored to the cell plasma membrane (740 a.a), where the small catalytic active site is located (50). Rodent and human ACE2 genes are located in the X chromosome and both orthologous share 80% identity at the amino-acid level (27;51). The ACE2 gene encodes for a protein with a predicted molecular...
weight of 85kDa. However, ACE2 is expressed as a protein of 120kDa due to its N-glycosylated nature (50). ACE2 is primarily expressed in the kidney and heart, which in turn are the main organs involved in controlling blood pressure (50;52). ACE2 is also found in vascular smooth muscle cells, gastrointestinal tract, liver, pancreas, retina, central nervous system, bone marrow and lymphoid tissues, although at lower levels when compared to heart or kidney (27;53). In the latter case, ACE2 is located in the apical brush border of renal proximal tubules, a characteristic localization also observed in the polarized Madin-Darby Canine kidney (MDCKII) epithelial cells (27;51;54). ACE2 also localizes in glomerular podocytes, renal capillary endothelium, distal tubules and collecting ducts (27). ACE2 acts as a carboxypeptidase rather than as a dipeptidase by cleaving only a single amino acid from the carbon-terminal side of Ang II to form a vasodilator peptide Ang (1-7) as well as of Ang I to form a biologically inactive peptide Ang (1-9) (49;50). Subsequently, Ang (1-7) undergoes degradation by removing two amino acids from its C-terminal via ACE resulting in Ang (1-5) that undergoes further degradation producing either Ang (3-5) or Ang (1-4). ACE2 is the main renal Ang (1-7) forming enzyme because its catalytic efficiency for generation of Ang (1-7) is $2.2\times10^6$ M per s which is dominant and higher than other Ang (1-7) generating peptides (NEP, PEP, and ACE)(27). Even though ACE2 has 400 times higher catalytic activity toward Ang II, ACE2 also cleaves apelin-13, dynorphin A and bradykinin efficiently (51). Consequently, recognition of the two pathways of Ang (1-7) production via ACE2, a counter-regulatory enzyme to ACE, has opened a door into new areas for understanding of renal and cardiovascular physiology as well as provided new potential targets and therapeutic agents (51;54;55).
**Neprilysin**

Neprilysin (NEP) is also known as neutral endopeptidase, CD10 or less commonly as CALLA-common acute lymphoblastic leukemia antigen or enkephalinase (56). NEP is a membrane-bound zinc-dependent metalloendopeptidase. NEP was first identified as a tumor-specific antigen of acute lymphoblastic leukemia, then as one of the renal podocyte antigens (57-59). NEP is expressed in renal podocytes and proximal tubular epithelial cells, as well as, in normal and malignant hematopoietic cells and in smooth muscle of blood vessels (60;61). The NEP gene encodes 4 different transcripts due to alternative inclusion of 4 distinctive exonic regions located in exons 1 and 2. This regions are predicted to imprint unique N-terminal sequences to NEP (61).

NEP generates Ang (1-7) from Ang I, and degrades atrial and brain natriuretic peptides as well (62;63). Accumulating evidence suggest that decreased expression of NEP in podocytes is involved in the pathogenesis of human membranous nephropathy (57;60). Renal NEP activity is markedly reduced in STZ diabetic rats (64). In addition, downregulation of NEP contributes to lung injury and inflammatory response after exposure to diesel exhaust emissions in the airways of normal mice and in cultured human epithelial BEAS-2B cells (65). Decreased levels of NEP are involved in blood pressure regulation, consequently, new dual combined therapies targeting ACE and NEP have been proven effective in reducing blood pressure, yet this medication have been stopped due to relatively high incidence of angioedema (66;67). The new generation of ARBs is intended to inhibit NEP aiming at reducing blood pressure without developing of angioedema (66-68). Controversially, Ang (1-7) formation was decreased by a prolylendopeptidase (PEP) and ACE2 inhibitors but not by NEP inhibitors, in cultured human glomerular endothelial cells (hGEnCs) (63). The dual ECE/NEP inhibitor, SLV338, preserves kidney function and reduces mortality in severe acute ischemic renal failure in male Wistar rats.
SLV338 also prevents hypertensive renal tissue damage in a blood pressure independent manner in L-NAME-treated male Sprague Dawley rats (69). Taken together, these results suggest that a role for NEP in the pathogenesis of chronic kidney disease and hypertension even though more studies are needed to elucidate its impact in the pathogenesis of diabetic nephropathy.

**Renoprotection of ACE2**

It is widely accepted that ACE2 plays a key renoprotective role in diabetic nephropathy due to its ability to catalyze the enzymatic degradation of Ang II, a vasoconstrictor/ inflammatory peptide, to Ang (1-7), a vasodilator/anti-inflammatory peptide (50;70). Studies involving Akita mice (38), STZ diabetic mice (32), STZ diabetic rats (49;71), and other mice models of chronic kidney disease (72) demonstrated decreased renal ACE2 protein expression and activity in this animal models. Further, deletion or pharmacological inhibition of ACE2 in Akita and STZ diabetic mice, respectively, results in increased albuminuria and deteriorated renal function (73;74). In addition, administration of human recombinant ACE2 or mouse recombinant ACE2 to diabetic Akita mice or STZ diabetic rats reduced albuminuria and kidney injury (75;76). Interestingly, a clinical study has demonstrated a decrease in renal tubular and glomerular ACE2 expression in type 2 diabetic patients with nephropathy compared to healthy subjects (77;78). Further, various other studies demonstrated increased renal ACE2 protein and mRNA expression in Akita and \( db/db \) diabetic mice (74;76;79) as well as activity in STZ and \( db/db \) diabetic mice (80). In spite of renal ACE2 expression controversy, the previous results have suggested that ACE2 is an endogenous renoprotective enzyme, which make ACE2 as a new promising target for preventing the onset and retarding the progression of diabetic nephropathy.
ADAM17 and ACE2 shedding

ADAMs are multidomain integral membrane proteins belonging to the metzincin subfamily of proteases (81). ADAM17, also known as tumor necrosis factor-α-converting enzyme (TACE) or CD156q, is a zinc-dependent protease and the most active “sheddase” of the ADAMs family. (82-84) ADAM17 is synthesized as an inactive zymogen, which is proteolytically activated by two proteases: proprotein-convertase PC7 and furin (85;86). The metalloprotease domain mediates ectodomain cleavage resulting in the release of several transmembrane proteins, a phenomenon known as ‘shedding’. The disintegrin domain of ADAM17 is involved in adhesive activities (84-87). The role of ADAM17 in the regulation of RAS is suggested by some studies demonstrating increased ADAM17 levels in mice treated with Ang II (88). Several studies have implicated the involvement of ADAM17 in a broad spectrum of diseases including renal, cardiovascular, diabetes, cancer and autoimmune diseases, a phenomenon probably related to the large variety of substrates that ADAM17 is able to process (82;89-93). ADAM17 shares sequence similarity and potential structural topology features with ADAM10 and both enzymes are inhibited by TIMP3 (see next section). However, neither ADAM17 nor ADAM10 are involved in the shedding of ACE (85;94-96). ADAM17, unlike ADAM10, has a high level of expression that is associated with inflammatory activities during immune response whereas ADAM10 is associated with neurodegenerative disorders and cancers. In addition, TIMP1 inhibits ADAM10, but not ADAM17 (90;96). Accumulating evidence suggests that upregulation of ADAM17 activity results in increased insulin resistance and hyperglycemia (92;93;97). Furthermore, in vitro studies performed in stably transfected HEK293, Huh7 and human respiratory airway epithelial cells demonstrated the role of ADAM17 in the ectodomain shedding of ACE2, whereas ablation of ADAM17 expression using specific RNA duplexes
decreased ACE2 shedding (98;99). Moreover, studies conducted on CHO cells established that ADAM17 is able to cleave the ectodomain ACE2 at the peptide sequence between Arg [708] and Ser [709] (100). Increased expression of ADAM17 coincided with reduced ACE2 activity in 3T3-L1 adipocytes (101). Administration of a pharmacological inhibitor of ADAM17 decreases insulin resistance and chronic kidney injury (88;91). Further, a clinical study conducted on type 2 diabetic patients showed downregulation of TIMP3 leading to high circulating levels of ADAM17 resulting in an increased insulin receptor resistance (102). ADAM17 activity is significantly higher in the liver of mice fed high-fat diets, a known maneuver to induce insulin resistance (97). ADAM17 is highly expressed in adipocytes and elevated level of its expression has been strongly linked to obesity and insulin resistance (103). In summary, ADAM17 has been implicated in a variety of syndromes, some of them characterized by chronic kidney disease (CKD). Therefore, ADAM17 may constitute a promising target for novel therapy of CKD.

TIMP3 (an endogenous inhibitor of ADAM17)
The tissue inhibitors of metalloproteinases (TIMPs) are endogenous inhibitors of matrix metalloproteinases (MMPs) including ADAM17 (104). TIMP3 is the physiological inhibitor of ADAM17, which, as mentioned above, has a crucial role in pathogenesis of various renal diseases, including diabetic nephropathy (105;106). Indeed, decreased levels of TIMP3 results in increased ADAM17 activity in mice harboring heterozygous insulin receptor mutations (92). In addition, renal ADAM17 activity is increased in TIMP3 KO Akita mice (107), which are characterized by a single inactivating mutation in the insulin gene (see below). Furthermore, TIMP3 is decreased in STZ mice, and in kidney biopsies from diabetic patients (106). Clearly, TIMP3 is involved in many pathological conditions. Although the mechanism of action is not
known, it is believed that TIMP3 blocks the release of tumor necrosis factor (TNF), an inflammatory mediator involved in kidney fibrosis, tubular atrophy and ischemia-perfusion injury, from cell surface via inhibition of ADAM17 function (108;109). Therefore, targeting ADAM17 by specific inhibitors may provide a new therapeutic strategy for preventing progression of diabetic nephropathy (110-112).

**Diabetic Nephropathy**

Diabetic nephropathy (DN) is a microvascular and progressive renal impairment caused by chronic hyperglycemia. DN is the main cause of morbidity and mortality among diabetic patients (113). DN is still the major leading cause of end stage renal disease (ESRD), which is the stage that requires kidney dialysis or transplantation (114). DN is diagnosed in more than 40% of patients with type 1 diabetes (115). In addition to microalbuminuria, the early pathological manifestations include glomerular mesangial cells hypertrophy, hyperperfusion and basement membrane thickening. As the disease progresses, albuminuria and tubulointerstitial fibrosis increases and effective glomerular filtration rate (eGFR) reduces (116;117). However, a study has shown that tubular damage precedes glomerular damage in the development of DN (118). Renal RAS, in particular Ang II, plays a crucial role in the pathogenesis of DN (119;120). Ang II is a potent vasoconstrictor that promotes the synthesis of prosclerotic cytokine transforming growth factor-β (TGF-β), stimulates the production of chemoattractant protein-1 (MCP-1), reactive oxygen species and the upregulation of podocyte autophagic genes (LC3-2, beclin-1) which increases renal damage and impairing its function (26). Treatment with ACEIs and ARBs is an efficient therapy which prevents progression of DN (121). Clinical studies have demonstrated that ARBs or ACEi decreases albuminuria and hypertension in patients with type 1
diabetic nephropathy (122). In spite of the fact that ARBs are considered very effective in reducing proteinuria compared to ACEi, the combination therapy of them in diabetic nephropathy is still debatable (26). Substantial clinical evidence has demonstrated a link between constant maintenance of euglycemia and blockage of RAS in preventing or retarding development of DN in early diagnosed type 1 diabetic patients (123). Furthermore, clinical studies involving patients with type 1 and 2 diabetes have shown that standard control of hyperglycemia delays onset and slows the progression of DN. In addition, a prospective diabetes study has shown that intensive glycemic control did not reduce total cardiovascular-related mortality rate compared to standard control of regimen (124). Therefore, it is believed in that controlling hyperglycemia should be the objective of therapeutic strategies to prevent or at least retard the onset of DN.

**Diabetic animal models of nephropathy**

Development of an ideal animal model of diabetes will undoubtedly facilitate our understanding of underlying pathogenic mechanisms of various complications including DN (125;126). Mice are attractive models to study DN because specific genes can be manipulated with relative ease (126;127).

Akita mice are an excellent genetic model of type 1 diabetes, making them useful for studying diabetic complications and specifically, diabetic nephropathy. Akita mice have a heterozygous spontaneous mutation in the insulin 2 gene \((Ins2^{Akita})\) that leads to a disruption in the disulfide bond of insulin molecule, resulting in insulitis and reduced pancreatic \(\beta\)-cells mass and failure. Akita mice develop spontaneous hyperglycemia, hypoinsulinemia, increased albuminuria, significant renal histopathological structural abnormalities and kidney dysfunction. Interestingly, Akita mice of different genetic background, for instance; C57BL/6, DBA/2, F1 and FVB/NJ,
exhibit variable levels of albuminuria suggesting genetic-specific susceptibility to diabetic kidney disease (128).

Streptozotocin (STZ)-induced diabetic mice and rats are chemical models of type 1 diabetes (129). STZ, an antibiotic isolated from *Streptomyces achromogenes*, is a toxic glucose analogue and a powerful alkylating agent that accumulates in pancreatic β-cells causing their destruction (130;131). A single high dose of STZ (60mg/kg) in rats or multiple small doses (40mg/kg) in mice result in hyperglycemia and albuminuria within five days (129-131). Interestingly, STZ-induced eNOS−/− diabetic mice, a type 1 diabetic mouse model with deficiency in eNOS activity, exhibit hyperglycemia, 10-fold increase in albuminuria, suggesting that the degree of albuminuria may be dependent upon particular animal models or may related to the genetic background (126;132). Indeed, non-obese diabetic (NOD) mice, another type 1 diabetes model, are characterized by hypoinsulinemia, hyperglycemia, modest albuminuria and mild renal histopathological changes (133). Type 1 diabetic OVE26 mice are a transgenic mouse model of DN, characterized by increased expression of calmodulin in pancreatic β-cells resulting in severe and early onset of hyperglycemia, profound hypoinsulinemia, albuminuria along with hypertension (126;134;135).

The obese hyperinsulinemic *db/db* mice, is a monogenic model of type 2 diabetes. It is characterized by a mutation in the leptin receptor leading to abnormal cellular signaling involved in leptin hormone action. This disturbance of leptin-mediated signaling results in leptin resistance, hyperleptinemia, hyperphagia, hyperglycemia and severe kidney dysfunction (136;137). The *ob/ob* mouse model has similar characteristics to the *db/db* model. However, the former is characterized by a deficiency in leptin rather than its receptor. These mice exhibit obesity, hyperinsulinemia, severe hyperglycemia, increase in renal mesangial matrix deposition
and urinary albumin excretion (133). In summary, inbred strains of mice exhibit substantial differences in the levels of hyperglycemia and albuminuria, declining renal function, renal histopathological changes, pathogenesis course of DN and the absence of ESRD. Therefore, to circumvent the nonspecific toxicity and side effects of STZ model of type 1 diabetes, Akita mice were used in the present study, the mutant model of type 1 diabetes, which closely mirror human DN course in order to enhance our understanding and accelerate our progress toward early diagnosis and treatment for this disease.

**Urinary biomarkers**

Urine is an important source of biomarkers because it is easily obtained and analyzed in non-invasive fashion. Urinary proteins are used as biomarkers include plasma proteins that pass through via glomerular filtration as well as proteins secreted by renal tubules and urinary tract, for instance; ureter, urinary bladder and urethra (138). Therefore, urinary biomarkers can directly reflect the pathogenesis of diabetic urological diseases (139). The advance and development of the proteomic technique permitted the identification of several proteins as potential biomarkers. Current studies of urinary biomarkers are focusing on diabetic nephropathy disease and renal rejection after transplantation (140).

**Albuminuria**

Albuminuria is defined as a persistent increase in the excretion of albumin in the urine. This parameter is a well-known kidney risk marker in patients with diabetes or hypertension (141;142). Microalbuminuria is the primary biomarker used in the clinical diagnosis of diabetic kidney disease. However, albuminuria due to diabetic nephropathy usually develops in late stages of the disease (19;143). Based on urinary albumin-to-creatinine ratio (UACR),
microalbuminuria is considered when 30-299.9 mg albumin per g creatinine is detected in spot urine specimen. If UACR equals or exceeds 300 mg/g, it is known as macroalbuminuria, which is observed in diabetic patients with severe kidney damage (144;145). In addition, approximately 55% of diabetic nephropathic patients with significant decline in GFR are non-albuminuric (UACR is less than 30 mg/g) so albuminuria detection is not the ideal screening test for DN (146). Control of blood pressure was effective for preventing onset and progression of microalbuminuria to macroalbuminuria in type 2 diabetic patients, but it did not prevent the development of end stage renal disease as shown in the ADVANCE trial (147). Therefore, there is a need for a more sensitive and specific marker for early prediction of DN.

**Urinary RAS components**

Soluble ACE2 protein activity and expression has been detected in human and sheep urine (148;149). Most of urinary ACE2 activity is derived from proteolytic shedding of its ectodomain, rather than desquamation of cells from the urinary tract (49). The use of ACE2 as a marker for the clinical diagnosis of chronic kidney disease (CKD) has been recently established in clinical studies where urinary levels of ACE2 protein expression and activity were significantly increased in patients with CKD compared to healthy subjects, in CKD patients with diabetic nephropathy when compared to those without nephropathy (148), and in diabetic renal transplant patients compared to healthy controls (150). Furthermore, a strong positive correlation between urinary ACE2 mRNA expression and proteinuria levels in type 2 diabetic patients with nephropathy has been recently established (151). Consequently, urinary ACE2 levels can reflect diabetic intrarenal changes, which could be used as a potential early biomarker of diabetic kidney diseases (49). Angiotensinogen (AGT) is found in high levels in the urine of primary hypertensive patients compared to control subjects (152). In addition, there is also an elevation of
urinary AGT in type 1 STZ diabetic mice (153), in type 1 diabetic childhood patients (154), in type 2 diabetic patients (155), in chronic hypertensive patients (156) and in patients with chronic kidney disease (157). In the same vein, a recent study has reported that urinary renin and its precursor, prorenin, were found to be elevated in diabetic patients. However, both parameters are mainly derived from plasma filtration in the urine rather than from renal tubules or glomeruli, making these parameters of little value as biomarker of DN (158). Therefore, the scientific community is trying to find out a specific and surrogate new urinary biomarker which can predict the early stage of DN.

Other urinary biomarkers

A variety of urinary biomarkers have been evaluated to predict acute kidney injury and diabetic nephropathy; for example: [1] neutrophile gelatinase-associated lipocalin (NGAL) and osteopontin (OPN) are considered potential urinary biomarkers with predictive value for acute kidney injury in premature infants (159); [2] detection of high levels of urinary haptoglobin may predict type 2 diabetic patients who are more prone to develop nephropathy (160); [3] urinary 8-oxo-7,8-dihydro-2-deoxyguanosine (8-oxodG) is a DNA oxidation marker presents in the urine and of potential use to determine pathophysiology of diabetes and related complications. There is an increase in urinary 8-oxodG levels in type 2 diabetic patients with microvascular complications such as retinopathy and nephropathy (161); [4] urinary mesenchymal mRNA related markers such as desmin, FSP-1, and MMP-9 are found in glomerular podocytes of DN and may be used for monitoring DN progression. Urinary mRNA levels of α-SMA, fibronectin, and MMP-9 are significantly higher in DN patients compared to healthy subjects (162); [5] type 2 diabetic patients with nephropathy have 3-fold increase in the urinary concentrations of their γ-glutamyltransferase and alkaline phosphatase enzyme activities compared to patients without
(163); [6] elevated levels of human zinc-α₂-glycoprotein in urine can be used to predict the development and progression of DN with normoalbuminuria (146); [7] abundant levels of mindin in urine can be used as marker to identify podocyte injury in DN (164); [8] elevated levels of urinary beta 2 microglobulin can be used as markers to identify patients with DN (165); [9] presence of higher levels of urinary liver-type fatty acid-binding protein (LFABP) indicated renal tubular damage and can be used as potential marker in tubulointerstitial damage of DN (166;167); [10] microvesicle-bound dipeptidyl peptidase-IV (DPP IV) concentration increased in the urine before the existence of albuminuria in patients with DN (168); [11] elevated concentration of urinary Pigment epithelium-derived factor (PEDF) can be used as a potential biomarker in predicting DN in type 2 diabetes (169); [12] presence of adiponectin in the urine can be used as a potent biomarker to discriminate early stage of glomerular vascular damage of DN (170); [13] a recent study has reported an increase in activity of urinary heparanase, endo-β (1,4)-glucuronidase that cleaves heparin sulphate of glomerular basement membrane, in patients with type 1 and 2 diabetic nephropathy (171). Clearly, many different biomarkers have been proposed to have predictive value, however, some of them are difficult to detect, developed in late stage of the disease or not organ specific marker.

**Biosynthesis of insulin**

Insulin is a polypeptide anabolic hormone, made up of two chains (A chain and B chain) connected by two disulfide bonds. This is the active and circulating form of insulin in the blood, which acts on cellular receptors and exerts major actions on hepatic, adipose and muscle tissue. Insulin is first synthesized in the pancreatic β-cells in the form of a larger peptide chain called proinsulin within the rough endoplasmic reticulum (172;173). During the packaging mechanism, a Golgi apparatus enzyme, protease, converts the folded proinsulin to insulin and C-peptide.
chain by hydrolyzing the single peptide chain at two locations. Insulin and C-peptide chain are then secreted into the blood in equimolar amounts by exocytosis (174-176). Insulin secretion is mainly regulated by blood glucose levels through a negative feedback mechanism. In this regard, an increase in blood glucose, usually after digesting a meal rich in carbohydrates, is detected by the β-cells within the islets of Langerhans, resulting in increased insulin secretion, glucose uptake and utilization as well as decreased blood glucose. The mechanism of insulin secretion occurs in two phases. First, there is an immediate phase, which involves a sharp peak and lasts for several minutes, and this is followed by a slow rising phase which lasts for a couple of hours (177;178).

**Renoprotection of insulin**

It has been affirmed that patients with type 1 diabetes are at a higher risk of developing incipient and overt nephropathy even though they are normoalbuminuric (179;180). Indeed, a study has reported that incidence of DN among patients with type 1 diabetes decreased consistently as a result of improved hyperglycemic control (181;182). In addition, a comprehensive study revealed that microvascular and macrovascular complications of type 1 diabetes are influenced by genetic variability as well as environmental factors including a person’s socioeconomic status (183). Accumulating studies have demonstrated that chronic glycemic control with insulin treatment retards diabetic microvascular complications, including DN in type 1 diabetic patients (184;185). Further, a clinical study has reported that sustained subcutaneous insulin infusion therapy decreases the glycosylated haemoglobin (HbA1C) and prevents development of DN in type 1 diabetic patients (186). Continuing in the same vein, a study has showed that keeping the HbA1c less than < 6.5%, fasting blood glucose (FBG) <110mg/dL with insulin treatment can retard the onset and advancement of DN in patients with type 1 diabetes (187). Moreover, insulin
treatment slows the progression of established DN in patients with type 2 diabetes (188;189) and has a potential role in retarding the onset of DN in newly diagnosed type 2 diabetic patients (190;191). Type 1 and 2 diabetic patients treated with insulin normalized blood glucose levels with minimum weight gain (192). Clearly, insulin therapy ameliorates renal histopathological abnormalities in STZ diabetic rats (193), decreases urinary albumin excretion and improves kidney function in STZ diabetic mice (153). Insulin treatment also improves glycemic control in transplanted insulin islets in Akita mice (194). Therefore, normalizing hyperglycemia with anti-diabetic medications is a fundamentally important in preventing the onset and retarding the progression of DN regardless to other antihypertensive medications.
2. HYPOTHESIS AND SPECIFIC AIMS

2.1. Hypothesis

In diabetic Akita mice, there is an increased shedding of ACE2, which is associated with increased renal ADAM17 protein expression. Normalizing hyperglycemia with insulin reduces urinary ACE2 excretion, renal ADAM17 protein expression and improves kidney function.

2.2. Specific aims

1. To test the hypothesis that in a mouse model of type 1 diabetes, there is an increase in urinary ACE2 excretion.

2. To test the hypothesis that normalizing hyperglycemia with insulin decreases ACE2 shedding and ameliorates albuminuria in Akita diabetic mice.

3. To test the hypothesis that hyperglycemia increases renal ADAM17 and decreases TIMP3 protein expression in Akita diabetic mice and that normalizing hyperglycemia with insulin will correct this dysfunction.
3. MATERIALS AND METHODS

**Study design.** Male (9 wk old) diabetic Akita mice (C57BL/6-Ins2Akita/J) and their age-matched wild type mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed individually in plastic cages at room temperature (22°C) with 12:12 hour light: dark cycle for 20 weeks. Animals had free access to water and standard 18% protein rodent chow. Mice (10 wk age) were randomly assigned into three groups: 1) control, 2) Akita untreated, 3) Akita treated with insulin.

Insulin treatment was delivered using LinBit a sustained-release insulin implant (0.1 Unit/day/implant; Lin-Shin Canada Inc, Toronto, Canada). These implants release insulin for at least 30 days. Following a brief anesthesia with isoflurane, insulin implants were immersed in betadine solution and implanted subcutaneously with a 12-gauge needle under the mid dorsal skin. The LinBit insulin implants are made from a mixture of insulin and micro-recrystallized palmitic acid.

Blood glucose, body weight, food intake, water intake and urine output were monitored weekly. All experiments were conducted in accordance to the guidelines of the Wright State University Animal Care and Use Committee.

3.1. **Body composition measurement**

Body composition was measured using $^1$H magnetic resonance spectroscopy (EchoMRI-100, Echo Medical system, Houston, TX, USA). After the calibration of the apparatus, mice body weights were measured individually. Briefly, each mouse was introduced in a clean and transparent plastic cylinder and held in position with a plastic plunger to prevent movements.
The cylinder containing the animal was then placed into the apparatus to determine fat content, lean mass and total body water measurements. At the end of the measurement each mouse was returned to the respective cages.

3.2. Measurement of blood glucose levels

Blood glucose concentration was determined by using a glucometer (FreeStyle Lite® Blood Glucose Monitoring System, Abbott, CA, USA) and FreeStyle® Blood Glucose Test Strips. A gentle cut was made at the tip of the mouse’s tail to draw venous blood samples for measurement. Values were recorded and expressed in mg/dL.

3.3. Urine collection

Mice were placed individually in metabolic cages for 24h urine collection with free access to food and water. Protease inhibitors (Roche Diagnostics, IN, USA) were added to prevent protein degradation. Urine samples were collected in two steps every 12h and kept at 4°C until the 24h collection was completed. Samples were centrifuged at 3,000 x g for 5 min at 4°C to remove cellular debris and supernatants were aliquoted and stored at -80°C until use.

3.4. Western blot analysis

Urine samples and kidney protein extracts (lysates) were obtained from non-diabetic wild type, Akita diabetic and Akita diabetic treated with insulin mice. Kidney lysates were obtained after homogenization on ice using Complete Lysis-M EDTA-Free buffer (Roche Applied Science, IN, USA) containing 2.5 mmol/L PMSF and protease inhibitors. Tissue homogenates were centrifuged at 10,000x g for 10 minutes at 4°C to remove cellular debris. Urine samples (2-10µl)
were normalized to creatinine that determined by using an enzyme-linked immunoassay (see below), and kidney protein lysates (50µg) were separated on 10% sodium dodecyl-sulfate polyacrylamide gel and electroblotted to polyvinylidene fluoride membranes (Millipore, MA, USA) (With Bio-Rad transfer apparatus, Hercules, CA). Each membrane was incubated overnight at 4°C with the following specific primary antibodies: goat anti-ACE2 (1:1000, R&D Systems, MN, USA), rabbit anti-ADAM17 (1:500, Enzo Life Sciences, NY, USA) or goat anti-TIMP3 (1:200, Enzo Life Sciences, NY, USA) followed by incubation with HRP-conjugated donkey anti-goat (1:2000 R&D Systems, USA) or donkey anti-rabbit (1:20000, Jackson ImmunoResearch, USA) secondary antibodies. Signals were detected using supersignal chemiluminescent substrate (Thermo Scientific, IL, USA) and visualized using Fujifilm image analyzer (LAS 3000, image Quant, CA, USA).

3.5. Kidney histology and immunohistochemistry

Tissues were collected from mice anaesthetized by injecting ketamine/xylazine mixture (100:8 mg/kg) and perfused intracardially with ice cold 1x PBS to flush out the blood followed by 4% paraformaldehyde (PFA) for 10 min. Kidneys were fixed in 4% PFA overnight at 4°C and sent to AML laboratories (Baltimore, MD, USA) for paraffin mounting, microtomy and staining. For immunofluorescence experiments, paraffin sections were deparaffinized by washing in xylene for 5 min and subsequent hydration with 100%, 95%, 70%, 50% and 30% ethanol. Sections were then rinsed with double-distilled water (ddH2O) and 1x PBS. Slides were then placed in plastic containers in the presence of 10 mM sodium citrate (pH 8.5) and transferred to a water bath at 95°C for 60 minutes. Slides were then blocked in 3% normal horse serum (diluted in PBS containing 0.1% Triton-X) for 1hr at 4°C. Primary antibodies used were: goat anti-ACE2, rabbit
anti-ADAM17 diluted in 3% normal horse serum. Sections were incubated with primary antibodies overnight at 4°C. After this period, the sections were washed with 1x PBS three times and incubated with donkey anti-goat Fluorescein-conjugated and anti-rabbit Cy3-conjugated secondary antibody (1:100, Jackson ImmunoResearch, PA, USA), respectively. Slides were allowed to air-dry and mounted using Vectashield-mounting medium (Vector, Burlingame, CA, USA). Images were obtained using a conventional fluorescence microscope (Optronics, Goleta, CA) as well as FV1000 Confocal Microscope (Olympus, PA, USA). MetaMorph software (Molecular Devices, CA, USA) was used for quantitation of fluorescent signals.

3.6. **ACE2 enzymatic activity assay**

ACE2 activity was measured by using a fluorogenic substrate 7-Mca-APK quenched by Dnp group (Biomol International, NY, USA). The fluorogenic substrate is specific for ACE2. After cleavage of the fluorogenic substrate, the 2, 4-dinitrophenyl moieties were removed to avoid quenching, resulting in falsely increased fluorescence. ACE2 activity was measured in presence of (10µM lisinopril), an ACE inhibitor to prevent interference from endogenous ACE. The assay consisted as follows: Two microliter kidney lysate (20-30µg) or 1-10µl urine samples (20-30µg) were incubated with 100µl of the reaction buffer (50 mM Tris, 5 mM ZnCl₂, 150 mM NaCl₂ and 10 µM lisinopril) and 4 mM Mca-APK (Dnp). 96-well plates were read 0, 1, 2, 3 and 4 hours post-incubation. Fluorescence was detected at 393nm after excitation by using a Fusion™ Packard plate reader. Results were expressed as pmoles/hr/ug protein of cleaved substrate for kidney samples and pmoles/hr/mg creatinine for the urine samples.
3.7. **Urinary albumin assay**

Urinary albumin excretion was determined by using Mouse Albumin ELISA Quantitation Set (Bethyl Laboratories, Montgomery, TX, USA) following the instructions of the manufacturer. Briefly, 96 well plates were coated with (100μl) goat anti-mouse albumin antibodies diluted in carbonate-bicarbonate buffer (1:100) for 1 hour at RT. After incubation, the dilution was suctioned and each well washed with wash buffer (250μl, TBS-T) 4 times. Plates were then incubated overnight at 4°C after adding (200μl) blocking buffer (Tris buffered saline with 1% BSA). The next day, wells were washed 4 times with (250μl, TBS-T). Standards were diluted according to the kit’s protocol. Urine samples (1μl) were diluted with (500μl) of conjugate buffer. Then, (100μl) of diluted standards and urine samples were loaded into each well and incubated for 1 hour at RT. After washing as indicated before, diluted HRP conjugated secondary antibodies (100μl) (1:35000 in conjugate buffer) were added to each well and incubated for 1 hour at RT. Wells were then washed 4 times with (250μl, TBS-T). Equal volumes of TMB substrate A and substrate B (KPL, Gaithersburg, MD, USA) were mixed for 1 hour in the dark, and then 100μl of this substrate mixture were added to each well and incubated in dark for 30 minutes. The reaction was stopped by adding 100μl of 2N H₂SO₄ to each well. Measurements were taken at 450nm by using a Fusion R Packard plate reader.

3.8. **Urinary creatinine assay**

Evaluation of urinary and plasma creatinine levels was carried out with a kit purchased from Quidel (San Diego, CA, USA). The principle of this assay is based on a modified Jaffe reaction in which alkaline picrate produces a colored solution in presence of creatinine as well as a direct proportion between the amount of creatinine in the sample and intensity of color production.
Urine, plasma samples and standards were diluted with distilled water (1:40), and then (50µl) of them were loaded into 96 well plate followed by adding (150µl) color reagent (7 ml picric acid + 1ml 1N NaOH). This mixture was incubated for 30 minutes at RT. Measurements were taken at 450nm by using a Fusion² Packard plate reader.

3.9. Plasma hormones and lipid measurement
Trunk blood was collected in ice-chilled heparinized tubes. Blood was centrifuged at 10,000 x g for 10 minutes at 4° C. Plasma was separated, aliquoted and stored at 80° C. Plasma samples were analyzed for insulin, glucagon, adiponectin, leptin and triglyceride levels at the Mouse Metabolic Phenotyping Centre (Cincinnati, OH, USA).

3.10. Statistics analyses
Statistical analysis was carried out with statistica software (Version.10) and Graph pad prism (5.01). All data was presented as mean ± SEM. An unpaired student’s t-test was used to evaluate the difference between two groups. However, for more than two groups one-way analysis of variance (ANOVA) was used. The differences in metabolic parameters (blood glucose, body weight, food intake, water intake, body fat, lean body mass and total body water) were assessed by repeated measures two-way analysis of variance. A probability value less than 0.05 was considered significant. If a significant difference was recognized, a Bonferroni’s multiple comparison test was performed.
Table 1: Age dependent changes in metabolic parameters of wild type, Akita and Akita mice treated with insulin.

<table>
<thead>
<tr>
<th>Mice strain</th>
<th>Wild type</th>
<th>Akita</th>
<th>Wild type</th>
<th>Akita</th>
<th>Akita +insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (weeks)</td>
<td>10</td>
<td>10</td>
<td>30</td>
<td>30</td>
<td>30</td>
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<tr>
<td>Group size (n)</td>
<td>10</td>
<td>10</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Duration of treatment (weeks)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Blood glucose (mg/dL)</td>
<td>121 ± 2</td>
<td>347 ± 9*</td>
<td>115 ± 2</td>
<td>450 ± 9*</td>
<td>124 ± 6*</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>22 ± 0.5</td>
<td>20 ± 0.3*</td>
<td>30 ± 1</td>
<td>26 ± 0.5*</td>
<td>27 ± 0.1</td>
</tr>
<tr>
<td>Food intake (g/day/mouse)</td>
<td>4 ± 0.2</td>
<td>6 ± 0.3*</td>
<td>5 ± 0.1</td>
<td>14.7 ± 1.2*</td>
<td>6.2 ± 0.6*</td>
</tr>
<tr>
<td>Water intake (ml/day/mouse)</td>
<td>5.5 ± 0.3</td>
<td>21 ± 1.9*</td>
<td>7.5 ± 0.2</td>
<td>44.7 ± 2.5*</td>
<td>7 ± 0.5*</td>
</tr>
<tr>
<td>Body fat (g)</td>
<td>1.8 ± 0.2</td>
<td>1.6 ± 0.2*</td>
<td>4.9 ± 0.3</td>
<td>2.1 ± 0.1*</td>
<td>4 ± 0.2*</td>
</tr>
<tr>
<td>Lean mass (g)</td>
<td>15.5 ± 0.3</td>
<td>14.5 ± 0.2</td>
<td>21.9 ± 0.4</td>
<td>20.0 ± 0.6</td>
<td>20.8 ± 0.5</td>
</tr>
<tr>
<td>Total body water (g)</td>
<td>13.2 ± 0.3</td>
<td>12.4 ± 0.2</td>
<td>18.7 ± 0.3</td>
<td>17.6 ± 0.4</td>
<td>17.3 ± 0.3</td>
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</tbody>
</table>

- Values represent mean ± SEM. *p<0.05 vs. age-matched wild type mice, and # p<0.05 vs. age-matched untreated Akita mice were considered statistically significant.
Table 2: Plasma hormones and lipid parameters of wild type, Akita and Akita mice treated with insulin.

<table>
<thead>
<tr>
<th>Mice strain</th>
<th>Wild type</th>
<th>Akita</th>
<th>Wild type</th>
<th>Akita</th>
<th>Akita + insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (weeks)</td>
<td>10</td>
<td>10</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Group size (n)</td>
<td>10</td>
<td>10</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Duration of treatment (weeks)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Plasma insulin (ng/dL)</td>
<td>ND</td>
<td>ND</td>
<td>2.3 ± 0.2</td>
<td>0.2 ± 0.04*</td>
<td>3.4 ± 0.4#</td>
</tr>
<tr>
<td>Plasma glucagon (ng/mL)</td>
<td>ND</td>
<td>ND</td>
<td>0.02 ± 0.003</td>
<td>0.09 ± 0.01*</td>
<td>0.04 ± 0.006#</td>
</tr>
<tr>
<td>Plasma leptin (ng/mL)</td>
<td>ND</td>
<td>ND</td>
<td>2.5 ± 0.2</td>
<td>0.4 ± 0.1*</td>
<td>2.3 ± 0.1#</td>
</tr>
<tr>
<td>Plasma adiponectin (µg/mL)</td>
<td>ND</td>
<td>ND</td>
<td>12.9 ± 0.8</td>
<td>7.5 ± 0.5*</td>
<td>12.4 ± 0.4#</td>
</tr>
<tr>
<td>Plasma triglycerides (mg/dL)</td>
<td>ND</td>
<td>ND</td>
<td>98.9 ± 7</td>
<td>318.4±17.5*</td>
<td>89±5.1#</td>
</tr>
<tr>
<td>Plasma cholesterol (mg/dL)</td>
<td>ND</td>
<td>ND</td>
<td>105.2 ± 7</td>
<td>102.6 ± 5.6</td>
<td>94.6 ± 8</td>
</tr>
</tbody>
</table>

- Values represent mean ± SEM *p<0.05 vs. age-matched wild type mice, and # p<0.05 vs. age-matched untreated Akita mice were considered statistically significant. ND means not determined.
Table 3: Age dependent changes in renal function parameters of wild type, Akita and Akita mice treated with insulin.

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>Akita</th>
<th>Wild type</th>
<th>Akita</th>
<th>Akita + insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (weeks)</td>
<td>10</td>
<td>10</td>
<td>30</td>
<td>30</td>
<td>30</td>
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<tr>
<td>Group size (n)</td>
<td>10</td>
<td>10</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Duration of treatment (weeks)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Albuminuria (µg/day)</td>
<td>23.5 ± 1.1</td>
<td>118.9 ± 11.4*</td>
<td>21.8 ± 6.4</td>
<td>185.6 ± 25.3*</td>
<td>46.5 ± 19.1#</td>
</tr>
<tr>
<td>Plasma creatinine (mg/dL)</td>
<td>ND</td>
<td>ND</td>
<td>0.5 ± 0.0</td>
<td>0.9 ± 0.1*</td>
<td>0.5 ± 0.0##</td>
</tr>
<tr>
<td>Urine output (ml/day)</td>
<td>1.0 ± 0.2</td>
<td>12 ± 1.6*</td>
<td>1.1 ± 0.1</td>
<td>33.2 ± 2.4*</td>
<td>3.2 ± 0.4##</td>
</tr>
</tbody>
</table>

- Values represent mean ± SEM. *p<0.05 vs. age-matched wild type mice, and #p<0.05 vs. age-matched untreated Akita mice were considered statistically significant. ND means not determined.
Figure (1). Time related changes in blood glucose levels in WT mice (green line), Akita diabetic mice (black line) and Akita diabetic mice treated with insulin (dotted line). Repeated measurements two-way ANOVA using a Bonferroni’s posthoc test showed that treatment caused a significant decrease in blood glucose levels of Akita + Insulin mice \([F (2, 20) = 1076.245], p<0.0001\). Similarly, duration of treatment showed significant decrease in blood glucose levels of Akita + Insulin mice \([F (8, 80) = 43.172], p<0.0001\). Data are represented as mean ± SEM of group size (n=8-10).
**Figure (2).** Time related changes in body weight measurement in WT mice (green line), Akita diabetic mice (black line) and Akita diabetic mice treated with insulin (dotted line). Repeated measurements two-way ANOVA using Bonferroni’s posthoc test showed that treatment had no effect on body weights of Akita + Insulin mice. Similarly, duration of treatment showed no effect on body weights of Akita + Insulin mice. Data are represented as mean ± SEM of group size (n=8-10).
Figure (3). Time related changes in food intake measurement in WT mice (green line), Akita diabetic mice (black line) and Akita diabetic mice treated with insulin (dotted line). Repeated measurements two-way ANOVA using a Bonferroni’s posthoc test showed that treatment significantly decreased food intake of Akita + Insulin mice \([F (2, 20) = 102.599], \ p < 0.0001\). Similarly, duration of treatment showed significant decrease in food intake of Akita + Insulin mice \([F (8, 80) = 8.877], \ p < 0.0001\). Data are represented as mean ± SEM of group size (n=8-10).
Figure (4). Time related changes in water intake measurement in WT mice (green line), Akita diabetic mice (black line) and Akita diabetic mice treated with insulin (dotted line). Repeated measurements two-way ANOVA using a Bonferroni’s posthoc test showed that treatment significantly decreased water intake of Akita + Insulin mice $[F (2, 20) = 107.7754], p<0.0001$. Similarly, duration of treatment showed significant decrease in water intake of Akita + Insulin mice $[F (8, 80) = 20.4599], p<0.0001$. Data are represented as mean ± SEM of group size (n=8-10).
Figure (5). Time related changes in absolute body fat measurement in WT mice (green line), Akita diabetic mice (black line) and Akita diabetic mice treated with insulin (dotted line). Repeated measurements two-way ANOVA using a Bonferroni’s posthoc test showed that treatment showed significant increase in absolute body fat of Akita + Insulin mice $[F(2, 18) = 45.954], p<0.0001$. Similarly, duration of treatment showed significant increase in absolute body fat of Akita + Insulin mice $[F(8, 72) = 7.552], p<0.0001$. Data are represented as mean ± SEM of group size ($n=8-10$).
Figure (6). Time related changes in absolute lean mass measurement in WT mice (green line), Akita diabetic mice (black line) and Akita diabetic mice treated with insulin (dotted line). Repeated measurements two-way ANOVA using a Bonferroni’s posthoc test showed that treatment had no effect on absolute lean mass of Akita + Insulin mice. Similarly, duration of treatment showed no effect on absolute lean mass of Akita + Insulin mice. Data are represented as mean ± SEM of group size (n=8-10).
Figure (7). Time related changes in total body water measurement in WT mice (green line), Akita diabetic mice (black line) and Akita diabetic mice treated with insulin (dotted line). Repeated measurements two-way ANOVA using a Bonferroni’s posthoc test showed that treatment had no effect on total body water of Akita + Insulin mice. Similarly, duration of treatment showed no effect on total body water of Akita + Insulin mice. Data are represented as mean ± SEM of group size (n=8-10).
**Figure 8.** Plasma insulin values determined in samples obtained from WT mice (white bar), Akita diabetic mice (black bar) and Akita diabetic mice treated with insulin (dotted bar). One-way ANOVA showed that 20 weeks after treatment commenced there was a significant increase in the plasma insulin of the Akita diabetic mice treated with insulin compared to untreated Akita diabetic mice. \# $p<0.001$ vs. untreated Akita mice, *$p<0.001$ vs. WT. Each bar represents mean ± SEM of group size (n=6-10).

**Figure 9.** Plasma glucagon values determined in samples obtained from WT mice (white bar), Akita diabetic mice (black bar) and Akita diabetic mice treated with insulin (dotted bar). One-way ANOVA showed that 20 weeks after treatment commenced there was a significant decrease in the plasma glucagon of the Akita diabetic mice treated with insulin compared to untreated Akita diabetic mice. \# $p<0.001$ vs. untreated Akita mice, *$p<0.001$ vs. WT. Each bar represents mean ± SEM of group size (n=6-10).
Figure 10. Plasma adiponectin values determined in samples obtained from WT mice (white bar), Akita diabetic mice (black bar) and Akita diabetic mice treated with insulin (dotted bar). One-way ANOVA showed that 20 weeks after treatment commenced there was a significant increase in plasma adiponectin of the Akita diabetic mice treated with insulin compared to untreated Akita diabetic mice. *\( p<0.001 \) vs. untreated Akita mice, *\( p<0.001 \) vs. WT. Each bar represents mean ± SEM of group size (n=6-10).

Figure 11. Plasma leptin values determined in samples obtained from WT mice (white bar), Akita diabetic mice (black bar) and Akita diabetic mice treated with insulin (dotted bar). One-way ANOVA showed that 20 weeks after treatment commenced there was a significant increase in the plasma leptin of the Akita diabetic mice treated with insulin compared to untreated Akita diabetic mice. *\( p<0.001 \) vs. untreated Akita mice, *\( p<0.001 \) vs. WT. Each bar represents mean ± SEM of group size (n=6-10).
Figure 12. Plasma triglyceride values determined in samples obtained from WT mice (white bar), Akita diabetic mice (black bar) and Akita diabetic mice treated with insulin (dotted bar). One-way ANOVA showed that 20 weeks after treatment commenced there was a significant decrease in the plasma triglyceride of the Akita diabetic mice treated with insulin compared to untreated Akita diabetic mice. *p<0.001 vs. untreated Akita mice, *p<0.001 vs. WT. Each bar represents mean ± SEM of group size (n=6-10).

Figure 13. Plasma cholesterol values determined in samples obtained from WT mice (white bar), Akita diabetic mice (black bar) and Akita diabetic mice treated with insulin (dotted bar). One-way ANOVA showed that 20 weeks after treatment commenced there was not significant change in the plasma cholesterol of the Akita diabetic mice treated with insulin compared to untreated Akita diabetic mice. Data are represented as mean ± SEM of group size (n=6-10).
Figure 14. Plasma creatinine values determined in samples obtained from WT mice (white bar), Akita diabetic mice (black bar) and Akita diabetic mice treated with insulin (dotted bar). One-way ANOVA showed that 20 weeks after treatment commenced there is a significant decrease in the plasma creatinine of the Akita diabetic mice treated with insulin compared to untreated Akita diabetic mice. \#p<0.001 vs. untreated Akita mice, *p<0.001 vs. WT. Each bar represents mean ± SEM of group size (n=6-10).
Figure 15. Renal ACE2 protein expression values determined in samples obtained from WT mice (white bar), Akita diabetic mice (black bar) and Akita diabetic mice treated with insulin (dotted bar). One-way ANOVA showed a significant increase in renal ACE2 protein expression of Akita diabetic mice compared to WT mice. Twenty weeks after treatment commenced there was a significant decrease in renal ACE2 protein expression of Akita diabetic mice treated with insulin compared to untreated Akita diabetic mice. *p<0.001 vs. untreated Akita diabetic mice, *p<0.001 vs. WT. Each bar represents mean ± SEM of group size (n=6-10).

Figure 16. Urinary ACE2 protein expression values determined in samples obtained from WT mice (white bar), Akita diabetic mice (black bar) and Akita diabetic mice treated with insulin (dotted bar). One-way ANOVA showed a significant increase in urinary ACE2 protein expression of Akita diabetic mice compared to WT mice. Two weeks after treatment commenced there was a significant decrease in urinary ACE2 protein expression of Akita diabetic mice treated with insulin compared to untreated Akita diabetic mice. #p<0.001 vs. untreated Akita diabetic mice, *p<0.001 vs. WT. Each bar represents mean ± SEM of group size (n=6-10).
Figure 17. Renal ADAM17 protein expression values determined in samples obtained from WT mice (white bar), Akita diabetic mice (black bar) and Akita diabetic mice treated with insulin (dotted bar). One-way ANOVA showed a significant increase in renal ADAM17 protein expression of Akita diabetic mice compared to WT mice. Twenty weeks after treatment commenced there was a significant decrease in renal ADAM17 protein expression of Akita diabetic mice treated with insulin compared to untreated Akita diabetic mice. *$p<0.001$ vs. untreated Akita mice, $p<0.001$ vs. WT. Each bar represents mean ± SEM of group size (n=6-10).

Figure 18. Renal TIMP3 protein expression values determined in samples obtained from WT mice (white bar), Akita diabetic mice (black bar) and Akita diabetic mice treated with insulin (dotted bar). One-way ANOVA showed that 20 weeks after treatment commenced there was no significant change in renal TIMP3 protein expression of Akita diabetic mice compared to WT mice or Akita diabetic mice treated with insulin. Each bar represents mean ± SEM of group size (n=6-10).
Figure 19. Renal ACE2 activity values determined in samples obtained from WT mice (white bar), Akita diabetic mice (black bar) and Akita diabetic mice treated with insulin (dotted bar). One-way ANOVA showed a significant increase in renal ACE2 activity in Akita diabetic mice compared to WT mice. Twenty weeks after treatment commenced there was a significant decrease in renal ACE2 activity of the Akita diabetic mice treated with insulin compared to untreated Akita diabetic mice. \(^p<0.001\) vs. untreated Akita mice, \(*p<0.001\) vs. WT. Each bar represents mean ± SEM of group size (n=6-10).

Figure 20. Urinary ACE2 activity values determined in samples obtained from WT mice (white bar), Akita diabetic mice (black bar) and Akita diabetic mice treated with insulin (dotted bar). One-way ANOVA showed a significant increase in urinary ACE2 activity in Akita diabetic mice compared to WT mice. Twenty weeks after treatment commenced there was a significant decrease in the urinary ACE2 activity of the Akita diabetic mice treated with insulin compared to untreated Akita diabetic mice. \(^p<0.001\) vs. untreated Akita mice, \(*p<0.001\) vs. WT. Each bar represents mean ± SEM of group size (n=6-10).
Figure 21. Urinary ACE2 activity values determined in samples obtained from WT mice (white bar) and Akita diabetic mice (black bar). There was a significant increase in urinary ACE2 activity of 10 weeks old Akita diabetic mice compared to 10 weeks old WT mice, *$p<0.001$ vs. WT. Each bar represents mean ± SEM of group size (n=10).

Figure 22. Urinary ACE2 activity was measured in presence and absence of the specific ACE2 inhibitor, MLN-4760 (µM.). One-way ANOVA showed a significant decrease in urinary ACE2 activity in presence of MLN-4760. $#p<0.001$ vs. Akita mice, *$p<0.001$ vs. WT. Each bar represents mean ± SEM of group size (n=10).
Figure 23. Age dependent changes in urinary ACE2 activity in WT mice (white bar) and Akita diabetic mice (black bar). One-way ANOVA showed a significant decrease in urinary ACE2 activity of 30 weeks old Akita diabetic mice compared to 10 weeks old Akita diabetic mice (#p<0.001), *p<0.001 vs. WT. Each bar represents mean ± SEM of group size (n=6-10).

Figure 24. Effect of normalizing hyperglycemia in Akita diabetic mice on urinary albumin excretion. One-way ANOVA showed that 20 weeks after treatment commenced there was a significant decrease in urinary albumin excretion in Akita diabetic mice treated with insulin (dotted bar) compared to untreated Akita diabetic mice (black bar). #p<0.001Vs untreated Akita mice, *p<0.001 vs. WT. Each bar represents mean ± SEM of group size (n=6-10).
Figure 25. Age dependent changes in urinary albumin excretion in WT mice (white bar) and Akita diabetic mice (black bar). There was a significant increase in urinary albumin excretion in 30 weeks old Akita diabetic mice compared to 10 weeks old Akita diabetic mice, \(^* p < 0.001, \# p < 0.001\) vs. WT. Each bar represents mean ± SEM of group size (n=6-10).

Figure 26. Relationship between food intake and blood glucose levels in WT, Akita diabetic and Akita diabetic mice treated with insulin. Regression analysis of food intake (gm/day) and blood glucose levels (mg/dL) showed a significant positive correlation between food intake and blood glucose.
Figure 27. Relationship between water intake and blood glucose levels in WT, Akita diabetic and Akita diabetic mice treated with insulin. Regression analysis of water intake (gm/day) and blood glucose levels (mg/dL) showed a significant positive correlation between water intake and blood glucose.

Figure 28. Relationship between urine output and blood glucose levels in WT, Akita diabetic and Akita diabetic mice treated with insulin. Regression analysis of urine output (ml/day) and blood glucose levels (mg/dL) showed a significant positive correlation between urine output and blood glucose.
Figure 29. Relationship between urinary ACE2 and albuminuria in WT, Akita diabetic and Akita diabetic mice treated with insulin. Regression analysis of urinary ACE2 activity (nmols/hr/mg Cr) and urinary albumin excretion (µg/day) showed a significant positive correlation between urinary ACE2 activity and urinary albumin excretion.

Figure 30. Relationship between urinary ACE2 and blood glucose levels in WT, Akita diabetic and Akita diabetic mice treated with insulin. Regression analysis of urinary ACE2 activity (nmols/hr/mg Cr) and blood glucose levels (mg/dL) showed a significant positive correlation between urinary ACE2 activity and blood glucose.
Figure 31. Relationship between urinary ACE2 and plasma glucagon levels in WT, Akita diabetic and Akita diabetic mice treated with insulin. Regression analysis of urinary ACE2 activity (nmols/hr/mg Cr) and plasma glucagon levels (ng/dL) showed a significant positive correlation between urinary ACE2 activity and plasma glucagon.

Figure 32. Relationship between urinary ACE2 and plasma triglyceride levels in WT, Akita diabetic and Akita diabetic mice treated with insulin. Regression analysis of urinary ACE2 activity (nmols/hr/mg Cr) and plasma triglyceride levels (mg/dL) showed a significant positive correlation between urinary ACE2 activity and plasma triglyceride.
Figure 33. Relationship between urinary ACE2 and plasma creatinine levels in WT, Akita diabetic and Akita diabetic mice treated with insulin. Regression analysis of urinary ACE2 activity (nmols/hr/mg Cr) and plasma creatinine levels (mg/dL) showed a significant positive correlation between urinary ACE2 activity and plasma creatinine.
Figure 34. Representative Periodic acid-Schiff (PAS) staining of 22 weeks old kidney sections in WT mice (white bar), Akita diabetic mice (black bar) and Akita diabetic mice treated with insulin(dotted bar). There was a significant increase of glomerular mesangial expansion (intense purple color) in Akita diabetic mice compared to WT mice (*p<0.001 vs. WT). Twelve weeks after treatment commenced there was a significant decrease in the glomerular mesangial expansion in Akita diabetic mice treated with insulin compared to untreated Akita diabetic mice (#p<0.001 vs. untreated Akita). Each bar represents mean ± SEM of group size (n=25 glomeruli per group of mice).
Figure 35 (A). Representative trichrome staining of 22 weeks old kidney sections in WT mice (white bar), Akita diabetic mice (black bar) and Akita diabetic mice treated with insulin (dotted bar). There was a significant increase of glomerular mesangial fibrosis (intense blue color) in Akita diabetic mice compared to WT mice (*p<0.001 vs. WT). Twelve weeks after treatment commenced there was a significant decrease in the glomerular mesangial fibrosis in Akita diabetic mice treated with insulin compared to untreated Akita diabetic mice (#p<0.001 vs. untreated Akita). Each bar represents mean ± SEM of group size (n=25 glomeruli per group of mice).
**Figure 35 (B).** Representative graph depicts glomerular surface area in WT mice (white bar), Akita diabetic mice (black bar) and Akita diabetic mice treated with insulin (dotted bar). There was a significant increase of glomerular surface area in Akita diabetic mice compared to WT mice (*p*<0.001 vs. WT). Twelve weeks after treatment commenced there was a significant decrease in the glomerular surface area in Akita diabetic mice treated with insulin compared to untreated Akita diabetic mice (#*p*<0.001 vs. untreated Akita). Each bar represents mean ± SEM of group size (n=25 glomeruli per group of mice).
Figure 36. Immunofluorescence staining with ACE2 in the kidney sections from WT mice (white bar), Akita diabetic mice (black bar) and Akita diabetic mice treated with insulin (dotted bar). Strong ACE2 staining (green) is in the cortical tubules in Akita diabetic mice compared to WT mice (*p<0.001 vs. WT). ACE2 staining in renal cortical tubules from insulin treated Akita diabetic mice was decreased compared with untreated Akita diabetic mice (#p<0.001 vs. untreated Akita).
Figure 37. Immunofluorescence staining with ADAM17 in the kidney sections from WT mice (white bar), Akita diabetic mice (black bar) and Akita diabetic mice treated with insulin (dotted bar). Strong ADAM17 staining (red) is in the cortical tubules in Akita diabetic mice compared with WT mice (*p<0.001 vs. WT). ADAM17 staining in renal cortical tubules from insulin treated Akita diabetic mice was decreased compared with untreated Akita diabetic mice (# p<0.001 vs. untreated Akita).
Figure 38. Angiotensin converting enzyme 2 (ACE2) and ADAM17 localization in renal cortical tubules: immunofluorescence staining of ACE2 (green) and ADAM17 (red) in a kidney from Akita mouse. Merging of both images shows stain in yellow indicating colocalization of ACE2 and ADAM17 in the cortical tubules.
4. RESULTS

4.1. General metabolic and body composition parameters

To examine the age-dependent changes in WT and Akita diabetic mice, the following parameters were monitored weekly.

a) Blood glucose: There was an age-dependent increase in the blood glucose levels of the Akita diabetic mice. Akita diabetic mice had significantly higher blood glucose levels compared to WT mice. Blood glucose levels were consistently increased over the 20 weeks study period (Table 1, \( *p<0.05 \), unpaired-t test). However there was no difference in the blood glucose levels of WT mice.

b) Body weight: Akita diabetic mice did not gain more body weight compared to WT mice (Table 1, \( *p<0.05 \)). Body weights of Akita diabetic mice were consistently less over time and age when compared to WT mice (Table 1, \( *p<0.05 \), unpaired-t test).

c) Food intake: Akita diabetic mice showed significantly higher food intake compared to WT mice (Table 1, \( *p<0.05 \), unpaired-t test). Food intake of Akita diabetic mice consistently increased over the 20 weeks study period (Table 1, \( *p<0.05 \), unpaired-t test). However there was no difference in food intake of WT mice.

d) Water intake: Akita diabetic mice showed significantly higher water intake compared to WT mice (Table 1, \( *p<0.05 \), unpaired-t test). Water intake of Akita diabetic mice consistently increased over the 20 weeks study period (Table 1, \( *p<0.05 \), unpaired-t test). However there was no difference in water intake of WT mice.

e) Body fat: Akita diabetic mice exhibited significantly lower body fat compared to WT mice (Table 1, \( *p<0.05 \)). Body fat of Akita diabetic mice was consistently less over time and age when compared to WT mice (Table 1, \( *p<0.05 \), unpaired-t test).
f) **Lean mass**: There was no significant difference in lean mass of Akita diabetic mice compared to WT mice (Table 1). Similar result was recorded over the 20 weeks study period.

g) **Total body water**: There was no significant difference in total body water of Akita diabetic mice compared to WT mice (Table 1). Similar effect was recorded over the 20 weeks study period.

h) **Urine output**: Urine output was significantly increased in Akita diabetic mice compared to WT mice (Table 3, *p*<0.05, unpaired t-test).

4.2. **Plasma hormone and lipid parameters**

To assess hyperglycemia related plasma hormone and lipid changes, the following parameters were measured.

a) **Plasma insulin**: 30 weeks old Akita diabetic mice were hypoinsulinemic. There was a significant reduction in plasma insulin levels in Akita diabetic mice compared to age-matched WT mice (Table 2, *p*<0.05).

b) **Plasma glucagon**: 30 weeks old Akita diabetic mice had significantly higher plasma glucagon levels compared to age-matched WT mice (Table 2, *p*<0.05).

c) **Plasma adiponectin**: There was a significant decrease in plasma adiponectin levels of 30 weeks old Akita diabetic mice compared to age-matched WT mice (Table 2, *p*<0.05).

d) **Plasma leptin**: There was a significant reduction in plasma leptin levels of 30 weeks old Akita diabetic mice compared to age-matched WT mice (Table 2, *p*<0.05).
e) **Plasma triglycerides:** There was a significant elevation in plasma triglycerides levels of 30 weeks old Akita diabetic mice compared to age-matched WT mice (Table 2, \(*p<0.05\)).

f) **Plasma cholesterol:** There was no significant difference in plasma cholesterol levels of 30 weeks old Akita diabetic mice compared to age-matched WT mice (Table 2).

### 4.3. Assessment of renal function

a) **Urinary albumin:** Akita diabetic mice demonstrated microalbuminuria and there was a significant increase in urinary albumin excretion in Akita diabetic mice compared to WT mice (Table 3, figure 25, \(*p<0.05\)). As disease progressed, microalbuminuria significantly increased in Akita diabetic mice compared to WT mice as recorded in 10 and 30 weeks old of age (Table 3, figure 25, \(*p<0.05\)).

b) **Plasma creatinine:** As kidney function declined, there was a significant elevation in plasma creatinine levels of 30 weeks old Akita diabetic mice compared to age-matched WT mice (Table 3, \(*p<0.05\)).

### 4.4. Enzyme activities measurement

To investigate the effect of hyperglycemia on renal and urinary ACE2 activity, kidney lysate and urine from WT and Akita diabetic mice were analyzed.

a) **Renal ACE2 activity:** There was a significant increase in renal ACE2 activity of the 30 weeks old Akita mice compared to their age-matched WT mice (Figure 19, \(*p<0.001\)).

b) **Urinary ACE2 activity:** There was a significant increase in urinary ACE2 activity of the 30 weeks old Akita mice compared to their age-matched WT mice (Figure 20, \(*p<0.001\)).
To determine the specificity of the ACE2 activity assay, urine samples were measured in presence and absence of the specific ACE2 inhibitor, MLN-4760.

c) Urinary ACE2 activity: There was a significant increase in urinary ACE2 activity of the 10 weeks old Akita diabetic mice compared to their age-matched WT mice (Figure 21, *p<0.001).

d) Urinary ACE2 activity-MLN(4760): One-way ANOVA showed MLN-4760 significantly reduced ACE2 activity in 10 weeks Akita diabetic+(MLN-4760) mice compared to Akita diabetic mice (Figure 22, #p<0.001).

To investigate the effect of long-term hyperglycemia on urinary ACE2 activity, urine from 10 and 30 weeks old Akita diabetic and WT mice were analyzed.

e) Urinary ACE2 activity: There was a decrease in the urinary ACE2 activity of 30 weeks old Akita diabetic mice compared to 10 weeks old Akita diabetic mice (Figure 23, #p<0.001).

4.5. Regression analysis

a) Blood glucose vs. food intake: There was a positive linear correlation between blood glucose (mg/dL) and daily food intake (gm/day) in WT, Akita diabetic and Akita diabetic treated with insulin mice (Figure 26, p<0.0001).

b) Blood glucose vs. water intake: There was a positive linear correlation between blood glucose (mg/dL) and daily water intake (gm/day) in WT, Akita diabetic and Akita diabetic treated with insulin mice (Figure 27, p<0.0001).

c) Blood glucose vs. urine output: There was a positive linear correlation between blood glucose (mg/dL) and daily urine output (ml/day) in WT, Akita diabetic and Akita diabetic treated with insulin mice (Figure 28, p<0.0001).
d) Urinary ACE2 activity vs. urinary albumin excretion: There was a positive linear correlation between urinary ACE2 activity (nmols/h/mg Cr) and urinary albumin excretion (µg/day) in WT, Akita diabetic and Akita diabetic treated with insulin mice (Figure 29, p<0.0001).

e) Urinary ACE2 activity vs. Blood glucose: There was a positive linear correlation between urinary ACE2 activity (nmols/h/mg Cr) and blood glucose (mg/dL) in WT, Akita diabetic and Akita diabetic treated with insulin mice (Figure 30, p<0.0001).

f) Urinary ACE2 activity vs. Plasma glucagon: There was a positive linear correlation between urinary ACE2 activity (nmols/h/mg Cr) and plasma glucagon (ng/dL) in WT, Akita diabetic and Akita diabetic treated with insulin mice (Figure 31, p<0.0001).

g) Urinary ACE2 activity vs. Plasma triglycerides: There was a positive linear correlation between urinary ACE2 activity (nmols/h/mg Cr) and plasma triglycerides (mg/dL) in WT, Akita diabetic and Akita diabetic treated with insulin mice (Figure 32, p<0.0001).

h) Urinary ACE2 activity vs. Plasma creatinine: There was a positive linear correlation between urinary ACE2 activity (nmols/h/mg Cr) and plasma creatinine (mg/dL) in WT, Akita diabetic and Akita diabetic treated with insulin mice (Figure 33, p<0.0001).

4.6. Renal protein expression of ACE2, ADAM17 and TIMP3

a) ACE2 protein expression: There was a significant increase in renal ACE2 protein expression of 30 weeks Akita diabetic mice compared to their age-matched WT mice (Figure 15, *p<0.001).

b) ADAM17 protein expression: There was a significant increase in renal ADAM17 protein expression of 30 weeks old Akita diabetic mice compared to their age-matched WT mice (Figure 17, *p<0.001).
c) **TIMP3 protein expression:** There was not a significant difference in renal TIMP3 protein expression of 30 weeks old Akita diabetic mice compared to their age-matched WT mice (Figure 18).

4.7. **Urinary protein expression of ACE2**

   a) **ACE2 protein expression:** There was a significant increase in urinary ACE2 protein expression of 12 weeks old Akita diabetic mice compared to their age-matched WT mice (Figure 16, *p*<0.001).

4.8. **Renal histopathological changes**

   a) **Renal PAS staining:** PAS stained kidney sections and graph showed an increase of the glomerular mesangial expansion in 22 weeks old Akita diabetic mice compared to their age-matched WT mice (Figure 34, *p*<0.001).

   b) **Renal Masson’s Trichrome staining:** Masson’s trichrome stained kidney sections graphs showed an increase of the glomerular mesangial fibrosis and glomerular surface area in Akita diabetic mice compared to their age-matched WT mice (Figure 35A,B, *p*<0.001).

4.9. **Immunohistochemistry of renal sections**

Immunohistochemistry analysis was performed in order to confirm Western blot results.

   a) **Renal ACE2 expression:** The expression of renal ACE2 significantly increased in the cortical tubules of 22 weeks old Akita diabetic mice compared to their age-matched WT mice (Figure 36, *p*<0.001).
b) Renal ADAM17 expression: The expression of renal ADAM17 significantly increased in the cortical tubules of 22 weeks old Akita diabetic mice compared to their age-matched WT mice (Figure 37, *p<0.001).

c) Colocalization of renal ACE2 and ADAM17: Merging of double immunostained images showed strong colocalization of renal ACE2 and ADAM17 in cortical tubules of 22 weeks old Akita diabetic mice (Figure 38).

4.10. Effect of insulin on metabolic parameters

To evaluate the effect of normalizing hyperglycemia with insulin on metabolic parameters, 10 weeks old Akita diabetic mice were treated with insulin implants (LinβitR) subcutaneously for 20 weeks.

a) Effect of insulin on blood glucose: Insulin treatment normalized the blood glucose levels in Akita diabetic mice compared to untreated Akita diabetic mice (Figure 1, p<0.0001).

b) Effect of insulin on body weights: Chronic insulin treatment showed no effect on body weights in treated Akita diabetic mice compared to untreated Akita diabetic mice (Figure 2).

c) Effect of insulin on food intake: Insulin treated Akita diabetic mice food intake decreased significantly compared to untreated Akita diabetic mice and fell to levels observed in wild type mice (Figure 3, p<0.0001).

d) Effect of insulin on water intake: Insulin treated Akita diabetic mice water intake decreased significantly compared to untreated Akita diabetic mice (Figure 4, p<0.0001).
e) **Effect of insulin on body fat:** Insulin significantly augmented accumulation of body fat in Akita diabetic mice compared to untreated Akita diabetic mice (Figure 5, \( p<0.0001 \)).

f) **Effect of insulin on lean mass:** There was not a significant increase in lean mass of insulin treated Akita diabetic mice compared to untreated Akita diabetic mice (Figure 6).

g) **Effect of insulin on total body water:** There was not a significant increase in total body water of insulin treated Akita diabetic mice compared to untreated Akita diabetic mice (Figure 7).

### 4.11. Effect of insulin on plasma hormone and lipid parameters

To examine the effect of normalizing hyperglycemia on type 1 diabetes-related plasma hormone and lipid changes, measurements were carried out using plasma from insulin treated Akita diabetic mice.

a) **Plasma insulin levels:** Plasma insulin levels were significantly increased in insulin treated Akita diabetic mice compared to untreated Akita diabetic mice (Figure 8, \( \# p<0.001 \)).

b) **Plasma glucagon levels:** Plasma glucagon levels were significantly reduced in insulin treated Akita diabetic mice compared to untreated Akita diabetic mice (Figure 9, \( \# p<0.001 \)).

c) **Plasma adiponectin levels:** Twenty weeks insulin treatment significantly increased the plasma adiponectin levels in treated Akita diabetic mice compared to untreated Akita diabetic mice (Figure 10, \( p<0.001 \)).

d) **Plasma leptin levels:** Insulin treatment significantly increased plasma levels of leptin in treated Akita diabetic mice compared to untreated Akita diabetic mice (Figure 11\( \# p<0.001 \)).
e) **Plasma triglyceride levels:** There was significant reduction in plasma triglyceride levels in treated Akita diabetic mice compared to untreated Akita diabetic mice (Figure 12, \( p < 0.001 \)).

f) **Plasma cholesterol levels:** Normalizing hyperglycemia with insulin had no effect on plasma cholesterol levels in treated Akita diabetic mice compared to untreated Akita diabetic mice (Figure 13).

**4.12. Effect of insulin on renal function**

a) **Urinary albumin:** Urinary albumin excretion was significantly reduced in insulin treated Akita diabetic mice compared to untreated Akita diabetic mice (Figure 24, \( p < 0.001 \)).

b) **Plasma creatinine:** Plasma creatinine levels were significantly attenuated in insulin treated Akita diabetic mice compared to untreated Akita diabetic mice (Figure 14, \( p < 0.001 \)).

**4.13. Effect of insulin on enzyme activities**

a) **Renal ACE2 activity:** There was a significant decrease in renal ACE2 activity in insulin treated Akita diabetic mice compared to untreated Akita diabetic mice (Figure 19, \( p < 0.001 \)).

b) **Urinary ACE2 activity:** There was a significant decrease in urinary ACE2 activity in insulin treated Akita diabetic mice compared to untreated Akita diabetic mice (Figure 20, \( p < 0.001 \)).
4.14. Effect of insulin on renal ACE2, ADAM17 and TIMP3 protein expression

a) **ACE2 protein expression:** There was a significant decrease in renal ACE2 protein expression of 30 weeks old Akita diabetic mice treated with insulin compared to untreated Akita diabetic mice (Figure 15, $p<0.001$).

b) **ADAM17 protein expression:** There was a significant decrease in renal ADAM17 protein expression of 30 weeks old Akita diabetic mice treated with insulin compared to untreated Akita diabetic mice (Figure 17, $p<0.001$).

c) **TIMP3 protein expression:** There was not a significant change in renal TIMP3 protein expression of 30 weeks old Akita diabetic mice treated with insulin compared to untreated Akita diabetic mice (Figure 18).

4.15. Effect of insulin on urinary ACE2 protein expression

a) **ACE2 protein expression:** There was a significant decrease in urinary ACE2 protein expression of 12 weeks old Akita diabetic mice treated with insulin compared to untreated Akita diabetic mice (Figure 16, $p<0.001$).

4.16. Effect of insulin on renal histopathological changes

a) **Renal PAS staining:** PAS stained kidney sections and graph showed that twelve weeks after treatment commenced there was a significant decrease in the glomerular mesangial expansion in Akita diabetic mice treated with insulin compared to untreated Akita diabetic mice (Figure 34, $p<0.001$).

b) **Renal Masson’s Trichrome staining:** Masson’s trichrome stained kidney sections and graph showed that twelve weeks after treatment commenced there was a significant decrease in the glomerular mesangial fibrosis and surface area in Akita diabetic mice treated with insulin compared to untreated Akita diabetic mice (Figure 35A,B, $p<0.001$).
4.17. Effect of insulin on immunohistochemistry of renal sections

a) Renal ACE2 expression: Insulin significantly decreased renal ACE2 protein expression in 22 weeks old treated Akita diabetic mice compared to untreated Akita diabetic mice (Figure 36, \( p<0.001 \)).

b) Renal ADAM17 expression: Insulin significantly decreased renal ADAM17 protein expression in 22 weeks old treated Akita diabetic mice compared to untreated Akita diabetic mice (Figure 37, \( p<0.001 \)).
5. Discussion

To date, there have been few studies addressing the role of ACE2 shedding in vivo (148). ADAM17 has been shown to increase the ectodomain shedding of ACE2 in several cell lines, in vitro (99). In the present study, Western blotting, and enzymatic activity assay documented a significant increase in urinary ACE2 protein expression and activity in Akita mice compared to wild type mice. The increased urinary ACE2 levels are unlikely to be originating from the plasma rather than shedding from renal tubules, since the ACE2 has a high molecular weight (~120kDa). In addition, increased urinary ACE2 levels were observed at an early stage of diabetes before signs of glomerular injury were evident. The present study also highlights the impact of hyperglycemia on renal ADAM17 expression, and its proteolytic role in the shedding of membrane-bound ACE2 (renal ACE2) into soluble ACE2 form (urinary ACE2), in a mouse model of type 1 diabetes.

Diabetic nephropathy is still the leading cause of end stage renal disease, and is diagnosed by presence of albuminuria within the range of microalbuminuria (195). In the present study, Akita mice developed significant, progressive and durable hyperglycemia through the 20 weeks study period, which was associated with renal functional and structural abnormalities. By 30 weeks of age, the level of hyperglycemia in Akita mice matched other studies conducted in this model with (C56BL/6) genetic background (38;107). Monitoring of the metabolic parameters demonstrated that Akita mice had a higher daily water intake (polydipsia), food intake (polyphagia) and urine output (polyuria), whereas body weight and fat composition were significantly less than the wild type mice. Additionally, as expected, analysis of plasma hormones and lipid profile revealed that Akita mice had severe insulin deficiency and reduced adiponectin and leptin concentrations. Conversely, there was an increase in plasma glucagon,
triglycerides and creatinine in Akita mice compared to wild type mice. Numerous studies have suggested the association between hyperglycemia and albuminuria in various genetic backgrounds of Akita mice (196). Our results are in agreement with those studies and demonstrate that as the disease progresses, hyperglycemia increases, resulting in a significant increase in albuminuria in 30 weeks compared to 10 weeks old Akita mice. Albumin excretion levels observed in our study were similar to the ones reported by others using Akita with (C56BL/6) background (38;107). Interestingly, albumin excretion in (C56BL/6) Akita mice is less robust as compared with 20 weeks old (FVB/NJ) Akita mice (196). Based on quantitative histomorphometric assessments, Akita kidneys developed a moderate increase in glomerular mesangial matrix expansion and glomerular fibrosis, which resembles the early histological lesions seen in humans with type 1 diabetes-induced nephropathy (38;196). In contrast to albuminuria, renal histopathological findings of the present study were similar with other Akita genetic strains (38;76;107;196).

As recent studies suggested the presence of urinary ACE2 in type 2 diabetic humans (148), we investigated in a similar manner whether Akita mice excrete ACE2. Indeed, the present study is the first to show increased urinary ACE2 in type 1 diabetic mice. The higher urinary ACE2 levels were associated with increased renal ACE2 and ADAM17 protein expression. Since ADAM17 has been shown to increase the ectodomain shedding of ACE2 in several cell lines (99), we proposed that the increased levels of urinary ACE2 may be due to its increase in renal expression and shedding mediated by ADAM17 instead of increases in ACE2 plasma levels and glomerular filtration rate. Therefore, urinary ACE2 may be a result of active ADAM17-mediated ectodomain shedding of renal ACE2 located in the tubular membrane facing the luminal site. This conclusion is supported by our findings that the molecular weight of urinary ACE2 was
20kDa lower than the molecular weight of kidney ACE2 and plasma ACE2 expression or activity was not detectable in Akita mice or wild type mice (result not shown). Work in our laboratory and others have confirmed the lack of ACE2 in plasma of \textit{db/db} diabetic mice or lean control mice (197), or in CKD patients or healthy subjects (148). The absence of detectable ACE2 activity in plasma has been attributed to the presence of endogenous ACE2 inhibitor (198), however, other studies have shown an elevation of serum ACE2 activity in type 1 diabetic patients (199), in serum of sheep (149), in plasma of STZ diabetic mice (70), and rats (64). The discrepancy between these results might be explained by differences between plasma or serum preparations, species, incubation time, type of buffer or substrate used in the ACE2 enzyme activity assays.

In the present study, Western blot analysis of urinary ACE2 detected immunoreactive bands at \(~70\text{kDa}\) in Akita mice suggesting that the soluble form of ACE2 (\(~70\text{kDa}\), urinary ACE2) is the shed fragment of membrane-bound ACE2 (\(~90\text{kDa}\), renal ACE2). The latter finding is in agreement with two recent clinical studies showing ACE2 expression as three bands in immunoblots of urine samples from patients with CKD and diabetic renal transplant recipients, the glycosylated form of ACE2 at \(~120\text{kDa}\), membrane-bound ACE2 at \(~90\text{kDa}\), and a cleaved fragment of ACE2 at \(~75\text{kDa}\) (148;150). To confirm whether an increase in urinary ACE2 expression is directly associated with an increase in urinary ACE2 activity, we quantified ACE2 enzymatic activity. In line with the Western blot results, there was a significant increase in urinary ACE2 activity in Akita mice compared to wild type mice (\(p<0.001\)), results we have recently confirmed in type 2 \textit{db/db} diabetic mice (197). In the present study, Akita mice were on neither ACEi nor ARBs, but in order to exclude the possibility of urinary ACE activity, Lisinopril (ACE inhibitor) was added to the buffer of ACE2 enzymatic activity assay, which did
not affect urinary ACE2 levels. This finding supports a clinical study that used ACEi or ARBs in patients with diabetic nephropathy, in which the urinary ACE2 levels were not altered (148). In contrast to urinary ACE2, microalbuminuria was decreased by ACEi in type 2 diabetic patients, but did not prevent the progression toward end stage renal disease as shown in the ADVANCE trial (147). On the other hand, MLN-4760, a pharmacological inhibitor of ACE2, significantly reduced urinary ACE2 activity in both Akita and wild type mice, which confirms the specificity of the conducted ACE2 activity assay in the current study. This finding is also supported by studies conducted in STZ diabetic mice and mice with CKD in which ACE2 activity decreased after four weeks of treatment with MLN-4760 (72;73).

To investigate the effect of aging on urinary ACE2 levels, urine specimens of 10 weeks old Akita mice were compared with 30 weeks old Akita mice. It was found that younger animals had higher urinary ACE2 activity than older animals. This finding indicates that with the progression of disease, the kidney is unable to maintain the same levels of renal ACE2 because it is depleted by shedding into the urine. It also reflects the possibility of using urinary ACE2 as an early biomarker of diabetic kidney disease. This notion is supported by several clinical studies that reported increased urinary ACE2 levels in patients with CKD and diabetic renal transplant recipients compared to healthy subjects (148;150). There was also a strong correlation between urinary mRNA expression of ACE2 and the degree of proteinuria in type 2 human diabetic patients compared to healthy subjects (151).

Additionally, the present study showed that urinary ACE2 levels appears to be positively correlated with albuminuria, blood glucose, plasma levels of glucagon, triglycerides, and creatinine. This supports our previous results conducted in type 2 \( \text{db/db} \) diabetic mice (197), and a recent clinical study (200). The relationship between urinary ACE2 levels and other risk factors
of kidney dysfunction imply that urinary ACE2 levels may be considered as an additional and independent risk factor of diabetic kidney disease.

It is well known that over activation of the RAS in both type of diabetes with subsequent abundant generation of Ang II plays an important role in the progression of diabetic nephropathy (201). Therefore, it is assumed that ACE2 has an endogenous renoprotective function due to its ability to degrade Ang II thereby reducing the deleterious Ang II-mediated effects in diabetes (202). The present study showed a significant increase in protein expression and activity of renal ACE2 in Akita mice compared to wild type mice. This finding is confirmed by Western blotting, immunostaining, and fluorogenic enzymatic activity assay. This finding is also in agreement with our previous study, which demonstrated an increase in renal ACE2 expression and activity in type 2 db/db diabetic mice compared to lean control mice (197). Increased renal ACE2 mRNA, protein expression or activity was also observed in various other studies including: renal ACE2 protein (74) and mRNA expression (76) in Akita mice, in db/db diabetic mice (79), as well as renal ACE2 activity in both db/db and STZ diabetic mice (80). These findings are in contrast to studies reporting on decreased renal ACE2 expression and activity in experimental models including Akita mice (38), STZ diabetic mice (32), STZ diabetic rats (49;71) and mice model of chronic kidney disease (72).

It should be noted that the present study also revealed for the first time the colocalization of renal ACE2 and ADAM17 in cortical tubules in type 1 diabetic mice supporting an active role of ADAM17 in the ectodomain shedding of ACE2 in diabetes. Some studies reported a decrease in renal glomerular ACE2 expression in db/db diabetic mice compared to lean control mice (203), and in patients with diabetic nephropathy compared to healthy subjects (77;78). In contrast, a recent study has shown an increase in glomerular ACE2 expression in STZ diabetic mice
compared to wild type mice (70). In spite of the controversy, we postulate that an increase of renal ACE2 in cortical tubules, but not in glomeruli, could be the earliest positive feedback response to hyperglycemia and a possible compensatory protective mechanism opposing the toxic effects of sustained hyperglycemia, elevated levels of Ang II, and increased ADAM17-mediated ACE2 shedding during the initial stages of kidney damage.

This study is also the first report that shows an increase of renal ADAM17 protein expression in type 1 Akita diabetic mice, which are confirmed by Western blotting and immunostaining. This finding supports our previous study conducted in type 2 db/db diabetic mice (197). In addition, studies conducted in STZ and OVE26 diabetic mice have shown a significant increase in renal ADAM17 activity compared to wild type mice (93;106). However, another study has shown that there was no significant difference in renal ADAM17 mRNA expression or activity between Akita and wild type mice (107). A 2005 study reported that ADAM17 mediates active ectodomain shedding of ACE2, but not ACE, in vitro (54). In addition, several studies conducted in different cell lines reported that overexpression of ADAM17 increases shedding of active extracellular domain of ACE2 in HEK293 cells and in Huh7 cells (99). Considered together, the colocalization of renal ACE2 and ADAM17 with previous reports on the involvement of ADAM17 in the shedding of the ACE2 ectodomain, the present study suggests that an increase of renal ADAM17 is involved in the shedding of the renal and enzymatically active ectodomain of ACE2 resulting in increased urinary ACE2 levels in a mouse model of type 1 diabetes-induced nephropathy. Our findings are supported by a clinical study in which the authors speculated that in patients with chronic kidney disease, ACE2 sheds directly from the proximal renal tubules into the urine via ADAM17, though at the time this notion was not confirmed experimentally (148).
Clinical studies conducted in patients with diabetic nephropathy reported that TIMP3, a physiological inhibitor of ADAM17, could contribute to the development and progression of DN (204;205). While this is unconfirmed, we speculate that hyperglycemia decreases renal TIMP3 protein expression results in increasing of ADAM17, because of losing its endogenous inhibition. Our speculation was supported by a recent study that reported a significant decrease in renal TIMP3 in STZ diabetic mice, and in kidney biopsies from type 2 diabetic patients (106). Moreover, a clinical study has reported that in type 2 diabetic patients TIMP3 is reduced, leading to elevated circulating levels of ADAM17 (102;105). Interestingly, Western blotting performed in the present study revealed no significant difference in renal TIMP3 protein expression in Akita mice compared to wild type mice. A study has shown an increase in renal TIMP3 protein expression of Akita mice when compared to wild type mice. In the same study, they also showed an increase in renal ADAM17 activity in TIMP3 KO Akita mice, but no difference in Akita compared to wild type mice (107). This discrepancy of increased renal TIMP3 expression while in ADAM17 activity or mRNA levels were unaltered in Akita kidneys was not addressed in the previous study. However, the present study identified a significant increase of renal ADAM17 independent of its endogenous inhibitor (TIMP3) in Akita mice, which could be due to a direct effect of hyperglycemia or through other indirect pathways.

Finally, the present study investigated the effect of normalizing hyperglycemia on renal ADAM17, ACE2, TIMP3 expression, urinary ACE2 and albumin excretion. Akita mice were treated by subcutaneous insertion of a sustained release insulin implant, in the mid-dorsal position lateral to the spine, every 5 weeks throughout the study period of 20 weeks. Insulin treatment normalized hyperglycemia and other metabolic parameters, including water intake, food intake, and urine output. It also increased body fat composition without significant
difference in body weight. The above results are confirmed by previous study conducted in STZ diabetic mice treated with insulin implants (32). Furthermore, we observed a significant reduction in plasma glucagon, triglycerides and creatinine to almost normal levels and an increase in plasma insulin, adiponectin, and leptin levels in treated Akita mice. Moreover, there was a significant attenuation in urinary albumin excretion and renal histopathological lesions of treated Akita mice. As was anticipated, previous findings showed the important role of glycemic control in the delayed onset or reduced progression of diabetic nephropathy. The latter is asserted in experimental and clinical studies, conducted in STZ diabetic mice, non-obese diabetic mice, and type 1 diabetic patients treated with subcutaneous insulin (32;206;207). In addition, we tested the effect of normalizing hyperglycemia on urine and renal ACE2. Interestingly, treatment with insulin decreased both urinary and renal ACE2 protein expression and activity in treated Akita mice. However, another study has shown the opposite effect of insulin treatment on renal ACE2 protein expression in STZ diabetic mice (32). Accordingly, we also tested the effect of normalizing hyperglycemia on renal ADAM17 and TIMP3 protein expression. Treatment with insulin decreased renal ADAM17 protein expression in treated Akita mice. As we expected, treatment with insulin had no effect on renal TIMP3 protein expression in Akita mice, suggesting that hyperglycemia has no immediate impact on ADAM17 via TIMP.

In conclusion, our results suggest that hyperglycemia increases renal ACE2 and ADAM17 expression in coalition with a rise in urinary ACE2 excretion most likely due to increased shedding of renal ACE2 mediated by ADAM17. The euglycemic effect of insulin decreases urinary ACE2 excretion, restores renal ACE2 and ADAM17 expression back to physiological levels and normalizes the rate of shedding. A strong positive correlation of urinary ACE2 concentrations with other independent risk factors of diabetic kidney disease suggests an
important clinical relevance for urinary ACE2 as a marker for diabetic renal impairment and an indicator for medical therapy intervention.
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