Université de Montréal

Caractérisation du gène de l'enzyme de conversion de l'angiotensine-2 dans le rein diabétique et implication dans le développement de la néphropathie diabétique et de l'hypertension

Par

Yixuan Shi

Programme de sciences biomédicales
Faculté de médecine

Thèse présentée à la faculté des études supérieures
en vue de l'obtention du grade de docteurès sciences (Ph.D.)
en sciences biomédicales

July 2014

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a été évaluée par un jury composé des personnes suivantes :

D[re] Jolanta Gutkowska
Présidente rapporteuse

D[r] John S.D. Chan
Directeur de recherche

D[re] Shao-Ling Zhang
Codirectrice de recherche

D[r] Stéphan Troyanov
Membre du jury

D[r] David Cherney
Examinateur externe

Résumé

De nombreuses études ont bien démontré que l'activation du système rénine-angiotensine (RAS) joue un rôle important dans le développement de l'hypertension et de la néphropathie diabétique (DN). La découverte de l'enzyme de conversion de l'angiotensine-2 (ACE2) et l'identification du récepteur MAS, spécifique pour l'angiotensine 1-7 (Ang 1-7), ont permis d'identifier deux nouveaux membres du RAS. L'axe ACE2/Ang 1-7/MAS contrebalance les effets de l'axe ACE/Ang II/AT1. Plusieurs évidences impliquent la contribution du RAS intrarénal dans la DN. Des études réalisées dans notre laboratoire avec des souris transgéniques surexprimant l'angiotensinogène de rat dans les cellules de leurs tubules proximaux rénaux (RPTCs) ont permis de démontrer l'importance du RAS intrarénal dans l'induction de l'hypertension et les dommages rénaux. Nous avons également observé que l'expression rénale de l'ACE2 et les niveaux urinaires d'ANG 1-7 sont plus faibles chez les souris Akita (diabète de type 1) et qu'un traitement avec des bloqueurs du RAS permet de normaliser l'expression de l'ACE2 et de prévenir le développement de l'hypertension dans le modèle des souris Akita. Dans un milieu diabétique, à la fois la glycémie et l'angiotensine II (Ang II) peuvent induire la génération des espèces réactives de l'oxygène (ROS), contribuant ainsi aux dommages rénaux. Afin d'explorer la relation entre les ROS, ACE2 et la DN, nous avons créé des souris Akita transgéniques surexprimant la catalase (Cat) dans les RPTCs, en croisant des souris Akita diabétique de type 1 à notre modèle de souris transgéniques surexprimant la Cat de rat dans les RPTCs. Dans une seconde étude, des souris Akita ont été traitées avec l'Ang 1-7 ou une combinaison d'Ang 1-7 et de son antagoniste, A779, afin

d'étudier la relation entre l'action de l'Ang 1-7, l'hypertension systolique (sHTN), le stress oxydatif, les dommages rénaux, ACE2 et l'expression du récepteur Mas.

Nos résultats ont montré que la surexpression de Cat atténue le stress oxydatif rénal; prévient l'hypertension, améliore le taux de filtration glomérulaire, l'albuminurie, l'hypertrophie rénale, la fibrose tubulo-interstitielle et l'apoptose tubulaire; et supprime l'expression des gènes profibrotiques et proapoptotiques dans les RPTCs des souris Akita Cat-Tg lorsque comparées aux souris Akita. De plus, la surexpression de Cat dans les RPTC des souris Akita normalise l'expression rénale de l'ACE2 et les niveaux urinaires d'Ang 1-7.

D'autre part, l'administration d'Ang 1-7 prévient l'hypertension systémique, normalise le ratio albumine/créatinine urinaire et atténue l'hyperfiltration glomérulaire des souris Akita, sans affecter la glycémie sanguine. De plus, le traitement avec l'Ang 1-7 atténue aussi le stress oxydatif et l'expression de la NADPH oxydase, Agt, ACE, TGF-β1 (transforming growth factor-β1) et collagène IV, tout en augmentant l'expression de l'ACE2 et du récepteur Mas dans les reins des souris Akita. Ces effets sont renversés par la co-admininstration d'A779.

Ces résultats démontrent que la surexpression de Cat prévient l'hypertension et la progression de la néphropathie, en plus de mettre en lumière l'importance du stress oxydatif intrarénal et l'expression de l'ACE2 comme facteurs contribuant à l'hypertension et les dommages rénaux observés dans le diabète. En outre, nos données suggèrent que l'Ang 1-7 joue un rôle protecteur dans l'hypertension et les dommages aux RPTC dans le diabète, principalement en réduisant les voies de signalisations du stress oxydatif dans les reins et en normalisant l'expression de l'ACE2 et du récepteur Mas. Nos résultats indiquent aussi que l'Ang 1-7 pourrait agir comme un agent thérapeutique potentiel dans le traitement de l'hypertension

systémique et les dommages rénaux observés dans le diabète. En conséquence, l'Ang 1-7 est responsable du rôle protecteur de l'ACE2 dans l'hypertension et la DN.

Mots-clés: Rein, système rénine-angiotensine, enzyme de conversion de l'angiotensine-2, catalase, hypertension, angiotensine 1-7, récepteur Mas, néphropathie diabétique, apoptose, fibrose tubulo-interstitielle, espèces réactives de l'oxygène, NADPH oxydase

Abstract

It is well accepted that renin-angiotensin system (RAS) activation plays an important role in the development of hypertension and diabetic nephropathy (DN). With the discovery of angiotensin-converting enzyme-2 (ACE2) and recognition of MAS as the receptor of Angiotensin 1-7 (Ang 1-7), new players in RAS, ACE2/Ang 1-7/MAS axis, have been identified to counteract the effect of ACE/Ang II/ AT1 axis. Evidence implicates the intrarenal RAS's contribution to DN. Previous studies from our laboratory using transgenic mice overexpressing rat Angiotensinogen (Agt) in their renal proximal tubular cells (RPTCs) have demonstrated the importance of the intrarenal RAS in renal damage and the induction of hypertension. We also recently observed that renal ACE2 expression and urinary Ang 1–7 were lower in type 1 diabetic Akita mice and that treatment with RAS blockers normalized ACE2 expression and prevented hypertension development in these Akita mice. In the diabetic milieu, both glycemia and angiotensin II (Ang II) can induce reactive oxygen species (ROS) generation, which contributes to kidney injury. To explore the relationship among ROS, ACE2 and DN, we created Akita transgenic mice overexpressing catalase (Cat) in RPTCs by crossbreeding type I diabetic Akita mice with our established transgenic mice overexpressing rat Cat in RPTCs. In another study, Akita mice were treated with Ang 1-7 or combination of Ang 1-7 and its antagonist, A779, to investigate the relations between Ang 1-7 action, systolic hypertension (sHTN), oxidative stress, kidney injury, ACE2 and Mas receptor expression.

Our results showed that overexpression of Cat attenuated renal oxidative stress; prevented hypertension; ameliorated glomerular filtration rate, albuminuria, kidney hypertrophy, tubulointerstitial fibrosis, and tubular apoptosis; and suppressed profibrotic and proapoptotic

gene expression in RPTCs of Akita Cat-Tg mice compared with Akita mice. Furthermore, overexpression of Cat in RPTCs of Akita mice normalized renal ACE2 expression and urinary Ang 1–7 levels.

On the other hand, Ang 1-7 administration prevented systemic hypertension, normalized urinary albumin/creatinine ratio and attenuated glomerular hyperfiltration without affecting blood glucose levels in Akita mice. Furthermore, Ang 1-7 treatment also attenuated oxidative stress and the expression of NADPH oxidase 4, Agt, ACE, transforming growth factor-β1 (TGF-β1) and collagen IV, and increased the expression of ACE2 and Mas receptor in Akita mouse kidneys. These effects were reversed by co-administration of A779.

These data demonstrated that Cat overexpression prevents hypertension and progression of nephropathy and highlight the importance of intrarenal oxidative stress and ACE2 expression contributing to hypertension and renal injury in diabetes. Furthermore, our data suggest that Ang 1-7 plays a protective role in hypertension and RPTC injury in diabetes, predominantly through decreasing renal oxidative stress-mediated signaling and normalizing ACE2 and Mas receptor expression. Our results also indicate Ang 1-7 as a potential therapeutic agent for treatment of systemic hypertension and kidney injury in diabetes. Therefore, Ang 1-7 mediates the major protective role of ACE2 in the hypertension and DN.

Key words: Kidney, renin-angiotensin system, angiotensin-converting enzyme 2, catalase, hypertension, Angiotensin 1-7, Mas receptor, diabetic nephropathy, hypertension, apoptosis, tubulointerstitial fibrosis, reactive oxygen species, NADPH oxidase

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List of Abbreviations

α-SMA: α-smooth muscle actin
ACE: angiotensin converting enzyme
ACEi: angiotensin converting enzyme inhibitor
ADA: American Diabetes Association
AGEs: advanced glycation end-products
Agt: Angiotensinogen
AMDCC: the Animal Models of Diabetic Complications Consortium
Ang I: angiotensin I
Ang II: angiotensin II
Ang 1-7: angiotensin 1-7
AR: aldose reductase
ARBs: angiotensin receptor blockers
AT1R: angiotensin II subtype I receptor
AT2R: angiotensin II sybtype 2 receptor
BENEDICT: Bergamo Nephrologic Diabetes Complications Trial
BP: blood pressure
Cat: Catalase
CDA: Canadian Diabetes Association
CKD: Chronic kidney Diseases
Co-Smad: common Smad
DAG: diacylglycerol
DISC: death-inducing signalling complex
DM: Diabetes mellitus
DN: Diabetic nephropathy
ECM: extracellular matrix
EC-SOD: extracellular superoxide dismutase
ELISA: Enzyme-Linked ImmunoSorbent Assay
EMT: epithelial-mesenchymal transition
eNOS: endothelial nitric oxide synthase
ER: endoplasmic reticulum
ERK: extracellular signal-regulated kinase
ESRD: end–stage renal disease
FADD: Fas-Associated via Death Domain
FasL: Fas ligand
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GBM: glomerular basement membrane
GFAT: glutamine: fructose-6 phosphate amidotransferase
GFR: glomerular filtration rate
GlcNAc: *N*-acetyl glucosamine
GPCR: G protein-coupled receptors
GPxs: Glutathione peroxidases
GSH: reduced glutathione
HE: hematoxylin-eosin

ICA: Islet cell antibodies
ICAM-1: intercellular adhesion molecule-1
IDF: International diabetes federation
IRPTCs: immortalized renal proximal tubular cells
JAK/STAT: Janus kinase/signal transducers and activation of transcription
JG apparatus: Juxtaglomerular apparatus
JNK/SAPK: c-Jun N-terminal kinase/stress-activated protein kinase
KAP: kidney androgen-regulated protein
KO: knockout
LAP: latency associated protein
MAPKs: mitogen-activated protein kinases
MCP-1: monocyte chemoattractant protein-1
MMPs: matrix metalloproteinases
mRNA: messenger RNA
NADPH: nicotinamide-adenine dinucleotide phosphate
NCC: NaCl cotransporter
NKCC2: Na^+-K^+-$2Cl^-$ cotransporter
NEP: neutral endopeptidase
NF-κB: nuclear factore-κB
NHE: sodium-hydrogen exchanger
NIDDK: National Institute of Diabetes and Digestive and Kidney Diseases
NKF: National Kidney Foundation
NO: nitric oxide
NOD: nonobese diabetic
NOX: NADPH Oxidase
Nrf2: Nuclear factor erythroid 2-related factor
$ONOO^-$: peroxynitrite anion
PA: plasminogen activator
PAI-1: plasminogen activator inhibitor-1
PARP: poly (ADP-ribose) polymerase
PKC: protein kinase C
PPARs: Peroxisome proliferator-activated receptors
PS: phosphatidyl serine
PTCs: proximal tubular cells
PTs: proximal tubules
RAGE: receptor for AGEs
RAP: receptor-associated protein
RAS: renin-angiotensin system
RIA: radioimmunoassay
ROS: Reactive Oxygen Species
RPT: renal proximal tubules
RPTC: renal proximal tubular cells
R-Smads: receptor-regulated Smads
SBP: systolic blood pressure
SD rats: Sprague Dawley rats
SGLT: sodium-coupled glucose transporters

SHR: spontaneous hypertension rat
SOD: superoxide dismutase
STZ: streptozotocin
T1D: type 1 diabetes
T2D: type 2 diabetes
TACE: tumor necrosis factor-α-converting enzyme
TAL: thick ascending limb
Tg: transgenic
TGF-β1: transforming growth factor-β1
TGF β RI: TGF-β receptor I
TGF β RII: TGF-β receptor II
TRADD: TNF receptor-associated death domain
TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF: vascular endothelial growth factor
VSMCs: vascular smooth muscle cells

Acknowledgements

Foremost, I would like to express my sincere gratitude to my advisor Dr. John S.D. Chan for the continuous support of my PhD study and research and for his patience, motivation, enthusiasm, perseverance and immense knowledge. I would also like to thank my co-director Dr. Shao-Ling Zhang for her academic direction.

I would also like to thank my committee members, Dr. Jolanta Gutkowska, Dr. Stéphan Troyanov and Dr. David Cherney for reading and commenting my thesis manuscript.

Moreover, I would like to express my gratitude to all the members of Dr. John S.D Chan and Dr. Shao-Ling Zhang's laboratory, specifically, Ms. Isabelle Chénier, Dr. Chao-Sheng Lo, Shiao-Ying Chang, Xinping Zhao, Min-Chun Liao and Yessoufou Aliou. I appreciate all the generous help from them.

I would like to express my sincere gratitude to Ju Jing Tan for proofreading my manuscript.

I also would like to thank all the friends – past and present – of my life for sharing my pressure and joy.

Last but not least, I am grateful to my family. The love and support from my wife and my son are my sources of energy.

Chapter 1: Introduction

1.1 Renal physiology and histology

1.1.1 Renal physiology

The kidney participates in whole-body homeostasis, regulating acid-base balance, extracellular fluid volume, electrolyte concentrations and blood pressure. It produces urine, in which various metabolic waste products are removed. Urine is continuously formed from an ultrafiltrate of plasma from which glucose, amino acids, water and other essential substances have been reabsorbed. Specific secretion also occurs to transfer the substances that are present in great excess or are natural poisons. The kidney also maintains the proper balance of water and minerals, including electrolytes, in the body, even though the dietary intake of water and salt may vary. In addition, the kidney can synthesize some hormones including 1,25 OH vitamin D, erythropoietin, and renin.

Nephron is defined as the essential structural and functional unit of the kidney. In the structural context, each nephron consists of a renal corpuscle (glomerulus and Bowman's capsule), and a tubule unit including proximal tubule, loop of Henle and distal tubule (Figure 1-1). There are approximately 1.7 to 2.4 million nephrons in both kidneys of normal adult.

1.1.2 Renal histology

At the beginning of the nephron, the glomerulus is a network of capillaries that receives its blood supply from an afferent arteriole of the renal circulation. The glomerulus is surrounded by Bowman's capsule, which is an expansion at the closed end of a renal tubule. The blood plasma is filtered through the capillaries of the glomerulus into the Bowman's capsule. The ultrafiltrate collects within the Bowman's capsule and then flows into the proximal tubule. The glomerulus drains into an efferent arteriole.

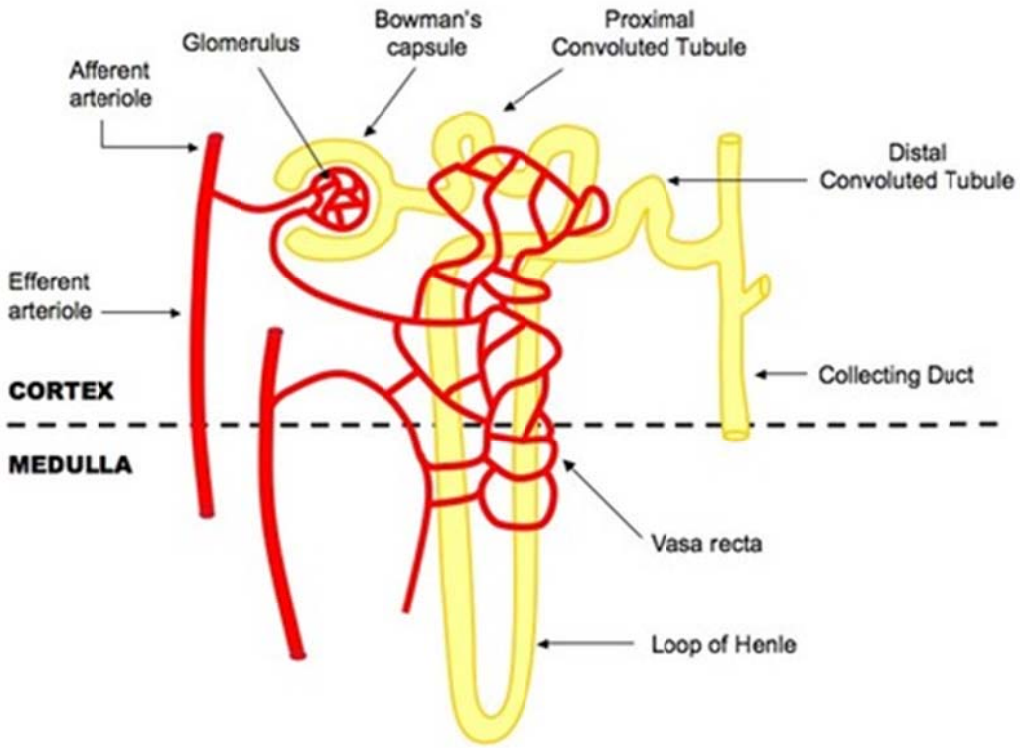

Figure 1-1. Diagram of nephron [1]

1.1.2.1 Glomerular filtration barrier:

According to the classical view, the glomerular filtration barrier consists of three interdependent layers: the innermost glomerular endothelial cells, the glomerular basement membrane (GBM), and the outermost podocytes (Figure 1-2). It displays a high conductance to small-to-mid-sized solutes in plasma but retains relative impermeability to macromolecules. The critical role of the podocyte in the prevention of proteinuria has been demonstrated by multiple examples of both inherited and acquired injury to the podocyte, especially the slit diaphragm domain [2, 3].

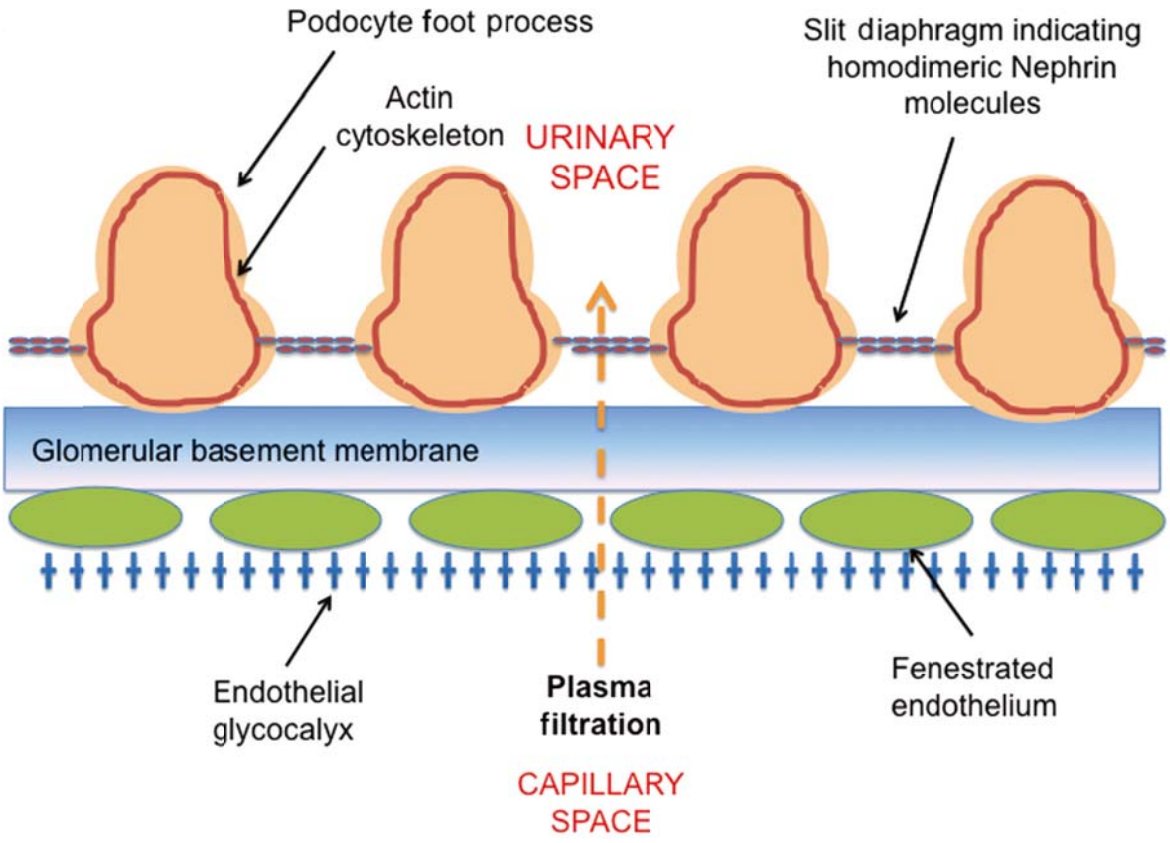

Figure 1-2. Schematic diagram of the glomerular filtration barrier [4], indicating functional components of the barrier (glycocalyx and endothelium on the capillary side, podocyte foot processes in cross-section and podocyte slit diaphragm on the urinary side) and glomerular basement membrane. The extracellular domains of nephrin are represented at the slit diaphragm, spanning the cell junction as a central component of the 'zipper' formation, and the cytoplasmic tail of nephrin links to cortical actin filaments.

1.2.2.2 Podocyte

The cell body sits in the urinary space, from which extend primary and secondary processes and, finally, fine interdigitating foot processes, which encircle the capillary on its outer side.

An almost universal finding in nephrotic disease states (in which large amounts of protein leak into the urine from the plasma) is flattening or effacement of these foot processes. The cell–cell junction between these foot processes is thought to be a key signalling and structural

domain. The slit diaphragm is a cell–cell junction that shares features of tight and adherens junctions [5] and displays anatomical regularity, with a gap of 40 nm thought to restrict the passage of proteins any larger than albumin. The gap is cross-linked by extracellular proteins, of which the transmembrane protein nephrin forms a major component, forming globular strands of 35 nm in length [6].

There is a consensus that podocyte response to injury follows a similar path regardless of the original cause of podocyte injury (genetic, environmental, or both). Podocyte injury leads to effacement of the podocyte foot processes, i.e. loss of membrane extension, which involves reorganization of the actin network. These early structural changes within the podocyte result in proteinuria. If these changes are not reversed, podocyte detachment and loss occurs with subsequent development of obliteration of the urinary space, segmental glomerulosclerosis, and finally, end-stage renal failure [7]. Growth factor receptors such as vascular endothelial growth factor (VEGF) and transforming growth factor β (TGF-β1), G protein-coupled receptors (GPCRs) such as the angiotensin type 1 receptor (AT1R), signalling through integrins, and ion channels, like the TRPCs, have been implicated in early podocyte injury [8].

Nephrin assembles into a zipper-like isoporous filter structure of the podocyte slit diaphragm, forming a part of the size-selective glomerular filtration barrier (Figure 1-3) [9]. Patients with nephrin mutations, which were originally found in cohorts of Finnish patients (Finnish-type nephrotic syndrome), display the earliest and most severe clinical phenotype, indicating an essential role for this protein in the intact filtration [10]. Abnormalities in nephrin expression have also been demonstrated in other human proteinuric renal diseases and in a range of experimental renal diseases associated with proteinuria [11, 12].

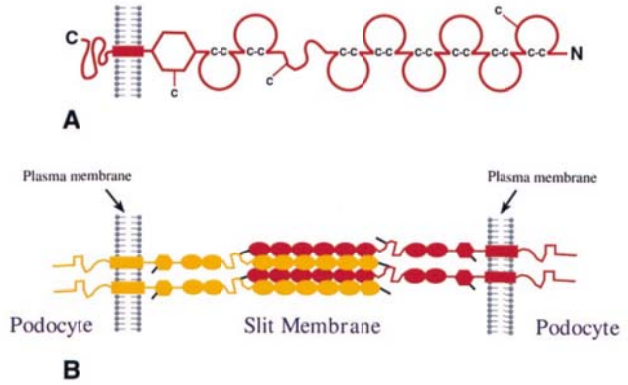

Figure 1-3. Hypothetical model of nephrin assembly to form the isoporous filter of the podocyte slit diaphragm. (A) Schematic domain structure of nephrin. (B) Possible mode of interdigitating association of four nephrin molecules in the slit between two foot processes [9].

1.1.2.3 Proximal tubule

The proximal tubule can be divided into two parts: the proximal convoluted tubule and the proximal straight tubule. It is a major site for reabsorption and for some secretion. The apical end of proximal tubule cell has a brush border of microvilli (Figure 1-4), which provides an increased surface area to accommodate the membrane channels that are responsible for absorbing small molecules from the filtrate in the tubular lumen into the cell.

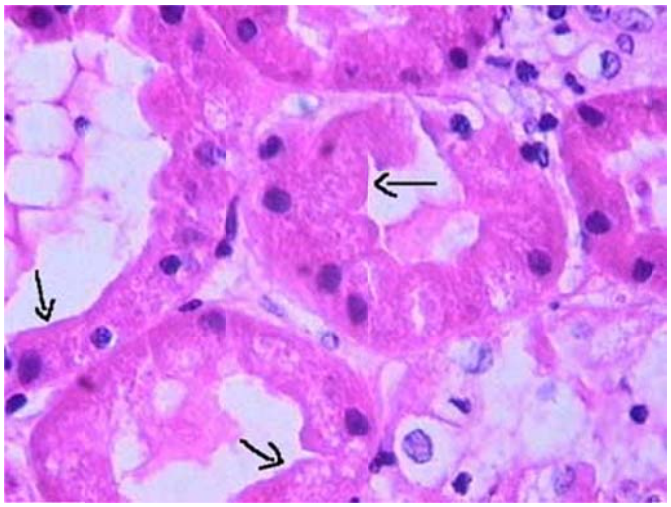

Figure 1-4. Proximal tubule. The arrows shows the brush border [13].

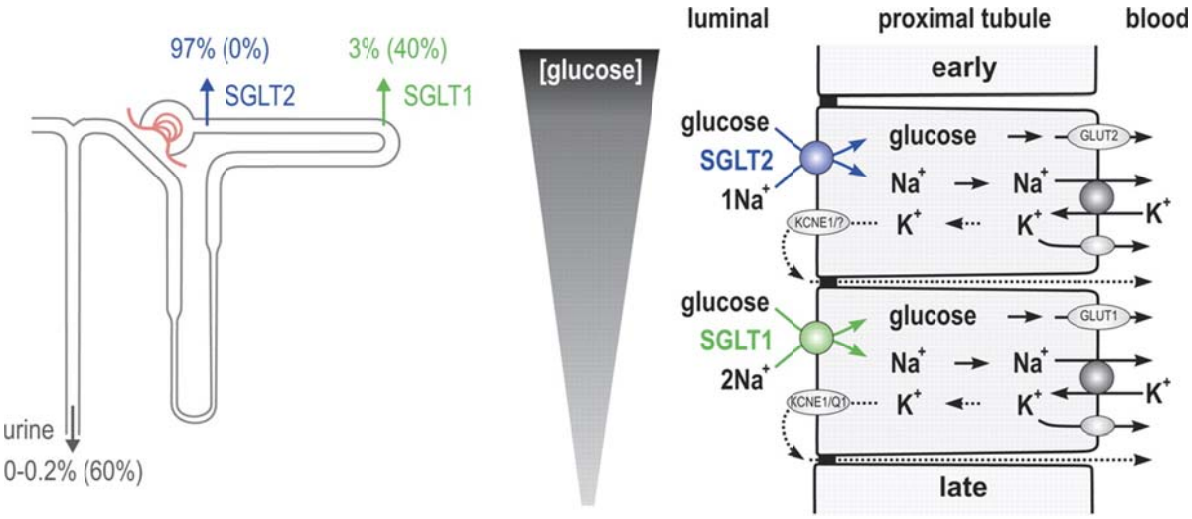

Figure 1-5. Glucose transport in the kidney. Under normoglycemia, ~97% of filtered glucose is reabsorbed via the Na^+-glucose cotransporter SGLT2 primarily in the early segments of the proximal tubule. A significant capacity of SGLT1 to reabsorb glucose in later segments of the proximal tubule is unmasked by SGLT2 inhibition (~40% of filtered glucose under normoglycemia; see numbers in parentheses), on the basis of the assumption that apical tubular glucose uptake in the kidney is primarily mediated by SGLT2 and SGLT1. The glucose transporters GLUT2 and GLUT1 mediate glucose transport across the basolateral membrane. Na^+-glucose cotransport is electrogenic, and luminal K^+ channels serve to stabilize the membrane potential [14].

Proximal tubule is responsible for absorption of 100% of glucose and amino acids, most water, salt and urea as well as secretion of ammonium, hydrogen ions and creatinine into the lumen. Under euglycemic conditions, ~97% of filtered glucose is reabsorbed via sodium-coupled glucose transporters2 (SGLT2) primarily in the early segments of the proximal tubule, whereas the rest is absorbed via SGLT1 in the late segments [15]. SGLT2 and SGLT1 are localized in the brush border membrane of the early and later sections of the proximal tubule, respectively. The Na^+/K^+-ATPase pump on the basolateral membrane of the proximal tubule cell uses ATP to move three Na^+ outward into the blood, creating a low intracellular Na^+

concentration. The electrochemical gradient thus created provides the driving force for ongoing transport of Na^+ into the cell across the apical membrane, allowing for glucose to be concurrently cotransported by SGLTs. The specific facilitative glucose transporter, GLUT, on the basolateral membrane then transports the glucose into the interstitium [16] (Figure 1-5).

The proximal tubule can only reabsorb a limited amount of glucose. Under normal conditions, the proximal tubule reabsorbs approximately 180 g of glucose from the glomerular filtrate each day. When the blood glucose level exceeds renal threshold of glucose (160–180 mg/dl), the proximal tubule becomes overloaded and begins to excrete glucose in the urine. This condition is termed glycosuria [17].

Sodium is freely filtered at the glomerulus. In the proximal convoluted tubule, 50% of the filtered sodium is reabsorbed. Although in the apical membrane there are 20 different sodium transporters, most of which couple to "substrates" (such as amino acids and carbohydrates), collectively they mediate only 10% of in sodium reabsorption the proximal tubule. The sodium-hydrogen exchanger, NHE3, mediates the majority of Na^+ reabsorption [18] (Figure 1-6B).

1.1.2.4 Other components of a nephron

(a) Henle's loop

Named after its discoverer, F. G. J. Henle, the loop of Henle's main function is to establish an osmotic gradient in the medulla of the kidney for water reabsorption. The loop of Henle has three distinct regions: the thin descending limb, the thin ascending limb and the thick ascending limb (TAL). In the TAL, almost all sodium transport results directly or indirectly from Na^+-K^+-$2Cl^-$ cotransporter (NKCC2) and ~20% of the filtered load is reabsorbed. The

loop of Henle as a whole reabsorbs 30–40% of the filtered sodium [18] (Figure 1-6C). After tubule fluid passes through Henle's loop, it becomes hypo-osmotic.

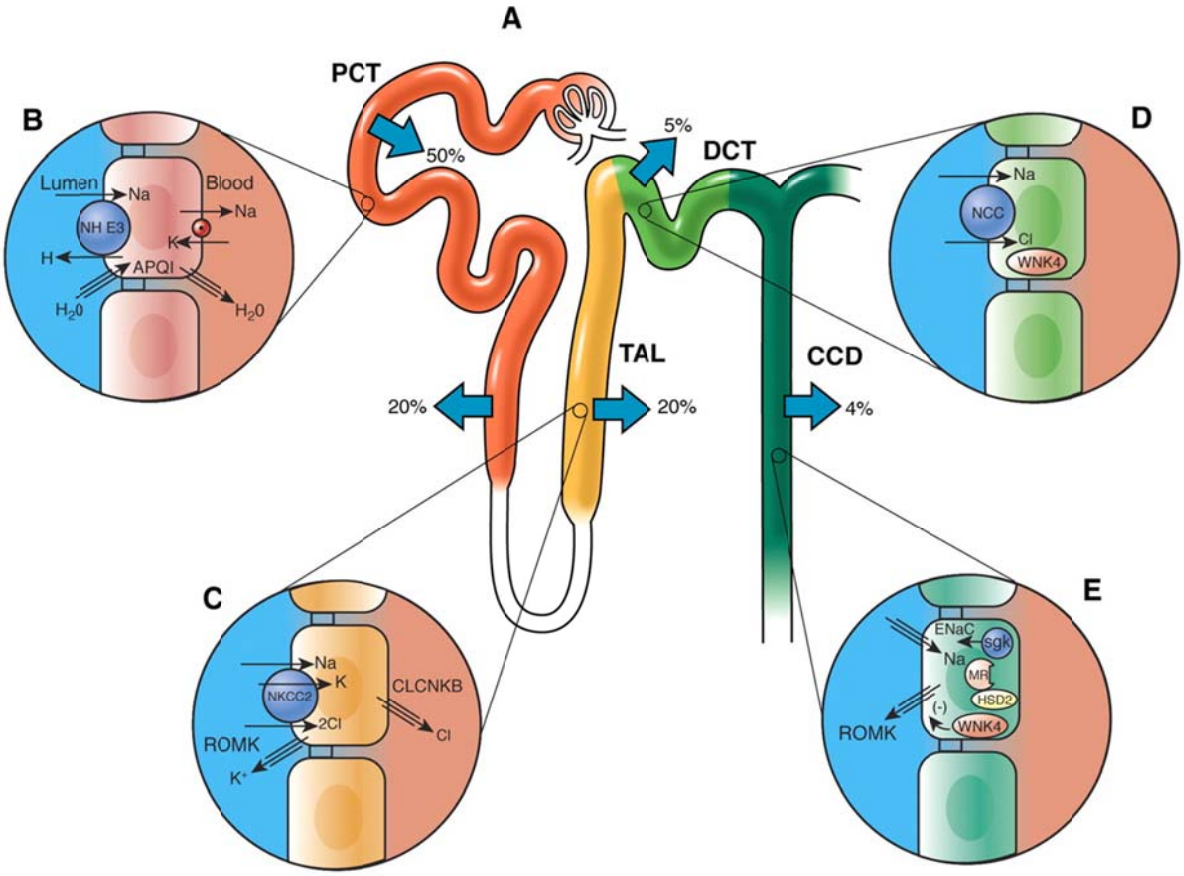

Figure 1-6. A: percentage sodium reabsorption over the length of the nephron. Principal mechanisms of sodium reabsorption are shown in the proximal tubule (B), the thick ascending loop of Henle (C), the distal convoluted tubule (D), and the collecting duct (E).

(b) Distal tubules

The distal tubule is lined with simple cuboidal cells that are shorter than those of the proximal convoluted tubule. Sodium reabsorption in the distal tubule is mediated by the thiazide-

sensitive NaCl cotransporter (NCC) (Figure 1-6D), which is regulated by aldosterone [19], and also, to a lesser extent, by sodium-hydrogen exchange (NHE2). After the distal tubule, the collecting duct connects the nephrons to the ureter.

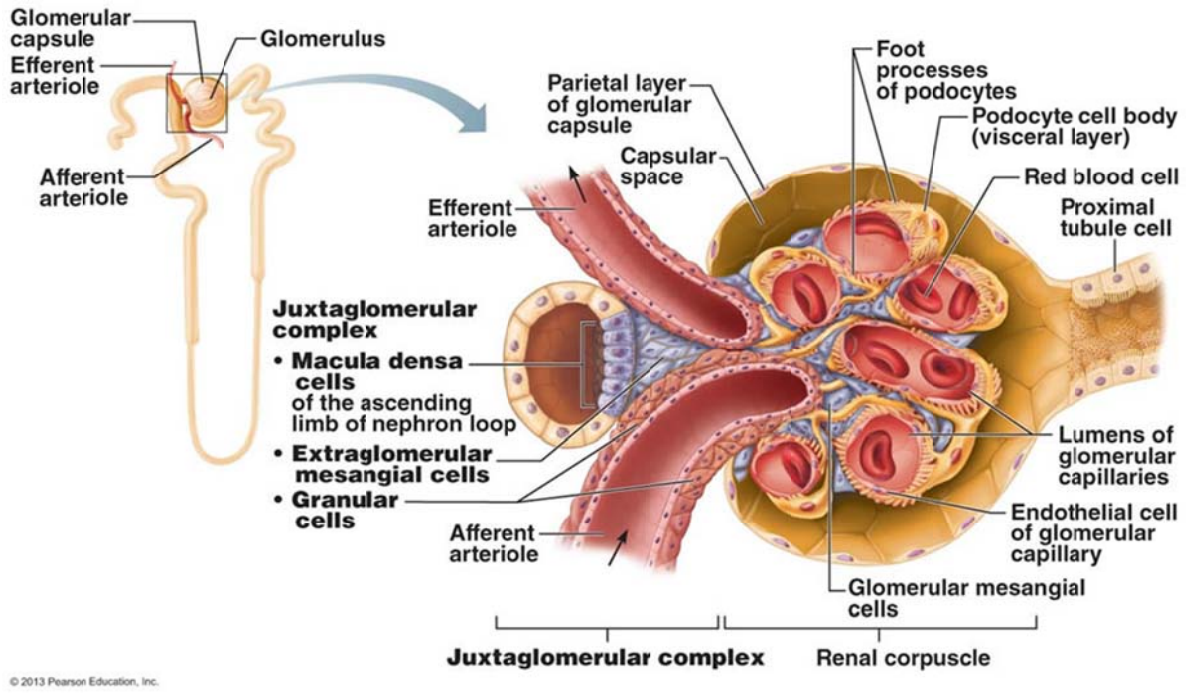

Figure 1-7. The juxtaglomerular apparatus [20]

(c) Juxtaglomerular (JG) apparatus

The JG apparatus is a specialized structure formed by the distal convoluted tubule and the glomerular afferent arteriole and its main function is to regulate blood pressure and the filtration rate of the glomerulus. It consists of JG cells, macula densa cells and extraglomerular mesangial cells (Figure 1-7). JG cells, also called Granular cells, are specialized smooth muscle cells of glomerular arterioles and can secrete renin. The macula densa senses any decrease in the sodium chloride concentration in the distal tubule and causes JG cells to

release renin. In response to increased sodium, the macula densa cells trigger contraction of the afferent arteriole, reducing blood flow to the glomerulus and thus also the glomerular filtration rate. Extraglomerular mesangial cells, also called Lacis Cells, are flat and elongated cells located near the macula densa, but their function remains unclear.

1.2 Pathological changes in kidney diseases

In the early stages of kidney disease, histological changes can vary because of many different causes. However, the pathological alterations of kidneys at end-stage are similar regardless of cause. The cortex is fibrotic, with most glomeruli becoming sclerotic and the affiliated tubule turning atrophic. There are scattered chronic inflammatory cell infiltrates, tubulointerstitial fibrosis and thickened arteries. Tubular lumens are dilated and filled with pink cast, giving an appearance of "thyroidization" (Figure 1-8).

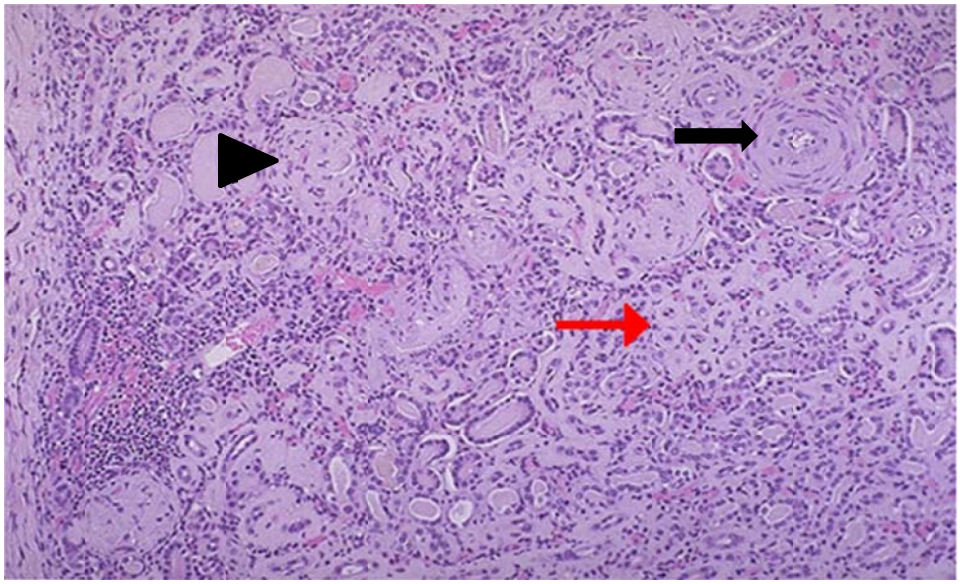

Figure 1-8. The microscopic appearance of end-stage renal disease (ESRD). Red arrow indicates cortex, black arrow indicates artery, and black arrow head indicates glomeruli. [21]

The kidneys in ESRD are usually small, firm, and pale and the surface is granular with more or less scattered, shallow cortical scars. Cross-section of the kidney reveals a thin cortex and blurred boundary between the cortex and medulla. (Figure 1-9) [21].

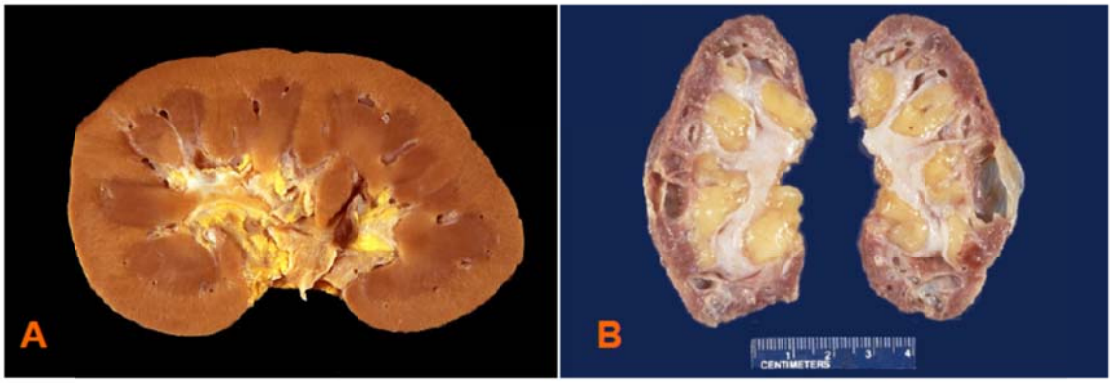

Figure 1-9. A: Normal kidney; B: Kidneys in ESRD [13]

1.3 Chronic kidney diseases (CKD)

1.3.1 CKD and GFR

CKD is defined as the presence of kidney damage (most commonly evidenced by protein in the urine or proteinuria, or, more specifically, elevated albumin: creatinine ratio in the urine or albuminuria; other blood or urine markers; or pathological abnormalities), or a decreased level of kidney function (as evidenced by reduced glomerular filtration rate or GFR), for a period of three months or more [22]. CKD can be divided into five stages, depending on how severe the kidney damages are, or the level of decrease in kidney function [23] (Table 1-1). When kidneys function at below 15 percent of their normal capacity, stage 5 CKD, also referred to as ESRD, wherein there is total or near-total loss of kidney function, they cannot effectively work, such as remove waste or excess fluid from the blood. When CKD develops into ESRD,

Table 1-1. The 5 stages of chronic kidney disease (CKD) [23]

The Five Stages of Chronic Kidney Disease		
	Kidney Function	Description
Stage 1	Kidney damage and more than 90%	Normal or High Function
Stage 2	Kidney damage and 60 to 89%	Mildly Decreased Function
Stage 3a	45-59%	Mildly to Moderately Decreased Function
Stage 3b	30-44%	Moderately to Severely Decreased
Stage 4	15 to 29%	Severely Decreased Function
Stage 5	Less than 15%	Kidney Failure
Adapted from the KDIGO Clinical Practice Guidelines for the Evaluation and Management of Chronic Kidney Disease: *Kidney Int Suppl.* 2013; 3:1-163.		

Table 1-2. Average estimated GFR by age [24].

TABLE : AVERAGE MEASURED GFR BY AGE IN PEOPLE WITHOUT CKD[2]	
AGE (Years)	AVERAGE MEASURED GFR (mL/min/1.73 m^2)
20-29	116
30-39	107
40-49	99
50-59	93
60-69	85
70+	75

dialysis or a kidney transplant is necessary to survive. However, since GFR declines gradually with age, even in people without kidney disease, it is necessary to take this into consideration when evaluating kidney function. Table 1-2 shows the average values of estimated GFR by decade in the general population [24].

1.3.2 Incidence, prevalence and causes of ESRD

Incidence is defined as the number of newly-diagnosed patients in a specific population during a given period of time. Prevalence of CKD is the number of CKD cases existing in a given population at a specific period of time (period prevalence) or at a particular moment in time (point prevalence).

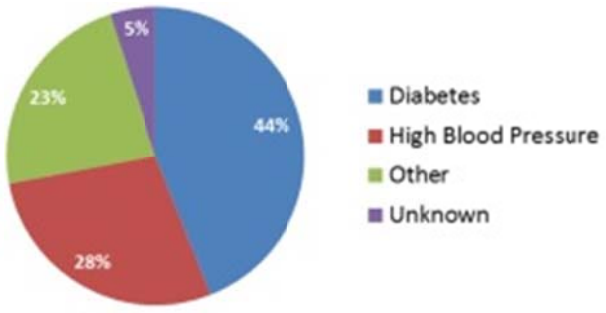

Figure 1-10. Causes of ESRD [13]

Total incident dialysis cases in USA rose 0.27 percent in 2010, to 114,083, while only 2,863 patients received a pre-emptive transplant as their first ESRD modality. The rate of new ESRD cases per million population, which has been relatively steady since 2000, fell 2.0 percent in 2010, to 348 [25]. As of December 31, 2010, prevalent population included 383,992 patients

on hemodialysis and 29,733 on peritoneal dialysis, as well as 179,361 with a kidney transplant; the total treated ESRD population thus rose to 593,086 [25].

Diabetes has become the most common single cause of end-stage renal disease (ESRD) in the U.S.A, accounting for ~44% of new cases of treated ESRD in 2011. The second most common cause is high blood pressure or hypertension [26] (Figure 1-10).

1.4 Diabetes Mellitus (DM)

1.4.1 Prevalence and cost of diabetes

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of a variety of organs, especially the kidneys, nerves, eyes, heart, and blood vessels [27]. The vast majority of cases of diabetes fall into two broad etiopathogenetic categories: type 1 diabetes (T1D) and type 2 diabetes (T2D).

T1D is characterized by the destruction of the pancreatic beta cells, resulting in absolute insulin deficiency. This is usually due to autoimmune-mediated destruction of the beta cells (type 1A). Islet cell antibodies (ICA) or other islet autoantibodies (such as antibodies to GAD65, insulin, IA-2) can be found in the serum [27]. However, some patients have no evidence of autoimmunity and have no other known cause for beta-cell destruction (type 1B).

T2D is by far the most common type of diabetes in adults and accounts for ~90–95% of those with diabetes. The patients have insulin resistance and usually have relative (rather than absolute) insulin deficiency. Most of them are obese, and obesity itself causes some degree of

insulin resistance. Even patients who are not obese by traditional weight criteria may have an increased percentage of body fat distributed predominantly in the abdominal region [27].

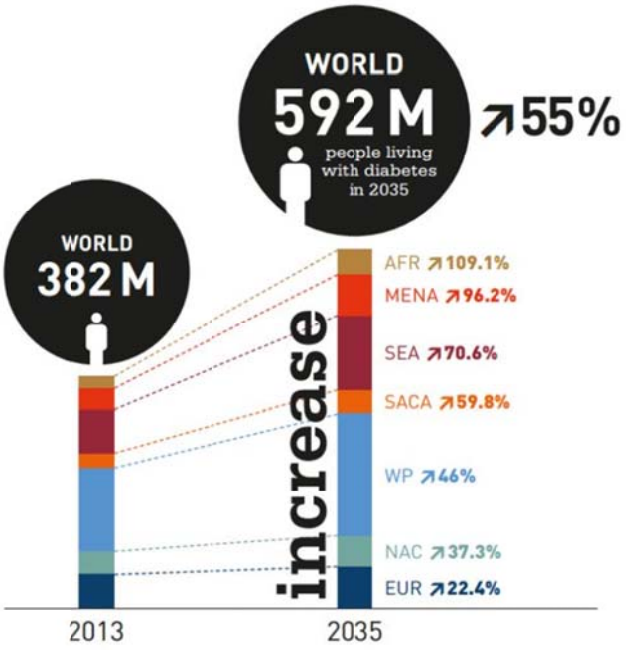

Figure 1-11. Global Prevalence of DM [28]. AFR: Africa, MENA: Middle East and North Africa, SEA: South-East Asia, SACA: South and Central America, WP: Western Pacific, NAC: North America and Caribbean, EUR: Europe.

An estimated 382 million people worldwide have diabetes in 2013, according to a new report from the International Diabetes Federation (IDF). The IDF expects that number to rise to 592 million by 2035 (Figure 1-11), when one in every 10 people will have the disease [28]. According to American Diabetes Society, 25.8 million children and adults in the United States—8.3% of the population—have diabetes [29]. In Canada, more than 3 million people have diabetes. From 2010 to 2020, another 1.2 million people are expected to be diagnosed with diabetes, bringing the total to approximately 3.7 million [30].

The global health expenditure on diabetes is expected to total at least US$ 376 billion in 2010 and US$ 490 billion in 2030. Globally, 12% of the health expenditures and US$ 1330 per person are anticipated to be spent on diabetes in 2010 [31]. Total costs of diagnosed diabetes in the United States in 2012 were $245 billion [29]. In Canada the economic burden of diabetes is expected to be about $12.2 billion in 2010 (measured in inflation-adjusted 2005 dollars), which is an increase of $5.9 billion or nearly double its level in 2000. The cost of the disease is expected to rise by another $4.7 billion by 2020 [30] (Figure 1-12). The direct cost of diabetes now accounts for about 3.5% of public healthcare spending in Canada.

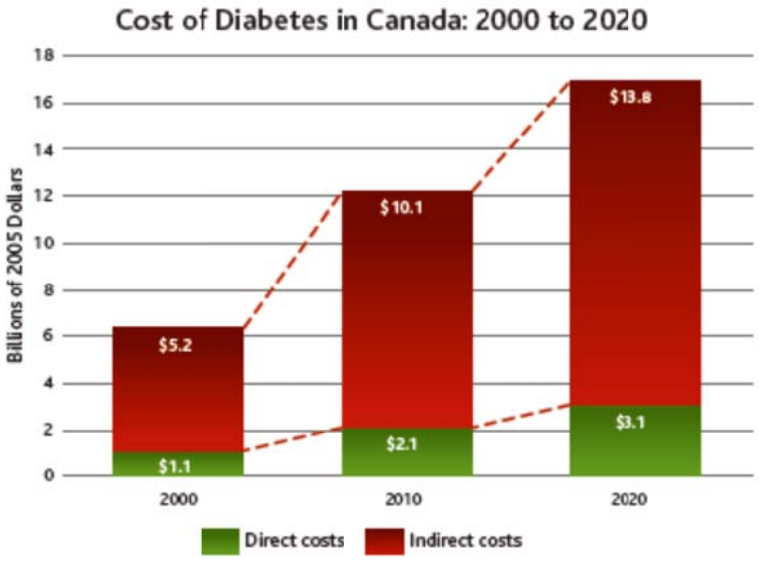

Figure 1-12. Cost of Diabetes in Canada [32]

1.4.2 Pathogenesis of diabetic complications

Diabetes is characterized by chronic hyperglycemia and the development of microvascular and macrovascular pathologies. The microvascular complications are due to damage to small

blood vessels and include nephropathy, retinopathy and neuropathy. The macrovascular complications affect larger blood vessels, including cardiovascular diseases, such as heart attacks and strokes. Many hypotheses have been proposed to explain underlying mechanisms. A large body of evidence indicates that oxidative stress is the common denominator link for the major pathways involved in the development and progression of diabetic micro- as well as macrovascular complications of diabetes [33].

1.4.3 Reactive Oxygen Species (ROS) and oxidative stress

ROS are composed of a series of oxygen intermediates, including the free radical superoxide anion (O_2^{-}), the highly reactive hydroxyl free radical ($HO^{\cdot}$), the nonradical hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^{-}$), hypochlorous acid (HOCl) and lipid radicals. Excessive amounts of ROS, overwhelming the detoxification capacity of various endogenous anti-oxidative defensive mechanisms, oxidize various tissue biomolecules, such as DNA, protein, lipids and carbohydrates, and this disastrous state has been commonly referred to as an oxidative stress [33]. In mammalian cells, potential sources of ROS include mitochondrial respiratory chain, NADH/NADPH oxidases, xanthine oxidase, NO synthase and other certain hemoproteins.

1.4.3.1 Mitochondrial origin of ROS

Glucose generates ATP, NADH and FADH2 via glycolysis and oxidative phosphorylation. The electrons from NADH or FADH2 are transferred to molecular oxygen (O_2) in the mitochondrial respiratory chains complex I – IV to generate ATP. During this process, most of the O_2 is reduced to water under normal physiological states, and less than 1% of O_2 is converted to superoxide anion, O_2^{-}. However, under mitochondrial dysfunctional or

hyperglycemic states there is an excessive leakage of electrons at two major sites, one at the complex I and another at the interface between coenzyme Q and complex III. This leads to an increase in the reduction of O_2 to O_2^- and increase in the production of ROS (Figure 1-13) [34].

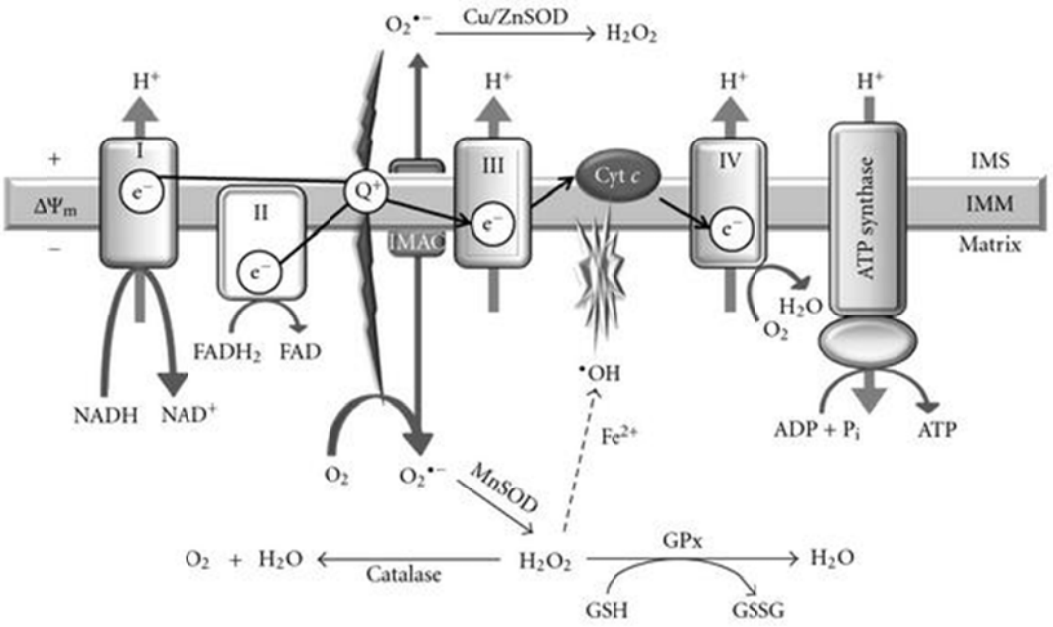

Figure 1-13. Mitochondrial ROS production [34].

1.4.3.2 NADPH oxidase

NAD(P)H oxidase is a multi-subunit enzyme that catalyzes the generation of O_2^- through the reduction of O_2 by using either NADPH or NADH. The prototype of this enzyme was originally found in neutrophils and phagocytic cells [35]. In these cells a large quantity of O_2^- is produced to kill the invading microorganisms. This enzyme has five units: membrane-associated $p22^{phox}$ and $p91^{phox}$ (also termed as NADH Oxidase 2 "NOX2"), cytosolic subunit

$p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and GTPase Rac1 or Rac2. Upon stimulation, these cytosolic subunits translocate to the cytochrome complex, leading to the formation of an active NAD(P)H oxidase multi-subunit complex, which transfers electrons to O_2 with the generation of O_2^- [36] (Figure 1-14).

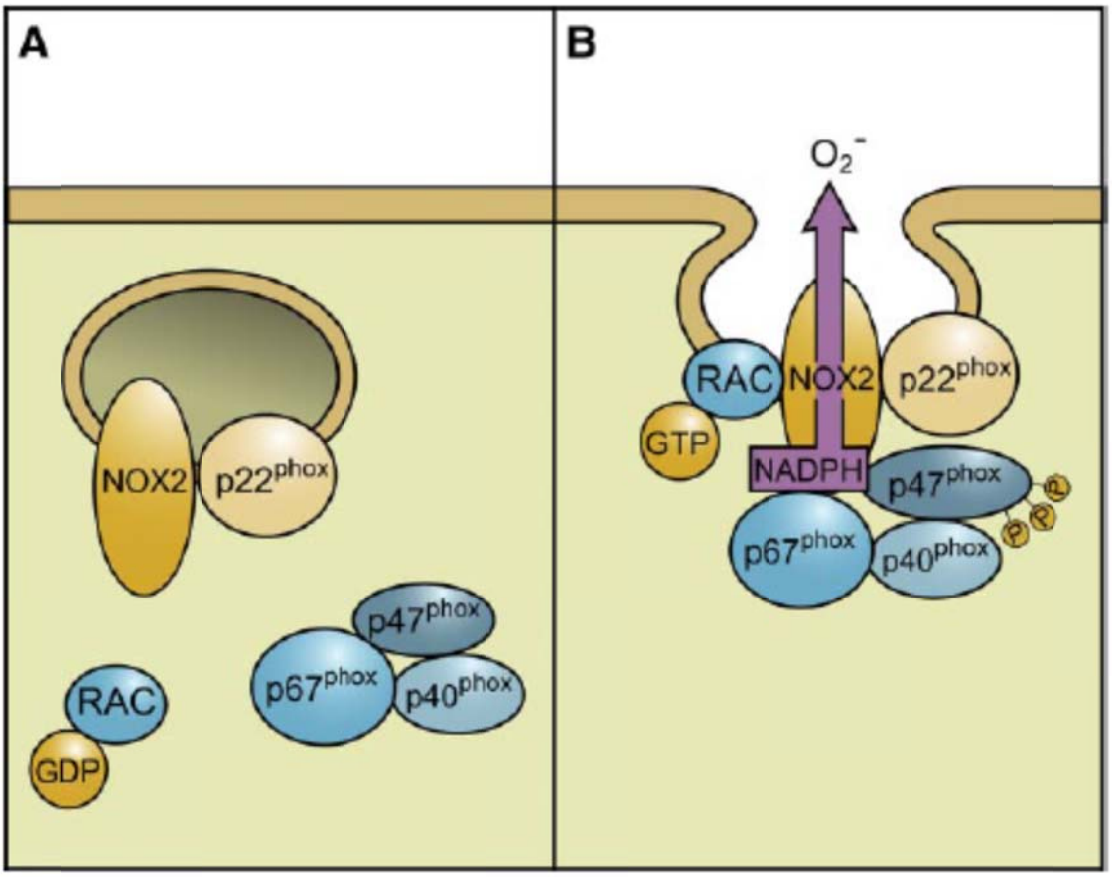

Figure 1-14. Assembly of the phagocyte NADPH oxidase NOX2 [37].

The new homologs along with gp91phox are now designated the Nox family of NADPH oxidases. This family has seven members: Nox1, Nox2, Nox3, Nox4, Nox5, Duox1 and Duox2 (Figure 1-15).

NOX1, NOX2, and NOX4 have all been identified within the kidney [38]. Renal cells differentially express NADPH oxidase subunits: p47phox, p67phox and p22phox are expressed in the mesangial cell, podocytes, endothelial cells, vascular smooth muscle cells,

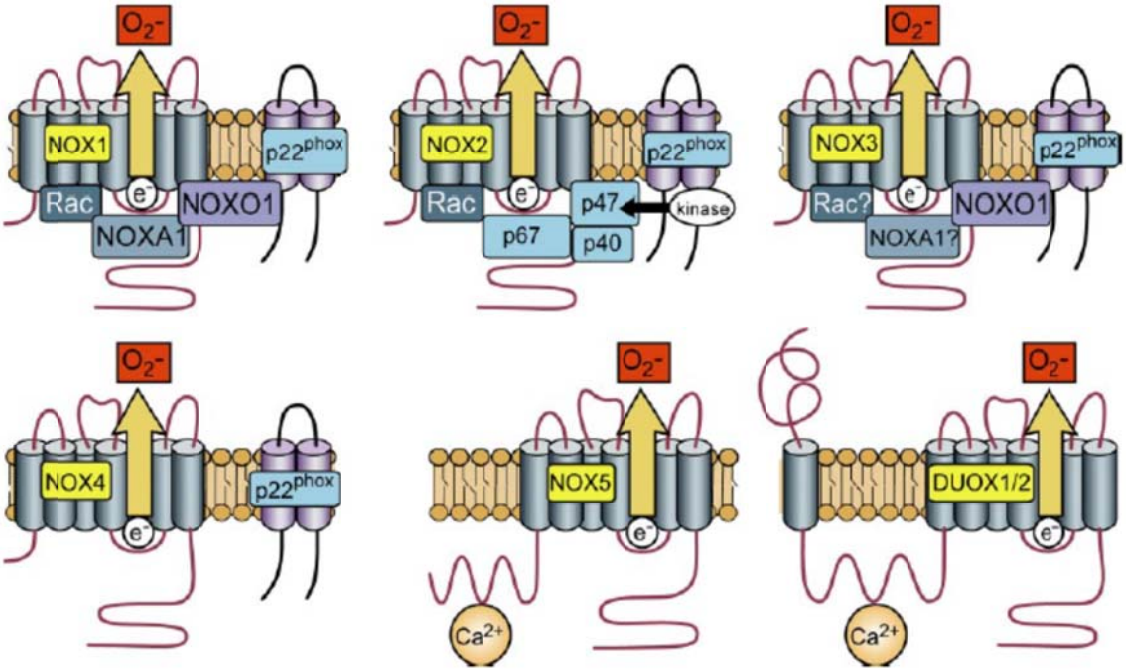

Figure 1-15. Activation of NADPH oxidase isoforms. Despite their similar structure and enzymatic function, NOX family enzymes differ in their mechanism of activation. *A*: NOX1 activity requires p22phox, NOXO1 (or possibly p47phox in some cases) and NOXA1, and the small GTPase Rac. *B*: NOX2 requires p22phox, p47phox, p67phox, and Rac; p47phox phosphorylation is required for NOX2 activation. Although not absolutely required, p40phox also associates with this complex and may contribute to activation. *C*: NOX3 requires p22phox and NOXO1; the requirement for NOXA1 may be species dependent, and the requirement of Rac is still debated. *D*: NOX4 requires p22phox, but in reconstitute systems, it is constitutively active without the requirement for other subunits. However, in native NOX4-expressing cells, activation, possibly including Rac, has been described. *E* and *F*: NOX5, DUOX1, and DUOX2 are activated by Ca^{2+} and do not appear to require subunits [37].

fibroblast, thick ascending limb, distal convoluted duct including macula densa cells and cortical collecting ducts; Nox-2 in podocytes, mesangial cell and endothelium; Nox-4 in glomerulus, proximal tubule and distal convoluted tubule; and Nox-3 in fetal kidney [39].

Of the renal Noxes, Nox4 is most abundantly expressed and hence was originally termed Renox [40, 41]. NOX4 is a p22phox-dependent enzyme, however it does not require cytosolic subunits for its activity [37]. NOX4 requires p22phox, but in reconstitute systems it is constitutively active without the requirement for other subunits. In native NOX4-expressing cells, however, a Rac requirement for activation has been documented [37].

The Nox5 gene is absent from the mouse and rat genomes, making the use of conventional animal models unfeasible. Unlike other Nox family members, Nox5 does not require membrane-bound or cytosolic components, such as p22phox or p47phox, for its activity, but is tightly regulated by changes in intracellular calcium levels [42]. Interestingly, Holterman and colleague [42] recently generated transgenic mice expressing human Nox5 in a podocyte-specific manner (Nox5pod^{+}). Nox5pod^{+} mice exhibited early onset albuminuria, podocyte foot process effacement, GBM thickening, interstitial fibrosis, and elevated systolic blood pressure (BP). Subjecting these mice to STZ-induced diabetes further exacerbated these changes. Furthermore, kidney biopsies from diabetic patients showed increased NOX5 expression compared with nondiabetic subjects.

1.4.3.3 SOD, Catalase, GPX and Nrf2

(a) SOD

Superoxide dismutase (SOD) is the main defense against $O_2\bullet^-$, catalyzing its dismutation to H_2O_2 and O_2. There are three isoforms in mammalian cells: SOD1 (Cu, Zn SOD) is located in

the cytoplasm, SOD2 (MnSOD) is in the mitochondria, and SOD3(extracellular SOD;EC-SOD) is extracellular [43]. Mitochondria are both a major source of ROS production from respiratory chains as well as a major target of ROS-induced cellular injury. Thus, mitochondrial Mn-SOD is thought to play an important role in cellular defense against oxidative damage by ROS [44]. Complete loss of the gene encoding MnSOD results in a lethal phenotype with some live births [45, 46], but heterozygous knockout mice have a normal lifespan [47]. Cu, Zn SOD is the major isoform of SOD in the renal cortex and glomeruli, accounting for more than 90% of total SOD activity in these tissues [48]. Deficiency of SOD1 accelerated renal injury in STZ-induced diabetic mice , while treatment of these mice with the SOD mimetic tempol for 4 weeks suppressed albuminuria, glomerular transforming growth factor β, collagen α1(IV), nitrotyrosine, and glomerular superoxide [48].

(b) Catalase (Cat)

Catalases are enzymes that catalyse the conversion of H_2O_2 into water and oxygen according to the equation: $2H_2O_2 \rightarrow 2H_2O + O_2$. They use either an iron or manganese cofactor with a high catalytic rate. Cat is encoded by a single gene, which is highly conserved among species. It is expressed in all mammalian tissues and can be found in the liver, kidneys and erythrocyte with high concentration [49]. High Cat activity is detected in peroxisomes. Cat is also found in the cytosol of erythrocytes [50]. Cat is a tetramer of four polypeptide chains, each over 500 amino acids long. Cat contains four porphyrin heme (iron) groups that allow the enzyme to react with the hydrogen peroxide[51]. In the kidney, Cat is localized predominantly in the cytoplasm of proximal tubules of the superficial cortex. Cat was not detected in the glomeruli, loop of Henle, distal tubules, and collecting ducts [52]. Cat deficiency in STZ-induced diabetic

mice increased mitochondrial ROS and fibronectin expression in response to free fatty acids, which were effectively normalized by catalase overexpression or N-acetylcysteine [53].

(c) Glutathione peroxidases (GPxs)

The "classical" glutathione peroxidase, now called GPx1, was first discovered as an erythrocyte enzyme that specifically reduces H_2O_2 by GSH, but later shown to reduce a broad scope of organic hydroperoxides [54]. There are eight distinct GPxs in mammals, most of which are selenoproteins. Only GPx1, 3 and 4 have been functionally characterized to some extent [49]. Low levels of both GPx1 and GPx3 are associated with the development of vascular disease. The patients of CVD with low erythrocyte GPx1 activities had increased recurrent events [55]. Lack of functional GPx1 accelerates diabetes-associated atherosclerosis via upregulation of proinflammatory and profibrotic pathways in apolipoprotein E (ApoE) knockout mice [56], whereas ebselen, a GPx mimetic, could attenuate diabetic nephropathy and diabetes-associated atherosclerosis in ApoE/GPx1 double knockout mice [57].

(d) Nuclear factor erythroid 2-related factor (Nrf2)

The genes encoding SOD, Cat and GPx contain antioxidant response elements (AREs) in their regulatory regions. Nrf2 is the principal transcription factor that binds to the ARE. Actin-tethered Keap1 is a cytosolic repressor that binds to Nrf2, keeps it in the cytoplasm, and promotes its proteasomal degradation [58]. Nrf2 activators modify specific cysteine residues of Keap1 and cause conformational changes that render Keap1 unable to repress Nrf2. Once released Nrf2 migrates into the cell nucleus, binds to AREs and activates the transcription of antioxidant genes [59]. Bardoxolone methyl is among the first orally available antioxidant

Nrf2 activators [60]. Bardoxolone methyl was first advanced into the clinic to assess its anticancer properties. In phase 1 trials that included cancer patients, bardoxolone methyl decreased serum creatinine levels, with a corresponding improvement in estimated glomerular filtration rate (eGFR) [61]. Phase 2 trials, including persons with T2D and stage 3b or 4 CKD, have shown that bardoxolone methyl can reduce the serum creatinine concentration for up to 52 weeks [62]. However phase 3 trials showed that among patients with T2D and stage 4 CKD, bardoxolone methyl did not reduce the risk of ESRD or death from cardiovascular causes. Moreover, significantly increased risks of heart failure and of the composite cardiovascular outcome prompted termination of the trial [62].

1.5 Diabetic Nephropathy (DN)

The earliest clinical evidence of nephropathy is the appearance of low but abnormal levels (≥30 mg/day or 20 μg/min) of albumin in the urine, referred to as microalbuminuria, and patients with microalbuminuria are referred to as having incipient nephropathy. Without specific interventions, ~80% of subjects with T1D who develop sustained microalbuminuria will progress to overt nephropathy or clinical albuminuria (≥300 mg/24 h or ≥200 μg/min) [63]. Recent findings showed that there existed another marker of early DN, early renal function decline (ERFD), i.e. the presence of a progressive loss of GFR over time even if it remains in the normal range [64]. There are three major histologic changes in the glomeruli in diabetic nephropathy: mesangial expansion; glomerular basement membrane thickening; and glomerular sclerosis [65]. An abnormality in late stage is Kimmelstiel-Wilson lesion and has been considered as a hallmark of DN (Figure 1-17). This lesion has a nodular appearance, with an acellular, hyaline matrix core, surrounded peripherally by sparse, crescent-shaped

mesangial nuclei [66]. Pathological changes in the tubulointerstitial compartment include tubular hypertrophy followed by thickening of the tubular basement membrane, interstitial fibrosis and tubular atrophy (Figure 1-17). Vascular changes typically include thickening and hyalinization of the afferent arterioles and interlobular arteries with effacement of their endothelia (Figure 1-16).

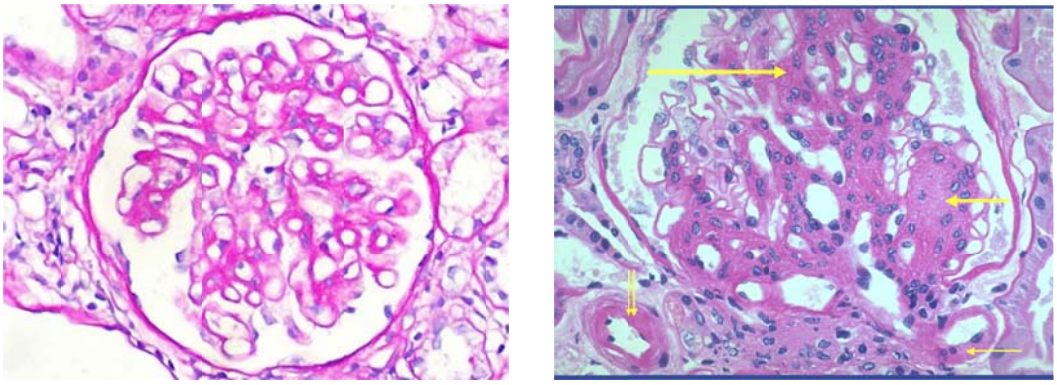

Figure 1-16. Left: Normal kidney (PAS staining). Right: Glomerulus from a type 1 diabetic (T1DM) patient with diffuse (long thick arrow) and nodular (short thick arrow) mesangial expansion and afferent (double thin arrows) and efferent (single thin arrow) arteriolar hyalinosis [67]

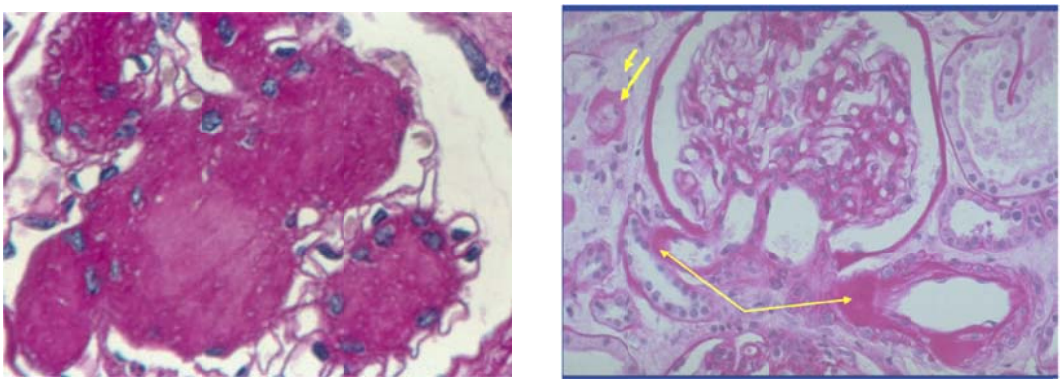

Figure 1-17. Left: Glomerulus from a T1DM patient with nodular (Kimmelstiel-Wilson) lesions. Right: Renal biopsy from a T2DM patient with hyalinosis of the afferent (right thin arrow) and efferent (left thin arrow) glomerular arterioles, interstitial expansion (short thick arrow) and tubular atrophy (long thick arrow). [67]

After 20 years of diabetes duration, around 30% of T1D patients develop DN. Over 5% of newly diagnosed T2D patients already have renal dysfunction. Another 25-40% of T2D patients will develop DN after 25 years of diabetes; they will also manifest a high tendency to progress to ESRD.

1.5.1 Pathogenesis of DN

Although the pathogenesis of DN is not completely understood, several factors are closely relevant to DN. Briefly, they are: (1) Hypertension and hemodynamic factors; (2) augmented ROS formation; (3) enhanced activity of the aldose reductase pathway; (4) increased formation of AGEs; (5) PKC activation; (6) Hexosamine pathway flux; (7) heightened activity of growth factors, TGF-β1; (8) activation of cytokines (e.g. angiotensin II or Ang II). The relationship of these major factors to the development of DN is discussed below.

1.5.1.1 Hypertension and DN

Diabetic patients who progressed to albuminuria had higher baseline arterial pressures than the patients who stayed normoalbuminuria [68]. Patients with T2D and hypertension have a sevenfold greater risk of progression to ESRD compared to patients with T2D and normal blood pressures [69].

Clinical studies have established that tight control of blood pressure can slow kidney disease progression in both T1D and T2D [70, 71]. Studies in recent years have focused on the primary prevention of microalbuminuria [72]. Tight achievement of recommended blood pressure goals may also prevent or delay the new onset of microalbuminuria and in doing so,

halt the development of later stages of overt nephropathy. As a result, new preventative strategies for hypertension management continue to be explored [73].

At diagnosis of T2D, hypertension is already present in 50% of patients. Sixty percent of hypertensive patients with T2D develop diabetic kidney disease. In T1D patients with normoalbuminuria, the prevalence of hypertension varied between that of the general population (4%) [74] and 19%, as reported in a cross-sectional Danish study [75]. In T1D, hypertension typically occurs in those with microalbuminuria or overt kidney disease. A large number of epidemiologic studies and clinical trials have established that high blood pressure (particularly systolic) is a risk factor for accelerated progression in proteinuric conditions such as diabetic CKD.

1.5.1.2 Oxidative stress in DN

The cell susceptibility to glucose-induced toxicity is determined by its expression of glucose uptake mechanisms and by the ability of these cells to downregulate glucose uptake in the setting of hyperglycemia [76]. Enhanced glucose uptake has been identified in many of the cell populations within the diabetic kidney, including glomerular epithelial cells, mesangial cells, and proximal tubular cells [77]. As mentioned before, more ROS have been generated by mitochondria.

Another important source of ROS is NADPH oxidase. Nox4 is the most abundant NOX in the kidney. The levels of both NOX4 and p22phox mRNA were increased in the kidney of streptozotocin (STZ)-induced diabetic rats as compared with control rats. Immunostaining analysis revealed that the expression levels of NOX4 and p22phox were clearly increased in both distal tubular cells and glomeruli from diabetic rats [78]. Nox4 protein expression was

increased in diabetic kidney cortex compared with non-diabetic controls and antisense oligonucleotides for Nox4 downregulated NOX4, reduced whole kidney and glomerular hypertrophy, and attenuated fibronectin expression [79]. Although Nox2 levels are not affected in the renal cortex from T2D *db*/*db* mice [80], Nox2 is increased in the cortex from T1D rats [79]. However, lack of Nox2 does not protect against diabetic kidney disease in T1D and this may be due to upregulation of renal Nox4 [81]. Data from Nox4 knockout mice are contradictory. In models of transient or permanent cerebral ischemia, Nox4-deficient mice and mice treated with the Nox4 inhibitor VAS2870 were protected from oxidative stress, neuronal apoptosis, and blood-brain barrier leakage [82]. On the contrary, Schroder et al [83] showed that in global Nox4 deficient mice, ischemia-induced angiogenesis is attenuated and that in tamoxifen-inducible Nox4 deficient mice, Ang II–mediated aortic inflammation, vascular hypertrophy, and endothelial dysfunction are exaggerated. Thus further studies are needed to unriddle the Nox4 paradox.

1.5.1.3 Aldose reductase and DN

In the setting of hyperglycemia there is increased shuttling of glucose metabolism into the sorbitol/polyol pathway [84]. The glucose is oxidized into sorbitol by the enzyme aldose reductase, a reaction that requires NAD(P)H. Sorbitol is subsequently reduced to fructose by sorbitol dehydrogenase, during which NADH is generated (Figure 1-18). Since NAD(P)H is required for the glutathione reductase reaction in which glutathione disulfide is reduced to glutathione, the consumption of NAD(P)H will finally reduce the levels of cellular glutathione and ultimately also the antioxidant activity. Furthermore, the newly generated NADH is utilized by the respiratory chain reaction resulting in excess generation of superoxide anion

[85]. In diabetic rats there is clear evidence of reduced proteinuria on treatment with aldose reductase inhibitor, Tolrestat [86].

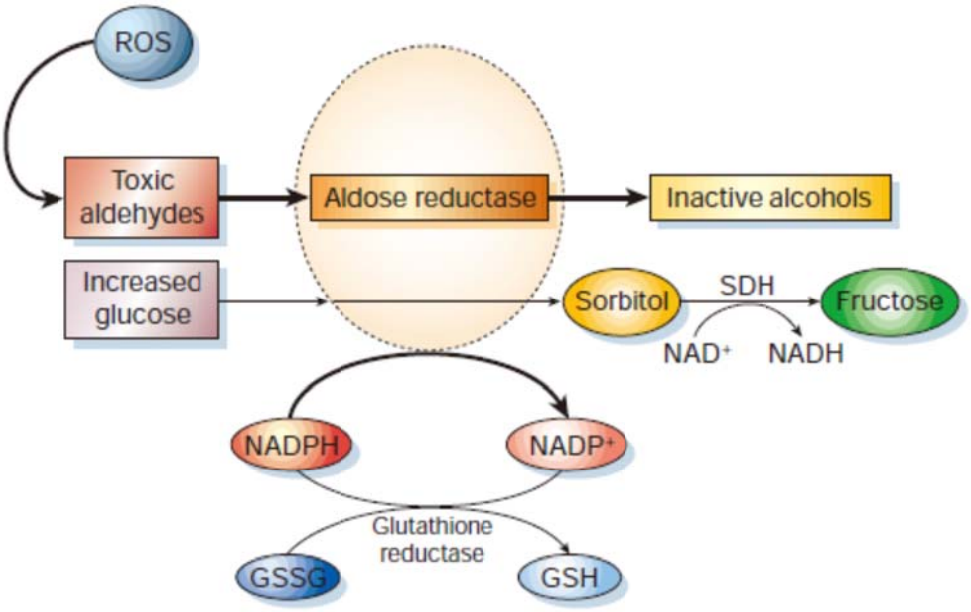

Figure 1-18. Aldose reductase and the polyol pathway [87]

1.5.1.4 Advanced Glycation End products (AGEs) and DN

AGEs are normally formed nonenzymatically by the interaction of carbonyl (aldehyde or ketone) groups of reducing sugars, such as glucose, and with lysine and N-terminal amino groups in a variety of proteins, lipids, and nucleic acids, which results in the formation of glycation products via the Maillard reaction (Figure 1-19). AGEs are involved in the development of the DN via two general mechanisms, receptor-dependent and receptor-mediated interactions [88]. The former mechanism includes modifications of various components of extracellular matrix (ECM) molecules and alterations of protein function, trafficking and breakdown [89]. Glycation of collagens leads to structural alterations,

including changes in surface charge and packing density, and results in increased collagen stiffness, reduced thermal stability, and resistance to proteolytic digestion [90]. Matrix cross-links may act as a sticky web nonspecifically trapping macromolecules and contributing to mesangial expansion. Cell-matrix interactions may also be disrupted by matrix glycation, resulting in changes in cellular adhesion [91], altered cell growth and loss of the epithelial cell phenotype [92].

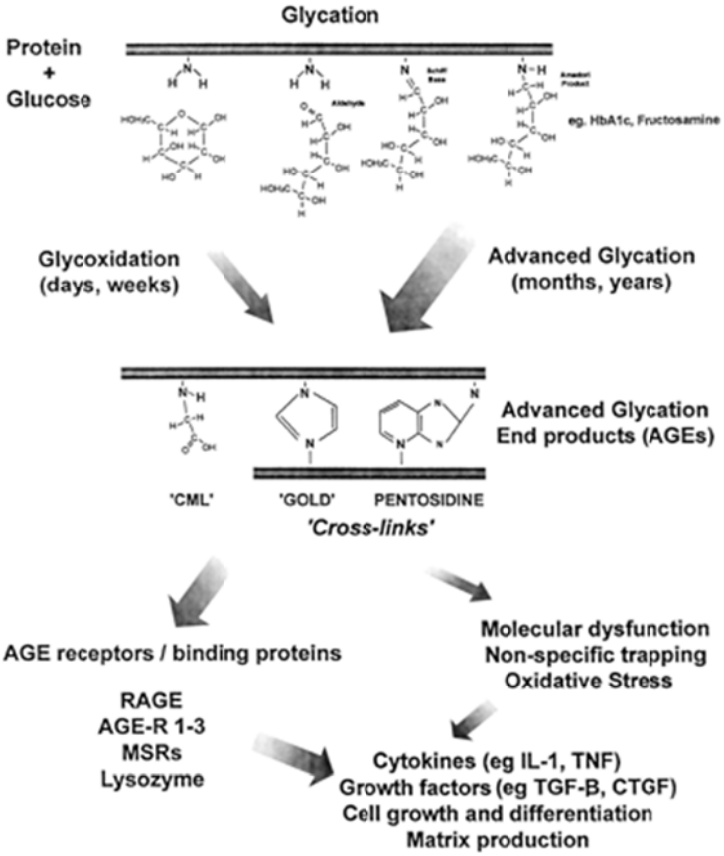

Figure 1-19. The formation of advanced glycation end products (AGEs), through a variety of mechanisms, leads to DN [93].

Although there are a number of AGE receptors, including lactoferrin, oligosaccharyl transferase complex protein 48 (AGE-R1) and 80K-H protein (AGE-R2), galectin-3 (AGE-

R3), the most well studied in the development of DN is the RAGE. The RAGE is a newly identified member of the immunoglobulin superfamily of cell-surface associated molecules [94, 95] and expressed on the surface of a variety of cell types, including smooth muscle cells, endothelia, monocytes, lymphocytes and neuronal cells [95].

1.5.1.5 Protein Kinase C (PKC) and DN

PKC, a family of serine/threonine kinases that consists of at least 15 isoforms, including PKC-α, -β1, -β2, -δ and -ε, has been documented to be activated in mesangial cells exposed to high glucose, as well as in the glomeruli of diabetic rats [96-99] (Figure 1-20).

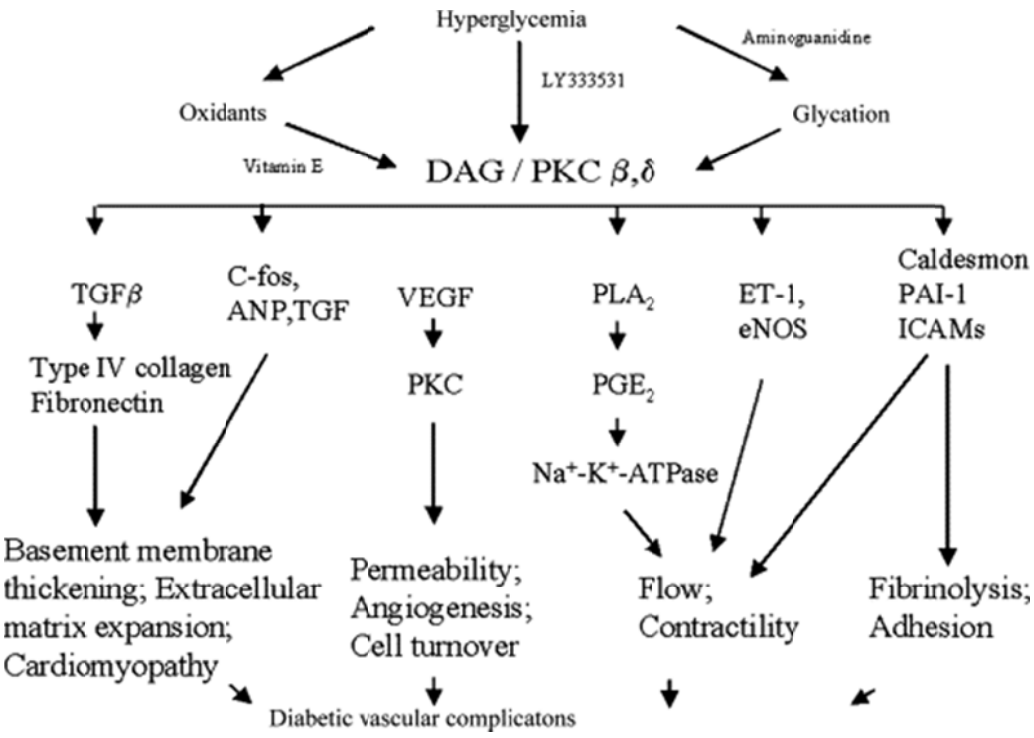

Figure 1-20. Physiological effects and cellular mechanisms of DAG–PKC activation induced by hyperglycemia [100].

In mesangial cells cultured in high concentration of glucose, enhanced fibronectin accumulation is mediated by a sustained activation of PKC and prevented by calphostin C (a potent inhibitor of PKC) [96, 97]. Some studies have demonstrated enhanced PKC activity in the glomeruli of STZ-induced diabetic rats [101, 102]. Furthermore, the PKCα isoform has been found to be significantly increased in the glomeruli, interstitial capillaries and endothelial cells of larger arteries in diabetic rats [98].

The implication of PKC causing elevated production of ECM and TGF-β is further supported by several reports showing that LY333531 (PKC*α*-selective inhibitor) prevents hyperglycemia-increased ECM production and TGF-β expression in mesangial cells [99]. Menne et al reported that protein kinase C alpha (PKC*α*)-deficient mice are resistant to the development of albuminuria under diabetic conditions [103]. They also found that PKCα is involved in reduction of nephrin surface expression and in PKCα$^{-/-}$ mice, hyperglycemia-induced downregulation of nephrin was prevented [104].

1.5.1.6 Hexosamine pathway flux and DN

Hexosamine pathway is required for proteoglycan synthesis and the formation of O-linked glycoprotein and provides the substrate, UDP-N-acetylglucosamine. When intracellular glucose is high, it enters the glycolytic pathway. The intermediate fructose-6-phosphate (Fruc-6-P) is converted to glucosamine-6-phosphate by the enzyme glutamine: fructose-6-phosphate amidotransferase (GFAT) (Figure 1-21). Glucosamine-6-phosphate is then converted to UDP-N-Acetylglucosamine. Specific O-GlcNAc transferases use this for post-translational modification of specific serine and threonine residues on cytoplasmic and nuclear proteins by O-linked N-Acetylglucosamine. O-GlcNAcylation of the transcription factor Sp1 augments

the synthesis of factors, such as TGF-β1 and plasminogen activator inhibitor-1 (PAI- 1) [87]. In addition to transcription factors, many other cytoplasmic and nuclear proteins are dynamically modified by *O*-linked GlcNAc. For example, endothelial nitric oxide synthase (eNOS) activity in arterial endothelial cells can be inhibited by O-GlcNAcylation at the Akt activation site of eNOS protein [105]. Thus, activation of the hexosamine pathway by hyperglycaemia may result in many changes in both gene expression and protein function, which collectively contribute to the pathogenesis of diabetic complications.

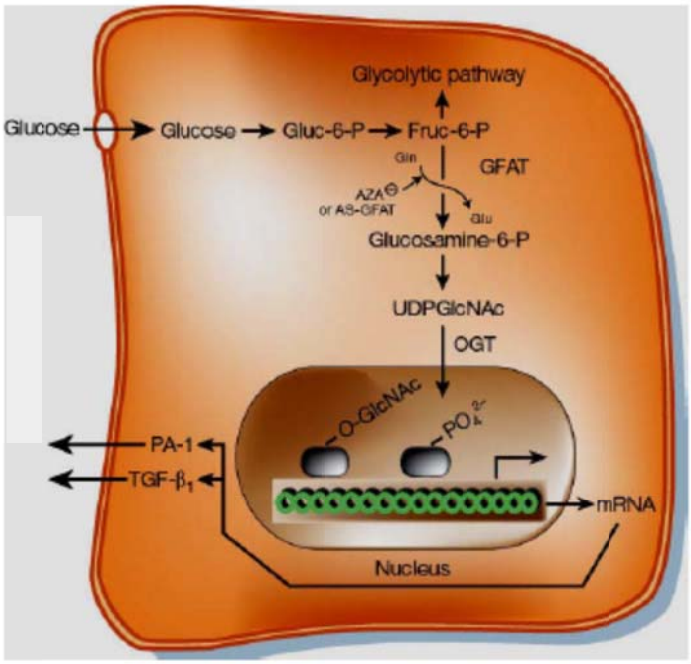

Figure 1-21. The hexosamine pathway [87]

A unified mechanism

Specific inhibitors of aldose reductase activity, PKC activation, AGE formation, and hexosamine pathway flux each ameliorate various diabetes-induced abnormalities in cell culture or animal models. Moreover, all the four abnormalities are rapidly corrected when

euglycemia is restored. Thus, it has been demonstrated that all of the 4 different pathogenic mechanisms described above stem from a single hyperglycemia-induced process, *i.e.* overproduction of superoxide by the mitochondrial electron-transport chain, which decreases the activity of the key glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [87]. When GAPDH activity is inhibited, the level of all the glycolytic intermediates that are upstream of GAPDH increase. This then increases the flux into the 4 pathways described above. ROS modifies the GADPH activity through polymers of ADP-ribose, which is made by poly (ADP-ribose) polymerase (PARP). Normally, PARP resides in the nucleus in an inactive form, awaiting DNA damage to activate it. When increased ROS induces DNA strand breaks, PARP is activated, thereby leading to the four pathway flux [105] (Figure 1-20).

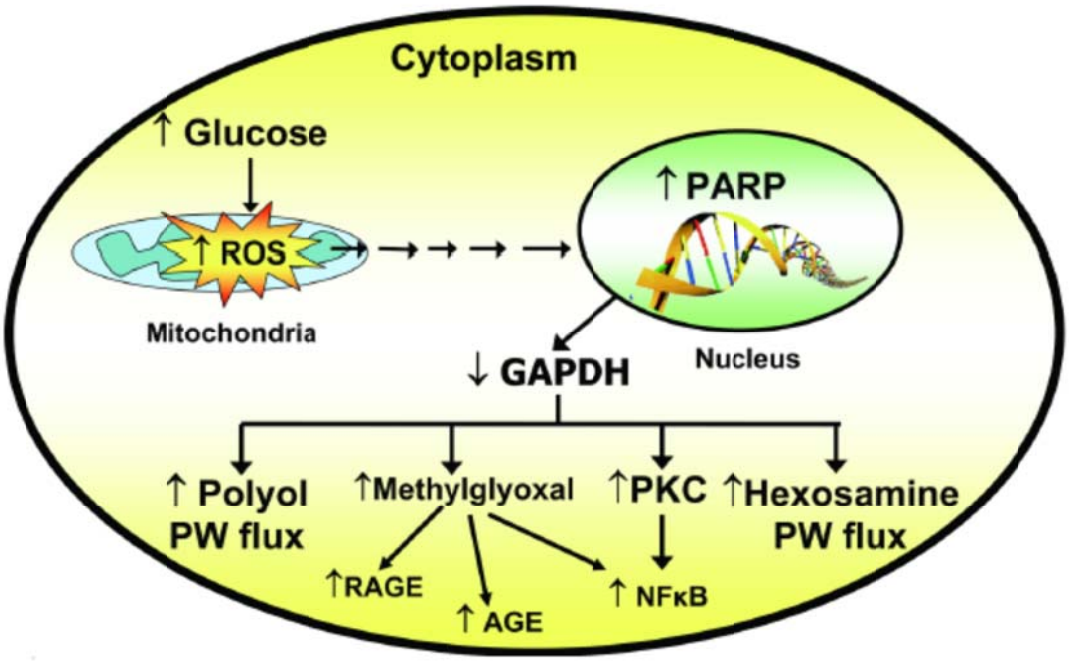

Figure 1-22. Schematic showing elements of the unifying mechanism of hyperglycemia-induced cellular damage [105]

1.5.1.7 TGF-β and DN

A characteristic feature in the biology of TGF-β s is that they are usually secreted from cells in latent forms, dimeric complex containing the C-terminal mature TGF-β and its N-terminal pro-domain, LAP (TGF-β latency associated protein) (Figure 1-23). The activation of latent TGF-β involves the disruption of the non-covalent interaction between the LAP and TGF-β, enabling TGF-β to bind to its signaling receptors [106].

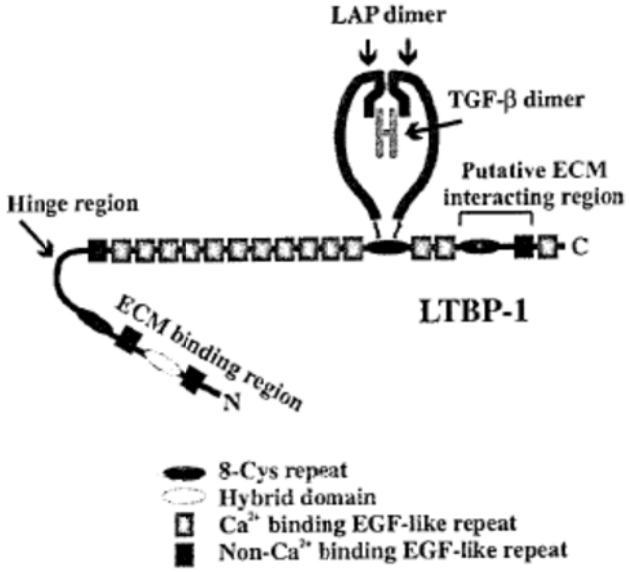

Figure 1-23. Schematic representation of the large latent TGF-β complex. The small latent complex contains the C-terminal mature TGF-β and its N-terminal pro-domain, LAP (TGF-β latency associated protein). This complex forms a disulphide-bonded complex with the third 8-Cys repeat of LTBP-1. ECM interacting regions as well as the hinge region are indicated. [106]

TGF-β initiates its cellular response by binding to its distinct receptor, TGF-β receptor II (TGF β RII). After ligand binding, the TGF-β RII activates the TGF-β RI kinase, which phosphorylates the receptor-regulated Smads (R-Smads), Smad2 and 3. The activated R-Smads form oligomeric complexes with the common Smad (Co-Smad), Smad4. These

oligomeric complexes then translocate into the nucleus and regulate the transcription of target genes by binding to DNA either directly or indirectly by interacting with various cofactors [107] (Figure 1-24).

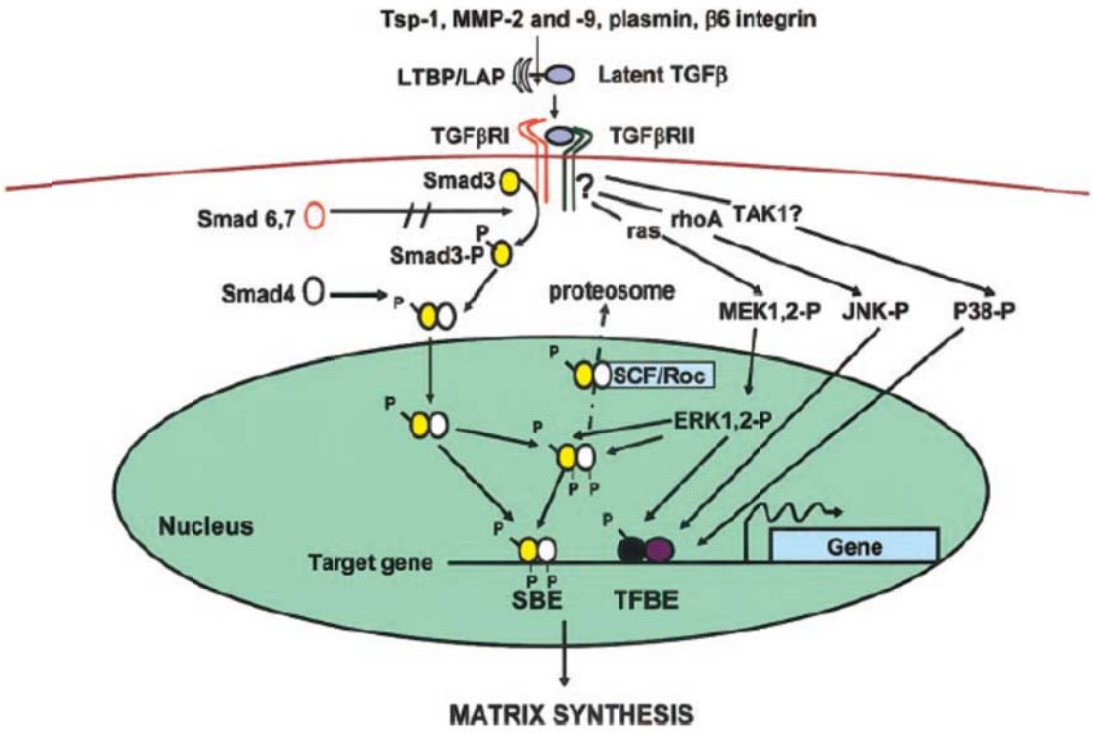

Figure 1-24. Model showing positive and negative controls on TGF-β signalling [108]. SBE: Smad binding element, TFBE: transcription factor binding element

TGF-β signalling is activated by a large number of mediators that have been identified to induce renal injury in DN. AGEs, ROS, DAG, PKC and Ang II are able to activate TGF-β signalling [108, 109]. TGF-β is recognized as the major cytokine responsible for the ECM pathobiology seen in DN and perhaps plays the most crucial role in DN pathogenesis [110].

TGF-β is one effector molecule that has been studied extensively as a major mediator of the hypertrophic and prosclerotic changes in diabetic kidney disease [111]. TGF-β1stimulates the synthesis of key extracellular matrix molecules including type IV collagen, type I collagen, fibronectin, and laminin [112]. TGF-β1 I also decreases matrix degradation by inhibiting proteases as well as activating protease inhibitors (*e.g.* Plasminogen activator inhibitor-1). In addition, TGF-β1 I promotes cell–matrix interactions by elevating integrins, the cell surface receptors for matrix [110].

1.5.2 Apoptosis and diabetic kidneys

1.5.2.1 General apoptosis pathways

Apoptosis is the nature's preprogrammed form of cell death. Apoptosis occurs normally during development, when damaged tissues are repaired, and as a homeostatic mechanism to maintain cell populations in tissues [113]. The classical understanding of apoptosis suggests that it provides a benign means for the necessary clearance of cells that are no longer needed or no longer functional. The current model holds that apoptotic cells do not elicit an immune response nor have any effect on surrounding cells.

The intrinsic apoptotic pathway is activated in response to various stimuli, such as DNA damage, hypoxia, and endoplasmic reticulum (ER) stress. This pathway is mainly regulated by the anti-apoptotic (Bcl-2, Bcl-XL), the pro-apoptotic (Bax, Bak), and the BH3-only proteins (Bad, Bid, Bim…). When Bax, Bid, and Bim are activated, they translocate to the mitochondria to induce apoptosis, either by binding to Bcl-2, Bcl-XL via BH3 domains and antagonizing their anti-apoptotic functions or through the permeabilization of the mitochondrial membrane. Permeabilization of the mitochondrial membrane releases

apoptogenic proteins, such as cytochrome c, leading to the formation of the apoptosome, to the activation of caspase 9 and ultimately to the activation of effector caspases -3 and -7 [114] (Figure 1-25).

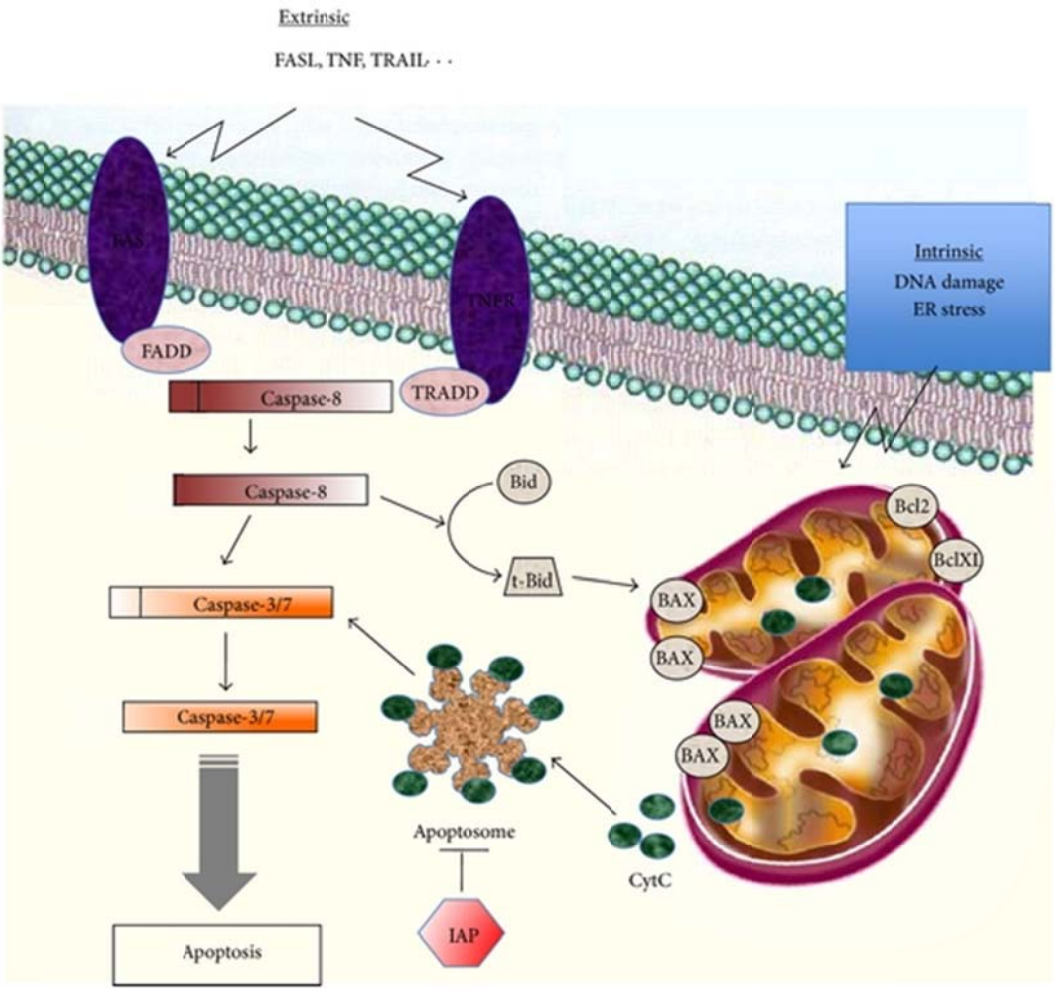

Figure 1-25. Intrinsic and extrinsic apoptosis pathways .[115]

The extrinsic pathway is initiated at the plasma membrane by specific transmembrane receptors, death receptors. For example, binding of Fas ligand (FasL) to the death receptors Fas-associated death domain (FADD) or TNFα to TNF receptor-associated death domain

(TRADD) recruits and activates caspase -8 to form a death-inducing signalling complex (DISC). DISC can transduce the death signal directly by activating the effector caspases-3 and -7, or indirectly via cleavage of Bid, which translocates to the mitochondria, leading to mitochondrial outer membrane permeabilization, subsequent release of cytochrome c, activation of caspase 9 and ultimately activation of caspase -3 and -7 [114, 115].

1.5.2.2 Apoptosis in diabetic glomeruli

Diabetes can cause the apoptosis of podocytes and mesangial cells. Susztak *et al*.[116] have shown that podocyte apoptosis increased markedly with onset of hyperglycemia in Akita mice and *db/db* mice. Podocyte apoptosis coincided with the onset of urinary albumin excretion and preceded significant podocytes loss in Akita (37% reduction) and *db/db* (27% reduction) mice. *In vitro* study demonstrated that high glucose led to activation of proapoptotic p38 MAPK and caspase 3 and to apoptosis of conditionally immortalized podocytes. They also found that ROS stimulated by high glucose play an important role upstream in this process and various inhibitors of ROS could reduce podocyte apoptosis and podocyte depletion *in vitro* and *in vivo*.

In human and murine renal mesangial cells, high glucose levels caused an increased Bax/Bcl-2 ratio, cytochrome c release from mitochondria and subsequent the proapoptotic caspase-3 activation [117, 118].

1.5.2.3 Apoptosis in diabetic tubules

Hyperglycemia by infusing 10% glucose into male Wistar rats for 300 minutes led to fragmentation in the DNA of proximal tubular cells [119]. Apoptosis has been found in renal

cortical tubular cells from STZ induced diabetic mice and in the renal cortex of diabetic *db/db* mice [120, 121]. Apoptosis was present in proximal and distal tubules as well as in interstitial cells in the biopsy specimens of human diabetic kidney [122]. *In vitro* study showed high ambient glucose downregulated the expression of the anti-apoptotic gene bcl-2 and bcl-xL and upregulated pro-apoptotic gene Bax to favor apoptosis in murine tubular epithelial MCT cells [120]. A significant apoptosis was also found in other proximal tubular cells, such as HK-2 cells and LLC-PK1 cells, when exposed to high glucose [123, 124].

1.6 The Renin-angiotensin system (RAS)

1.6.1 Systemic RASs

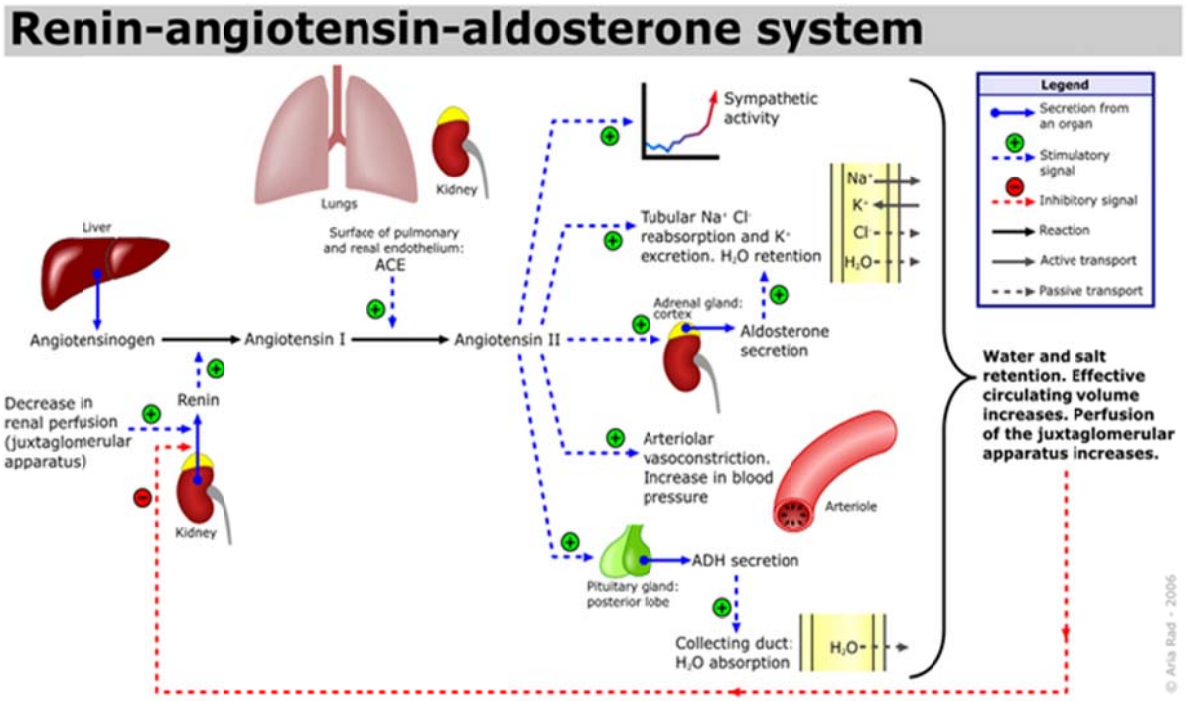

Figure 1-26. RAAS schematic [17]

Renin-angiotensin system is a hormone system that plays an important role in regulation of blood pressure, renal hemodynamics as well as fluid and electrolyte homeostasis [125]. The classical or circulating RAS consists of several major components including the precursor angiotensinogen (Agt), two critical enzymes (renin and angiotensin-converting enzyme, ACE), their bioactive product, angiotensin II (Ang II) together with its receptors (AT1 and AT2 receptors).

Generally speaking, kidneys secrete renin in response to low blood pressure, which stimulates the production of angiotensin I (Ang I) from Agt, which is synthesized from the liver. Circulating Ang I is, in turn, cleaved by the lung-derived ACE to produce Ang II, a physiologically active peptide of this system. Being a potent vasoconstrictor, Ang II elicits blood vessels to constrict, thus resulting in increased blood pressure (Figure 1-26).

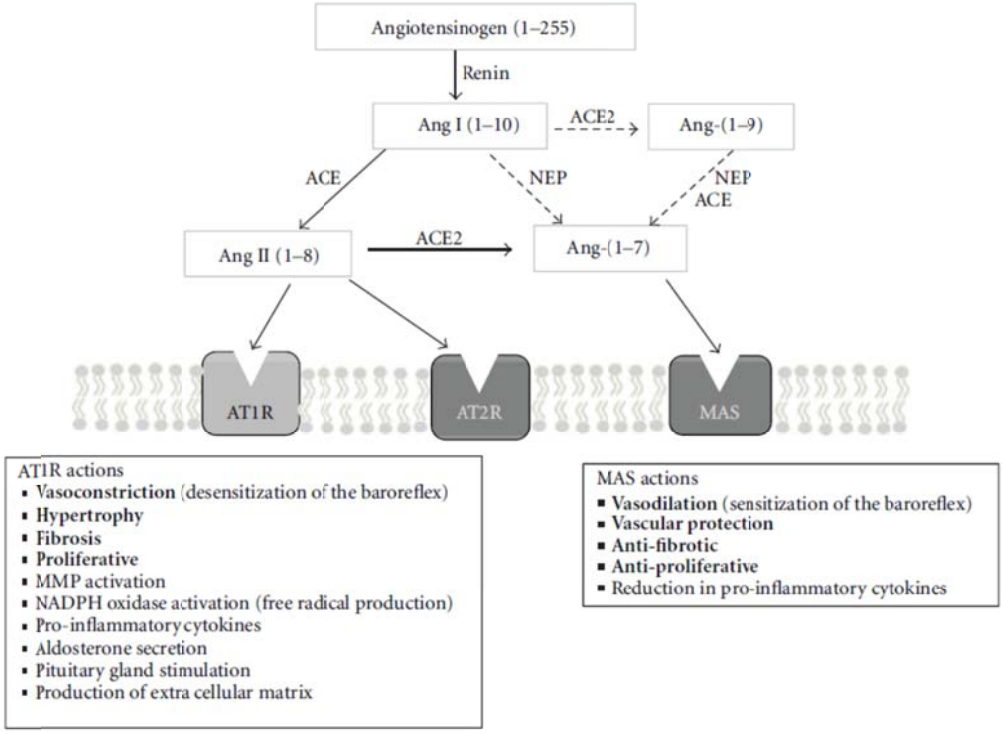

Figure 1-27. Schematic representation of ACE-Ang II-AT1R axis and ACE2-Ang-Mas axis [126]

In 2000, two independent groups [127, 128] discovered the new member of RAS, ACE2.

The main product of ACE2 is angiotensin-(1–7) (Ang 1–7), which is now recognized as a vasodilatory peptide. It has been established that Ang 1–7 acting on its own unique receptor (the Mas receptor) mediates vasodilation, antiproliferation, and apoptosis [129], therefore opposing the effects of Ang II. Thus, the new arm of the RAS, the ACE2-Ang-Mas axis, has been shown to be effective at counterregulating the effects of the classic ACE-Ang II-AT1R axis (Figure 1-27).

1.6.2 Local RASs

In parallel with the recognition of an increasing number of novel RAS components, emerging evidence has demonstrated the importance of local RAS in the brain, heart, adrenal glands, vasculature, and kidneys. In particular, the renal RAS is unique because all of the components of the RAS are present within the kidney [125].

Proximal tubular cells express both messenger RNA (mRNA) and protein for all components of the RAS [130-132] (Figure 1-28). In the lumen of the proximal tubule, Ang II levels are in the nanomolar range, which is 1,000-fold greater than those in plasma [133, 134]. In addition, the glomeruli also contain a local RAS. Components of the RAS have been detected in glomeruli in both humans and animals [135, 136] and Ang II concentrations in glomerular ultrafiltrate are significantly higher than in plasma [133, 137].

Increased intrarenal Ang II levels have been shown in several models of hypertension, including chronic Ang II infusions and two-kidney–one-clip Goldblatt hypertension [138]. Overproduction of intrarenal Ang II leads to the development of hypertension and renal injury [138, 139].

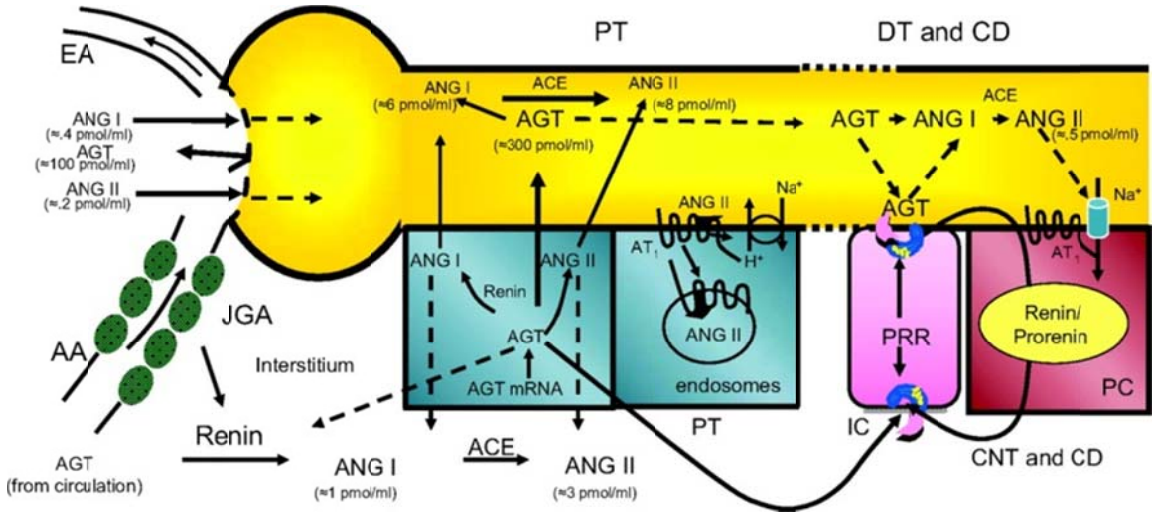

Figure 1-28. Cascade of intratubular RAS in Ang II–dependent hypertension. In Ang II–dependent hypertension, the kidney maintains intrarenal Ang II formation, enhanced proximal tubule AGT formation and spillover into distal nephron segments coupled with enhancement of CD renin and stimulation of tubular ACE. PT indicates proximal tubule; IC, intercalated cell; PC, principal cell; AA, afferent arteriole; EA, efferent arteriole.[140]

1.6.3 Intrarenal RAS activation

1.6.3.1 Animal models of RAS activation

To study the effects of RAS on the hypertension and kidney damage, genetically modified animals have been generated.

1.6.3.1.1 RAS KO mice

(a) Mice lacking renin

In the human and the rat, there is only a single renin gene. Some strains of mice, such as C57BL/6, also contain one renin gene (*Ren1*^{c}), whereas others, such as DBA2/J and 129, contain two (*Ren1*^{d} and *Ren2*) [141]. On one hand, knockout of the *Ren2* gene in 129/Ola mice

has no discernible effect on blood pressure [142]. On the other hand, knockout of *Ren1*^{d} in a two renin-gene strain results in the reduction of blood pressure in females, together with altered morphology of the macula densa of the kidney's distal tubule and with complete absence of juxtaglomerular cell granulation [143].

Knockout of Ren1 in a single renin-gene strain are hypotensive and polyuric, but heterozygotes are indistinguishable from wild-type [144].

(b) Mice lacking Agt

Angiotensinogen-deficient C57Bl/6 mice exhibited hypotension (66.9 mm Hg, compared with wild-type 100.4 mm Hg) [145], reduced survival rates of newborns, abnormal kidney morphology, and impaired blood–brain barrier function after cold injury [146]. Stec et al [147] generated a transgenic model in which exon 2 of the human Agt gene is flanked by loxP sites (hAgtflox) so that this region of the gene can be deleted by the Cre-recombinase (delivered by intravenous administration of adenovirus, which mainly infects the liver). Blood pressure decreased by 25 mm Hg from baseline by day 8 post-administration, paralleled by a significant acute decrease in circulating Agt within 5 days.

(c) Mice lacking ACE

The gene encoding ACE is composed of two homologous regions and codes for both a somatic and testis isoenzyme. Homozygous mice lacking both ACE isozymes suffer from not only low blood pressure, but also severe renal disease. For instance, the renal papilla is markedly decreased and the intrarenal arteries exhibit vascular hyperplasia associated with a perivascular inflammation. These animals cannot effectively concentrate their urine [148]. These manifestations suggest an essential role of ACE in systemic blood pressure, kidney development and function, and male fertility [148]. Krege et al [149] also created ACE-

deficient mice and found that all homozygous female mutants were fertile and males had blood pressures that were 15-20 mm Hg less than normal. Interestingly, Gonzalez-Villalobos et al [150] created a kidney-specific ACE knockout mouse and showed that the absence of intrarenal ACE can protect against hypertension. The absence of kidney ACE substantially blunts the hypertension induced by Ang II infusion (a model of high serum Ang II) or by nitric oxide synthesis inhibition (a model of low serum Ang II). Furthermore, the renal responses to high serum Ang II observed in control mice, such as intrarenal Ang II accumulation, sodium and water retention, and activation of ion transporters in the loop of Henle (NKCC2) and distal nephron (NCC, ENaC, and pendrin) as well as the transporter activating kinases OSR1 and SPAK, were effectively prevented in mice lacking kidney ACE.

(d) Mice lacking AT1RA and/or AT1RB

Whereas human possesses a single AT1 receptor, there are two AT1 receptor isoforms in rodents (AT1A and AT1B) that are products of separate genes (*Agtr1a* and *Agtr1b*). Knockout of the AT1A receptor (Agtr1a) gene in mice causes a drop in blood pressure in both heterozygotes and homozygotes [151]. Blood pressure can be further reduced in the deficient mice, by administration of losartan, suggesting that the AT1B receptor can contribute to blood pressure regulation in the absence of AT1A receptor. In the absence of AT1A receptors, the AT1B receptor contributes to the regulation of resting blood pressure [152].

Mice lacking both receptor subtypes exhibit phenotypes similar to those of mice lacking Agt, characterized by marked hypotension, low body weight gain and abnormal kidney morphology including delayed maturity in glomerular growth, hypoplastic papilla, renal arterial hypertrophy and increased early neonatal death [153].

(e) Mice lacking AT2R

The AT2 receptor targeted by gene deletion (AT2 knockout mice) has been reported by two groups in 1995 [154, 155]. Deletion of the AT2 receptor resulted in a significant increase in blood pressure and enhanced vascular sensitivity to Ang II in both models. However, AT2-deficient mice appeared to be normal, and no renal developmental anomalies were described. Moreover, these animals have normal GFR and the pressure-natriuresis relationship [156].

1.6.3.1. 2 Transgenic (Tg) mice overexpressing systemic RAS

(a) Mice overexpressing renin

The *Ren2* transgene from the 129/Ola strain of mouse was introduced into the genome of the rat and the expression of this gene causes severe hypertension by 8 weeks of age [157]. However, Tg rats harboring either the human renin gene or the human Agt gene do not develop hypertension, because of strict species specificity in the reaction between renin and Agt; therefore, human renin is unable to cleave mouse Agt and vice versa [158]. Thus, double human Tg mice were created and became a useful experimental model for studying human-specific enzyme kinetics and drug development [159].

(b) Mice overexpressing Agt

By injecting the entire rat Agt gene into the germ line of NMRI mice, the resulting Tg animals developed hypertension with a mean arterial blood pressure of 158 mm Hg in males and 132 mmHg in females. Total plasma Agt and plasma Ang II concentrations were about three times as high as those of negative control mice [160]. Using targeted gene disruption and duplication, the generated mice were genetically identical [(129 x C57BL6) F1] except for having one, two or three functional copies of the gene coding for Agt [161, 162]. Plasma

steady-state Agt levels increase progressively but not linearly with Agt gene copy number, ranging from zero in the zero-copy animals to approximately 145% normal in the four-copy animals. Blood pressures increase progressively and significantly with increases in the number of functional Agt gene copies, with a change in mean arterial pressure of approximately 8 mmHg per copy.

(d) Mice overexpressing AT1 receptor

Le et al [163] created mice with 3 and 4 copies of the *Agtr1a* gene locus on an inbred 129/Sv background. AT1A mRNA expression and AT1-specific binding of Ang II were increased in proportion to *Agtr1a* gene copy number. In female mice, but not in males, there was a highly significant positive correlation between blood pressure and AT1A receptor over-expression.

1.6.3.1. 3 Tg mice overexpressing intrarenal RAS

In additional to systemic RAS transgenic mice, animal models overexpressing intrarenal RAS have been generated. Ding et al [164] created Tg mice with human Agt gene specifically expressed in the proximal tubular cells of kidney. They employed kidney androgen-regulated protein (KAP) promoter to drive the expression of transgene.

The KAP was originally identified as an abundant 20 KDa polypeptide product derived from *in vitro* translation of mouse kidney RNA. The KAP mRNA represents the most abundant (~ 4% of the total poly(A) RNA) mRNA species that is induced by androgens in the mouse's kidney [165]. The KAP mRNA exhibited unusual sensitivity to low concentrations of the androgen-receptor complex. Using the technique of in situ hybridization with single stranded RNA probes, the renal proximal tubular cells were identified as the site of synthesis of KAP mRNA [165].

Lavoie et al [166] from the same group developed transgenic mice expressing both human renin and Agt specifically in RPTs with a modified vector, KAP2. In these mice, there was no secretion of hAgt and hRenin into the systemic circulation and plasma Ang II was not elevated. Double-transgenic female mice had a normal baseline blood pressure, which increased by 15 mmHg after 2 weeks of testosterone treatment, and returned to baseline after elimination of the testosterone pellet. This suggests that intrarenal production of Ang II can result in a systemic increase in arterial pressure. Therefore, their studies demonstrated for the first time that the intrarenal RAS can contribute to the regulation of systemic blood pressure.

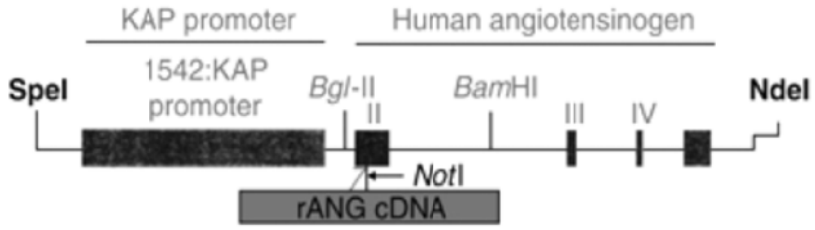

Figure 1-29. Schematic map of the kidney androgen-regulated promoter (KAP2)-rat Agt (rAgt) construct. The rAgt gene was inserted into the *Not*I sites in exon II. The transgene was excised as a SpeI and NdeI fragment for microinjection.

1.6.3.1. 4 Tg mice in the present study

Our laboratory created mice overexpressing Agt or Catalase (Cat), with the KAP2 vector. The KAP2 has a *Not*I insertion site downstream of the KAP promoter sequence, which allows for insertion of any cDNA with the *Not*I site at both ends of the cDNA. At the same time, the deletion of exon II is made that removed most of the Agt-coding potential contained in exon II, including 271 amino acids of Agt protein containing the starting codon, the secretory peptide, and the Ang I and Ang II peptides. Exons 3–5 of hAgt remain intact supplying distal

enhancer. Therefore, theoretically, replacement of the Agt coding region with any cDNA (inserted at the *Not*I site) should result in a proximal tubule-specific and androgen-regulated gene expression [167] (Figure 1-29).

1.6.4 RAS paradox

The beneficial effects of inhibitors of ACE or AT1R blockade in the prevention of diabetic renal disease imply that Ang II is a major mediator of progressive renal injury [168, 169]. However, the measurement of the circulating components of the RAS indicates their reduction in diabetic animals. [170]. This RAS paradox suggests intrarenal activation of the RAS in DN [61]. Several observations have showed that, in spite of normal or suppressed plasma renin activity, the intrarenal content of renin is increased. Early STZ-induced diabetes stimulated proximal tubule renin mRNA expression in the rat and this process was reversed with insulin therapy [171]. Our laboratory also demonstrated that rat IRPTCs stimulated by high-glucose expressed higher Agt levels, via PKC signal pathway [172].

1.6.4.1 Hemodynamic effects of Ang II

Ang II acts on AT1 receptor and exerts vasoconstriction effect. Micropuncture measures in rats infused with Ang II showed that it constricts both afferent and efferent arterioles, but has a greater effect on efferent arterioles than afferent ones. As a result, Ang II increases glomerular pressure [173, 174]. Control of glomerular hypertension by ACE inhibitors in Munich-Wistar rats prevented the development of proteinuria and structural lesions [175, 176]. In T2D patients with albuminuria, the resistance of efferent arterioles and the glomerular pressure were higher than in patients with normoalbuminuria. Treatment with cilazapril decreased

glomerular pressure and albuminuria[177]. These findings demonstrated that hemodynamic effects of Ang II play an important role in the development of DN.

1.6.4.2 Non-hemodynamic effects of Ang II

In addition to its hemodynamic effect, Ang II has multiple effects, including inducting hypertrophy and proliferation, increasing tubular reabsorption, promoting fibrosis and apoptosis.

Mesangial cell protein synthesis is stimulated by Ang II, with enhanced production of extracellular matrix proteins and elaboration of TGF-β1 [178]. In human mesangial cells, Ang II stimulated vascular permeability factor/vascular endothelial growth factor production, contributing to increased capillary permeability and proteinuria in glomerular diseases [179]. In another study, an AT1-receptor antagonist, but not the calcium channel blocker amlodipine, normalized the reduced nephrin expression in podocytes from spontaneously hypertensive rats with superimposed STZ-induced diabetes, suggesting Ang II in diabetes leads to suppression of nephrin protein expression in the podocyte slit diaphragm [180, 181].

In our laboratory, we created transgenic mice specifically overexpressing Agt in proximal tubular cells, where intrarenal Ang II was overproduced. Ang II overexpression led to tubular hypertrophy, tubular fibrosis, albuminuria and tubular apoptosis [182].

1.6.5 ACE2/ Ang 1-7/MAS axis

1.6.5.1 ACE2

Human and rodent ACE2 are similar proteins containing 805 amino acids, which include an N-terminal signal sequence, a single active-site catalytic region, and a C-terminal membrane

anchor segment [128]. ACE2 functions predominantly as a carboxymonopeptidase with a substrate preference for hydrolysis between proline and a hydrophobic or basic C-terminal residue [183]. The ACE2 gene maps to the human X chromosome. ACE2 shows 42% sequence identity with the catalytic domain of ACE [127, 128] (Figure 1-30). However, it is not sensitive to ACE inhibitor [128]. ACE2 is localized in a wide variety of tissues, including heart, kidneys, testes, liver and brain [128, 184, 185].

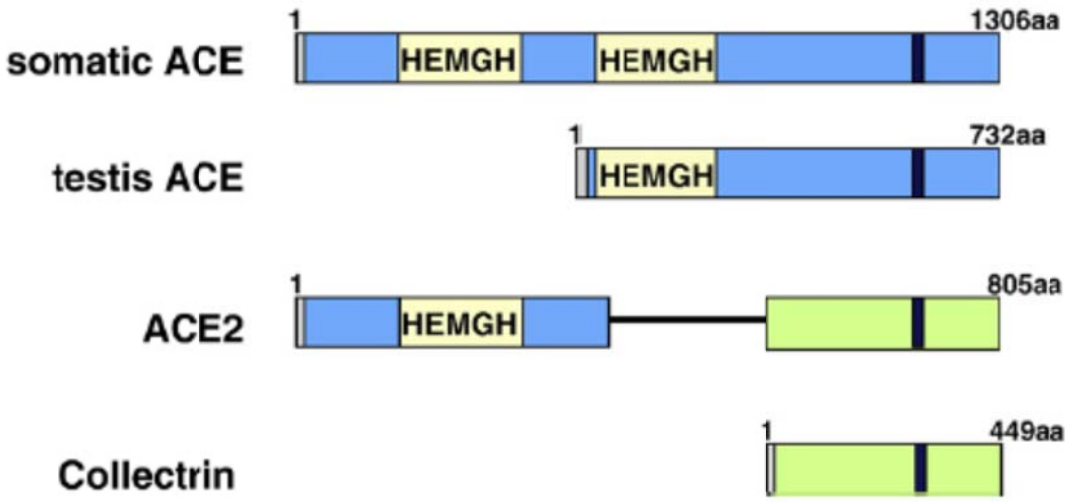

Figure 1-30. Domain structures of ACE, ACE2 and Collectrin. Each protein is a type I integral protein with a signal peptide, depicted in gray, and a transmembrane domain shown in black. The zinc-binding motif (HEMGH) repeats two times in ACE and once in ACE2, and is located within the homology region denoted by the yellow box. Regions of homology between ACE2 and Collectrin are denoted in green. The numbers refer to the amino acids in each human protein.[186]

In mouse kidneys, ACE and ACE2 are co-localized in the brush border of proximal tubules, but within the glomerulus these two enzymes are localized in distinct structures. Glomerular ACE2 is mainly present in podocytes and, to a lesser extent, in glomerular mesangial cells, whereas glomerular ACE is only present in endothelial cells [187]. In human kidneys, the

pattern of ACE2 expression is similar to that of mouse kidneys. In kidneys from healthy control subjects, Lely et al. [188] found ACE2 expression in tubular, glomerular visceral and parietal epithelial cells, and in vascular muscular smooth muscle cells and the endothelium of interlobular arteries as well.

In rat models of hypertension, renal ACE2 mRNA and protein are decreased [189]. Koka et al [190] observed low ACE2 levels in human hypertensive kidneys compared with normal human kidneys. Renal expression of ACE2 is downregulated in murine models of diabetes and in diabetic patients [187, 191-193].

Administration of human recombinant ACE2 to diabetic Akita mice significantly reduced albuminuria, associated with decreased blood pressure [194]. Similarly, intravenous administration of recombinant adenovirus carrying the mouse ACE2 gene to rats with STZ-diabetes was associated with diminished albuminuria and glomerulosclerosis, although systolic BP was also reduced [195]. Nadarajah et al [196] overexpressed ACE2 in podocytes in experimental diabetic nephropathy using Tg methods where a nephrin promoter drove the expression of human ACE2. After STZ-induced diabetes, ACE2 Tg mice are protected against the early development of albuminuria and show partial preservation of podocyte proteins and podocyte number, attenuated glomerular histological injury, and suppressed kidney cortical TGF-β1 expression. In 2009, a phase I clinical trial looking at recombinant ACE2 administration in humans (NCT00886353) was completed, demonstrating that administration of rhACE2 was well tolerated by healthy human subjects [197].

ACE2-deficient mice are viable as well as fertile, and are characterized by normal cardiac function and plasma levels of Ang II [198, 199]. However, baseline blood pressures of ACE2-deficient mice vary according to genetic strain (Table 1-3). In C57BL/6 mice, ACE2 knockout

is associated with a modest increase in blood pressure, whereas the deficiency of ACE2 has no effect on baseline blood pressures in 129/SvEv mice [198]. Deletion of the ACE2 gene in mice also causes late glomerulosclerosis and increased albuminuria [200]. These effects are reversed by treatment with the AT1 receptor antagonist irbesartan, suggesting that ACE2 deficiency causes renal injury via impaired degradation of Ang II and subsequent activation of AT1 receptors. Nevertheless, the potential independent effect of reduced Ang 1-7 generation on renal function in the ACE2 knockout mice has not been studied.

Table 1-3. Baseline cardiovascular phenotypes in ACE2 knockout mice [201]

	ACE2 mouse line				
Phenotype	Crackower *et al.*	Yamamoto *et al.*		Gurley *et al.*	
Genetic background	Mixed	Mixed	Mixed	129/SvEv	C57BL/6
Cardiac systolic function	Impaired	Normal	Normal	Normal	Normal
Heart weight	Normal	Normal	Normal	Normal	Normal
Blood pressure	Decreased	Normal	Variable	Normal	Increased
Plasma angiotensin II	Increased	Normal	Normal	n.d.	n.d.
Cardiac angiotensin II	Increased	Normal	n.d.	n.d.	n.d.
Renal angiotensin II	Increased	n.d.	n.d.	n.d.	n.d.

n.d., not determined.

1.6.5.2 Ang 1-7

Ang 1-7 is a biologically active heptapeptide that has been postulated to counterbalance the physiological actions of Ang II within the RAS. *In vivo* detection of Ang-1–7 was first reported in rat brain, adrenal and plasma in 1989 by Chappell et al.[172]. Several observations have demonstrated the generation of intrarenal Ang 1-7. The intrarenal levels of Ang 1-7 are comparable to levels of Ang II [202]. Furthermore, Ang 1-7 can be detected in the urine from experimental animals and human subjects [203, 204]. Untreated patients with essential

hypertension present decreased amounts of Ang 1–7 in the urine when compared with healthy volunteers [204].

In the nonrenal vasculature, Ang 1-7 exerts a vasodilatory effect by increasing the production of nitric oxide (NO), prostaglandins or endothelium-dependent hyperpolarizing relaxing factor [173, 176, 177]. Sampaio et al. [205] revealed the molecular basis for the NO-releasing activity of Ang 1-7. Using human aortic endothelial cells and Chinese hamster ovary cells stably transfected with Mas cDNA, they demonstrated that Ang 1-7 stimulates eNOS activation and NO production via Akt-dependent pathways. These effects were blocked by the Ang 1-7 receptor antagonist A-779, suggesting the involvement of Mas receptor signalling pathways.

Van der Wouden et al [175] studied the effects of Ang 1-7 on the renal vasculature *in vitro* and *in vivo*. They found that Ang 1-7 alone had no effect on the renal vasculature in any of the experiments, but antagonized Ang II in renal vessels *in vitro*. Ren et al showed that Ang 1-7 caused afferent arteriole dilatation due to the production of NO [206]. Infusion of Ang 1-7 into spontaneously hypertensive rats (SHR) normalizes systolic blood pressure [207]. In Wistar-Kyoto rats and SHR, Ang 1-7 has a regulatory role in the kidney vasculature including the attenuation of the contractile effect of Ang II, which is blocked by antagonism of the Mas receptor, cyclo-oxygenase inhibition, or NOS inhibition [208].

Antidiuretic effects of Ang 1–7 are associated in male Wistar rats with increases in urinary Na^+ concentration, urinary osmolality, and reduction in creatinine clearance. These effects are blocked by administration of A-779 or losartan [209]. In proximal tubular cells, Ang 1-7 inhibits sodium flux, an effect associated with activation of phospholipase A2 [210] and inhibits the ouabain-sensitive Na^+/K^+-ATPase [211].

In addition to its effects on renal hemodynamics and tubular transport, Ang1-7 may regulate cell growth in the kidney. In the proximal tubule, Ang 1-7 exhibits growth inhibitory properties and antagonizes the effects of Ang II and high glucose. It can inhibit Ang II–stimulated phosphorylation of three mitogen-activated protein kinases (MAPK) and production of TGF-β1 [212] (Figure 1-31).

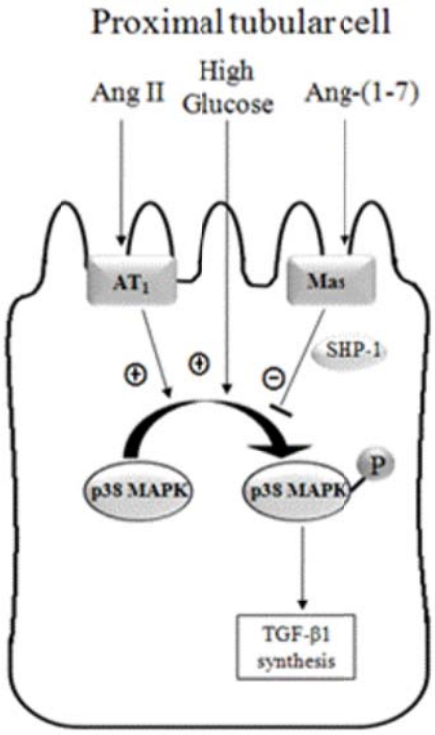

Figure 1-31.Signaling pathways for Ang 1-7 in proximal tubule [195]

1.6.5.3 Mas receptor

Mas receptor was discovered in 1986 and was originally described as a proto-oncogene [213]. Subsequent studies, however, revealed that the transforming activity could not be confirmed and may have been due to a unique genetic rearrangement in the transformed cells which may

have affected the imprinting of the neighboring genes [171, 214]. In 2003, Santos et al. finally identified Mas as a functional receptor for Ang 1-7 [169].

The analysis of the human *MAS* cDNA sequence revealed an open reading frame that codes for a 325-amino-acid protein [213]. The protein consists of seven membrane-spanning α-helices and hydrophilic N- and C-terminal ends. Mas is highly conserved except in its hydrophilic amino-terminal domain, with mouse and rat homologues sharing 97% identity, compared with 91% between the mouse and human forms [215].

Mas is ubiquitously expressed in many tissues, including kidney, heart, vasculature, brain, liver, spleen, testis and lung [215]. Within the kidney, Mas is localized in afferent arterioles, and also in proximal tubules, collecting ducts and the thick ascending limb of Henle's loop. It is detected within glomeruli from rat, but not from sheep [215]. In human mesangial cells, Mas protein expression has been detected by immunoblot [216].

The phenotype of the Mas knockout mouse appears to be highly dependent on strain. Mas-deficient mice on the mixed 129XC57BL/6 genetic background are healthy, grow normally, and show no apparent developmental anomaly [163]. Indeed, these mice are normotensive and display normal plasma levels of Ang II [163]. However, Mas knockout mice on a pure C57BL/6 genetic background exhibit impaired cardiac function that is partially due to an increase in collagen expression to a profibrotic phenotype [166]. In addition, C57BL/6 Mas knockout mice exhibit a renal phenotype that is characterized by sodium and water retention, glomerular hyperfiltration, microalbuminuria, and renal fibrosis [164]. Mas-deficient mice on the FVB/N genetic background display a cardiovascular phenotype that is characterized by increased arterial blood pressure, lower eNOS expression, reduced NO production, and impaired endothelial function, compared to wild-type mice [217]. In addition, the NADPH

oxidase catalytic subunit gp91 (phox) protein is expressed at a higher level in Mas-deficient mice compared to wild-type, whereas superoxide dismutase and catalase activities are reduced [217].

1.6.6 Clinical trials of RAS blockade in diabetic patients

RAS system plays a crucial role in the pathophysiology of DN. Clinical trials have established the efficacy of ACEi and ARBs in reducing the progression of DN. In addition to a reduction in systemic blood pressure, RAS blockade reduces proteinuria, thus slowing progression of DN [218].

The benefits of an ACEi in T1D diabetes can be demonstrated early in the course of the disease when microalbuminuria is the only clinical manifestation. The administration of captopril to 235 normotensive T1D patients with microalbuminuria decreased both albumin excretion and, at two years, progression to overt diabetic nephropathy when compared with patients treated with placebo [219, 220]. This effect is partly independent of its blood pressure-lowering effects [220]. A more pronounced benefit was demonstrated in the largest trial to date in T1D patients who already had overt nephropathy [221, 222]. Captopril treatment was associated with a 50% reduction in the risk of the combined end points of death, dialysis and transplantation independent of the small disparity in blood pressure between the groups [222].

In T2D, two major trials, IDNT (Irbesartan in Diabetic Nephropathy Trial) and the RENNAL (Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan), compared ARBs to placebo and demonstrated that ARBs are effective in reducing proteinuria and the incidence of a doubling of the plasma creatinine [223-225]. The ADVANCE trial (the Action

in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation) compared the use of a fixed combination of ACE inhibitor perindopril and the diuretic indapamide to placebo in over 11,000 patients with type 2 diabetes [226]. After a mean of 4.3 years, the patients treated with active therapy had a significant reduction in the rate of new onset of microalbuminuria (19.6 versus 23.6 percent) and in the combined endpoint of new onset of microalbuminuria, worsening of microalbuminuria, or new onset or worsening of macroalbuminuria [227].

1.7 Animal models of DN

An ideal animal model would reproduce most or all of the lesions of human DN [228]. In 2005, the nephropathy subcommittee of the Animal Models of Diabetic Complications Consortium (AMDCC) published an account of phenotyping standards, validation criteria, and observations about currently available murine strains and models of DN [229].

STZ is a compound that has a preferential toxicity toward pancreatic β cells because its glucose moiety is avidly transported into β cells by GLUT2. After it enters the β cells, STZ can damage pancreatic β cells, leading to hypoinsulinemia and hyperglycemia. STZ-treated models develop a modest degree of proteinuria and serum creatinine increase, as well as minimal mesangial matrix expansion, depending on the genetic background [230]. However, interpreting results in this model may be complicated by nonspecific toxicity of STZ. To circumvent this potential issue, one may use the insulin-2 *Akita* ($Ins^{2+/C96Y}$) mouse mutant model of T1D [231]. This point mutation causes misfolding of the insulin protein, toxic injury to pancreatic β cells, and impaired capacity to secrete insulin, resulting in T1D [232]. The mice are commercially available through the Jackson Laboratories.

Table 1-4. Selected mouse models of diabetes studied for DN [229]

Mouse model	Diabetes type	Advantages	Disadvantages
Streptozotocin	Type 1	Well-established, reproducible, timed; may be established in strains both resistant and susceptible to DN	Potential for nonspecific toxicity; strain-dependent dosing necessary; biohazard: potential mutagen
Ins2 Akita	Type 1	Commercially available (JAX); autosomal dominant mutation	Presently only C57BL/6 commercially available; C57BL/6 relatively resistant to nephropathy; hyperglycemia in females is mild
NOD Type	Type 1	Spontaneous development of β-cell failure may mimic pathophysiology of the disease in humans; available commercially	Unpredictable timing of diabetes development; no appropriate control strain; needs insulin therapy to survive long periods; multigenetic cause of diabetes precludes easy intercrosses
Db/db	Type 2	Available on multiple strains; available commercially	Infertile; autosomal recessive; mutation in leptin receptor is a very rare cause of obesity and T2D in humans
Ob/ob	Type 2	Exists in diverse inbred strains; available commercially	Infertility-can be circumvented with leptin; autosomal recessive; nephropathy uncharacterized; mutation of leptin is a very rare cause of obesity and T2D in humans
High-fat diet	Type 2	Onset can be determined by the investigator	Only C57BL/6 reported to be susceptible; hyperglycemia not prominent

The db/db mouse model is currently the most widely used mouse for DN in settings of T2D and exhibits many features similar to human DN. The *db* gene encodes for a G-to-T point mutation of the leptin receptor, resulting in abnormal splicing and defective signalling of the leptin [233]. The mice become obese around 3 to 4 weeks of age. Elevations of plasma insulin begin at 10 to 14 days and of blood sugar at 4 to 8 weeks [234]. After 16 weeks of age, there is a very consistent threefold increase in mesangial matrix expansion[235]. DN in these mice is characterized by albuminuria, podocyte loss, and mesangial matrix expansion [236]. Another T2 DN model, the *ob/ob* recessive obese mouse carries a mutation in leptin, the ligand for the leptin receptor. Renal lesions and function change in C57BL/6J *ob/ob* mice are said to be relatively mild [229]. High-fat diet induced obesity and insulin resistance in C57BL6 mice provide a commonly used approach to study T2 DN. However, hyperglycemia is not prominent in this model [229] (Table 1-4).

1.8 Objectives of the present study

Rationale: DN is the leading cause of all ESRD in North America [13]. Although there is no question that there are changes seen in the glomerulus in patients with DN, it is also generally accepted that tubulointerstitial changes are a prominent component of the disease [237]. Tubulointerstitial fibrosis and tubular atrophy may be a better predictor of renal disease progression than glomerular pathology [238].

Activation of RAS plays a critical role in the progression of DN, as can be seen in the effectiveness of ACEi and ARB on the DN [168]. The existence of a local intrarenal RAS is now well accepted and human and murine RPTCs express all components of the RAS [131,

136]. Our laboratory has reported that Tg mice that specifically overexpress Agt, the sole precursor of angiotensins in RPTCs, develop hypertension, albuminuria, and tubular apoptosis [239, 240]. Furthermore, Agt overexpression enhances tubular apoptosis in STZ-induced diabetic mice [182]. These findings indicate that intrarenal RAS activation and hyperglycemia act in concert to enhance hypertension and RPTC apoptosis in diabetes.

Enhanced ROS generation also has been implicated in the progression of DN [241]. Ang II is a potent stimulator of ROS production by heightened NADPH oxidase activity in mesangial cells and RPTCs [242, 243]. Cat, an antioxidant enzyme, converts H_2O_2 to water and oxygen and thus mitigates the toxic effects of H_2O_2. To study the mechanism (s) of Ang II action on ROS generation and kidney-dependent hypertension, double-transgenic mice specifically overexpressing Agt and Cat in their RPTCs were created in our laboratory. Overexpression of Cat prevented hypertension and renal injury in the Agt Tg mice [244]. Another animal model, db/db catalase Tg mouse, indicated a crucial role for intrarenal ROS in the progression of hypertension, albuminuria, interstitial fibrosis, and tubular apoptosis in T2D diabetes [245]. Thus, in the diabetic milieu, both glycemia and Ang II can induce ROS generation and these 3 factors play important roles in the development of hypertension and DN.

ACE2 shares 40–42% homology in the catalytic domain with ACE, although ACE2 is non-sensitive to ACEi [128]. ACE2 specifically cleaves Ang II into heptapeptide Ang 1–7, which acts on the membrane-bound MasR [169]. Ang 1–7 opposes many Ang II-mediated actions, including vasoconstriction, proliferation, hypertrophy and fibrosis [246]. Recently, administration of recombinant human ACE2 was demonstrated to attenuate Ang II-dependent and pressure overload-induced hypertension and myocardial remodeling as well as renal injury

in ACE2 KO mice [247-249], indicating an important counterregulatory role of ACE2 in Ang II-induced heart and kidney disease.

Our laboratory has recently observed that renal ACE2 expression and urinary Ang 1–7 were lower in T1D Akita mice and that dual RAS blockade normalizes ACE2 expression, prevents hypertension and ameliorates tubular fibrosis and apoptosis in Akita Agt-Tg mice [250]. The molecular mechanism(s) by which RAS blockade normalizes renal ACE2 expression, however, remains undefined. Furthermore, no one has reported how ROS and Cat regulate ACE2 in Akita mice till now. Therefore, the objectives of the present studies are to investigate:

1) Whether oxidative stress is involved in the development of hypertension and nephropathy in Akita mice via downregulation of renal ACE2 expression.

2) Whether Ang 1-7 is involved in the prevention of the development of hypertension and kidney injury in diabetic Akita mice.

Chapter 2: Article 1

Overexpression of Catalase Prevents Hypertension and Tubulointerstitial Fibrosis and Normalization of Renal Angiotensin-Converting Enzyme-2 Expression in Akita Mice

Am J Physiol Renal Physiol 304: F 1335-1346, 2013

Overexpression of Catalase Prevents Hypertension and Tubulointerstitial Fibrosis and Normalization of Renal Angiotensin-Converting Enzyme-2 Expression in Akita Mice

Yixuan Shi[1,4], Chao-Sheng Lo[1,4], Isabelle Chenier[1], Hasna Maachi[1], Janos G. Filep[2], Julie R. Ingelfinger[3], Shao-Ling Zhang[1†], and John S D Chan[1†]

[1]Université de Montréal
Centre de recherche
Centre hospitalier de l'Université de Montréal (CRCHUM)
Hôtel-Dieu Hospital
Pavillon Masson
3850 Saint Urbain Street
Montreal, Quebec
Canada H2W 1T8

[2]Université de Montréal
Centre de recherche
Maisonneuve-Rosemont Hospital
5415 boul. de l'Assomption
Montreal, Quebec
Canada H1T 2M4

[3]Harvard Medical School
Pediatric Nephrology Unit
Massachusetts General Hospital
15 Parkman Street, WAC 709
Boston, MA 02114-3117
USA

[1†] John S.D. Chan and Shao-Ling Zhang are joint senior authors
To whom correspondence should be addressed: John S. D Chan
Telephone: (514) 890-8000 ext. 15080; Fax: (514) 412-7204

[4]These authors contributed equally to this work
Running Title: Oxidative Stress and Ace2 in Diabetic Mice
Keywords: Catalase, Hypertension, Angiotensin-converting enzyme-2, Tubulointerstitial Fibrosis
Subject categories: Pathophysiology of renal disease and progression
Abstract: 243 words; Text: 3,889

2.1 Abstract

We investigated the relationship between oxidative stress, hypertension, renal injury and angiotensin-converting enzyme-2 (Ace2) expression in type 1 diabetic Akita mice. Blood glucose, blood pressure and albuminuria were monitored for up to 5 months in adult male Akita and Akita catalase (Cat) transgenic (Tg) mice specifically overexpressing Cat, a key antioxidant enzyme in their renal proximal tubular cells (RPTCs). Same-age non-Akita littermates and Cat-Tg mice served as controls. In separate studies, adult male Akita mice (14 weeks) were treated with Ang 1-7 (500 $\mu g/kg^{-1}.day^{-1}$, subcutaneously) ± A-779, an antagonist of Mas receptor (10 $mg/kg^{-1}.day^{-1}$) and euthanized at the age of 18 weeks. The left kidneys were processed for histology and apoptosis studies. Renal proximal tubules were isolated from the right kidneys to assess protein and gene expression. Urinary angiotensinogen (Agt), angiotensin II (Ang II) and Ang 1-7 were quantified by specific ELISAs. Overexpression of Cat attenuated renal oxidative stress, prevented hypertension, normalized RPTC Ace2 expression and urinary Ang 1-7 levels (both were low in Akita mice), ameliorated glomerular filtration rate, albuminuria, kidney hypertrophy, tubulointerstitial fibrosis and tubular apoptosis, and suppressed profibrotic and proapoptotic gene expression in RPTCs of Akita Cat-Tg mice compared with Akita mice. Furthermore, daily administration of Ang 1-7 normalized systemic hypertension in Akita mice, which was reversed by A-779. These data demonstrate that Cat overexpression prevents hypertension and progression of nephropathy and highlight the importance of intrarenal oxidative stress and Ace2 expression contributing to hypertension and renal injury in diabetes.

2.2 Introduction

Oxidative stress is implicated in the progression of diabetic complications. High glucose (HG, 25 mM D-glucose) induces the generation of reactive oxygen species (ROS) that contribute to apoptosis in podocytes, mesangial and tubular cells [1-3]. Angiotensin II (Ang II) also stimulates ROS generation via heightened nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase activity in various kidney cell types, whereas antioxidants provide renal protection, in part by ameliorating oxidative stress [4-6]. Taken together, these data strongly link oxidative stress, RAS activation and kidney injury to diabetes.

Human and murine renal proximal tubular cells (RPTCs) express all RAS components [7-10]. In the kidneys, angiotensinogen (Agt, the sole substrate of all angiotensins) is expressed predominantly in RPTCs and sequentially cleaved by renin and angiotensin converting enzyme (ACE) to yield active Ang II. Ang II is then cleaved by angiotensin converting enzyme-2 (Ace2) yielding angiotensin 1-7 (Ang 1-7), which has opposing actions to Ang II (See recent reviews, references [11, 12]).

Ace2 shares 40-42% homology with ACE but possesses distinct and different biochemical activities [13, 14]. Ace2 specifically cleaves Ang I and Ang II to generate Ang 1-9 and Ang 1-7, respectively. However, Ace2 is 400-fold more effective cleaving Ang II than Ang I, resulting in predominant Ang 1-7 formation [15, 16]. The identification of Mas as a receptor for Ang 1-7; genetic deletion of the Mas receptor leads to kidney injury [17], which established Ang 1-7 as a part of the RAS cascade. Recombinant human Ace2 administration attenuates Ang II-dependent and pressure-overload-induced hypertension, myocardial remodeling and renal injury in Ace2 knockout mice [18-20] supporting an important counterregulatory role for Ace2 in Ang II-mediated cardiac and renal pathology.

We previously examined the role of RAS components in renal injury, reporting for example, that transgenic (Tg) mice specifically overexpressing rat Agt in their RPTCs develop hypertension, albuminuria and kidney injury [21, 22] Further, in diabetic mice, hyperglycemia and intrarenal Agt overexpression act together to increase hypertension and kidney injury [23]. We also recently observed that renal Ace2 expression and urinary Ang 1-7 were lower in type 1 diabetic Akita mice and that treatment with RAS blockers normalized Ace2 expression and prevented hypertension development in these Akita mice [24]. The molecular mechanism(s) by which RAS blockade normalizes renal Ace2 expression, however, remains undefined.

Our present study investigated whether oxidative stress is involved in the development of hypertension and nephropathy in Akita mice via down-regulation of renal Ace2 expression. We used a cDNA encoding catalase (Cat), an antioxidant enzyme that by converting hydrogen peroxide (H_2O_2) to water and oxygen mitigates the toxic effects of H_2O_2. In the kidneys, Cat is localized to the cytoplasm of RPTCs and is not detectable in other parts of the nephron [25]. For these reasons, we created Akita Cat-Tg mice that overexpress rat Cat selectively in their RPTCs by cross-breeding heterozygous Akita mice with our established homozygous Tg mice overexpressing rat Cat in RPTCs [26]. Here we report that overexpression of Cat in RPTCs of Akita mice normalizes renal Ace2 expression and urinary Ang 1-7 levels, prevents hypertension, attenuates glomerular filtration rate (GFR), renal hypertrophy, tubulointerstitial fibrosis and tubular apoptosis, and suppresses profibrotic and apoptotic gene expression. Furthermore, daily administration of Ang 1-7 normalized systemic hypertension in Akita mice, which was reversed by the Mas receptor antagonist A-779.

2.3 RESEARCH DESIGN AND METHODS

Chemicals and Constructs

Ang 1-7 and the mas receptor antagonist, A-779 (d-Ala^{-7}-Ang I/II/1-7) were purchased from Bachem (Torrence, CA, USA). The following antibodies were used: bovine Cat polyclonal antibody and β-actin monoclonal antibody (Sigma-Aldrich Canada Ltd. ,Oakville, ON, Canada), polyclonal anti-heme oxygenase-1 (HO-1) (Assay Designs, Ann Arbor, MI, USA), monoclonal anti-collagen type IV antibody (Chemicon International, Temecula, CA, USA), polyclonal anti-TIM-1/KIM-1/HAVCR, anti-hLAP/TGFb1 and anti-Ace2 antibody (R&D Systems, Minneapolis, MN, USA), polyclonal anti-transforming growth factor-beta 1 (TGF-β1), monoclonal anti-aquaporin-1 (AQP-1), and anti-ACE antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). A rabbit polyclonal antibody against rAgt was generated in our lab (J.S.D.C.) [27]. This antibody is specific for intact rat and mouse Agt (55-62 kDa) and does not cross-react with pituitary hormone preparations or other rat or mouse plasma proteins. pKAP2 plasmid containing the kidney-specific androgen-regulated protein (KAP) promoter responsive to testosterone stimulation was a gift from Dr. Curt Sigmund (University of Iowa, Iowa, IA, USA) [28]. Oligonucleotides were synthesized by Invitrogen (Burlington, ON, Canada). Restriction and modifying enzymes were purchased from Invitrogen, Roche Biochemicals (Dorval, QC, Canada) or GE Healthcare Life Sciences (Baie d'Urfé, QC, Canada).

Generation of Akita Transgenic Mice Overexpressing Rat Cat

Tg mice (C57Bl/6 background) that overexpress rat Cat-HA (HA-tag, a sequence encoding amino acid residues 98-106 (YPYDVPDYA) of human influenza virus hemagglutinin)

in their RPTCs (line #688) driven by the KAP gene promoter were created in our lab (J.S.D.C.) and have been described elsewhere [26]. Homozygous Cat-Tg mice were then crossed with heterozygous Akita mice (C57BL/6-Ins2^{Akita}/J, Jackson Laboratories, Bar Harbor, ME, USA ((http://jaxmice.jax.org); N.B., homozygous Akita mice are infertile). Breeding was continued until Akita Cat-Tg mice were obtained. These mice are homozygous for the Cat transgene but heterozygous for the insulin2 gene mutation. The presence of the Cat-HA transgene in the Akita Cat-Tg mouse was confirmed by PCR of genomic DNA with specific primers against the Cat-HA transgene [26] and the mutated insulin2 gene [24, 29]. The mutation in the insulin2 gene was identified by *Fnu4H1* digestion (1 h at 37°C) of the PCR product revealing two fragments (280 bp and 140 bp) separated on 3% agarose gel electrophoresis.

Physiological Studies

Male adult non-Akita littermates, Akita, Cat-Tg and Akita Cat-Tg mice (8 mice per group) were studied. All animals had *ad libitum* access to standard mouse chow and water. Animal care and procedures were approved by the CRCHUM's Animal Care Committee.

Systolic blood pressure (SBP) was monitored in the morning with a BP-2000 tail-cuff pressure monitor (Visitech Systems, Apex, NC, USA) at least 2-3 times per week per animal, for 12 weeks [21-24]. The mice were habituated to the procedure for at least 15-20 min per day for 5 days before the first SBP measurements. SBP values are expressed as the mean ± SEM. All animals were housed individually in metabolic cages for 24 h prior to euthanasia at the age of 20 weeks. Body weight was recorded. Urine was collected and assayed for albumin and creatinine by ELISAs (Albuwell and Creatinine Companion, Exocell, Inc., Philadelphia,

PA, USA) [21-24]. Immediately following euthanasia, the kidneys were removed, decapsulated and weighed. The left kidneys were processed for histology and immunostaining, and the right kidneys were harvested for isolation of renal proximal tubules (RPTs) by Percoll gradient [21-24].

In separate studies, adult male Akita mice (age 14 weeks) were treated subcutaneously with Ang 1-7 (500 $\mu g/kg^{-1}.day^{-1}$) ± A-779 (10 $mg/kg^{-1}.day^{-1}$) and euthanized at age 18 weeks (6 mice per group). Controls were untreated non-Akita WT. SBP was measured thrice weekly [21-24].

The glomerular filtration rate (GFR) was estimated as described by Qi et al. [30], as recommended by the Animal Models of Diabetic Complications (AMDCC) (http://www.diacomp.org/) with slight modifications [31].

Agt, Ang 1-7 and Ang II Measurement

Mouse urinary Agt levels were assayed by ELISA (Immuno-Biological Laboratories, IBL America. Minneapolis, MN, USA) and normalized by urinary creatinine levels. Serum and urinary Ang II and Ang 1-7 levels were assayed by specific ELISAs for Ang II and Ang 1-7, respectively, following extraction with a kit (Bachem Americas Inc) [24, 32].

Histology

Kidney sections (4-5 sections, 3-4 μm thick, per kidney) from 8 animals per group were stained with PAS or Masson's trichrome, and assessed by light microscopy by two independent blinded observers. The collected images were analyzed and quantified using the NIH ImageJ software (http://rsb.info.nih.gov/ij/)[22-24, 32].

Mean glomerular volume on 30 random glomerular sections per mouse was assessed by the Weibel method [33] with Motics Images Plus 2.0 image analysis software (Motic Instrument, Richmond, BC, Canada) [22-24, 32]. Tubular luminal areas were measured on renal sections (6 animals/ group; 4 to 5 sections per kidney, 4 random fields per section, 10 tubules around the glomerulus per field) with Motics Image Plus 2.0 image analysis software [22-24, 32]. Outer cortical RPTs with similar cross-sectional views and clear nuclear structure were selected. Mean cell volume was estimated by the Nucleator method [34], as described previously [22-24, 32].

Immunohistochemical staining was performed according to the standard avidin-biotin-peroxidase complex method (ABC Staining System, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) [21-24, 32]. Immunostaining with non-immune normal rabbit serum in non-Akita mouse kidneys served as controls and no immunostaining was observed (photographs not shown). Oxidative stress in RPTs *in vivo* was assessed by dihydroethidium (DHE; Sigma) staining in frozen kidney sections [35]. In this assay, the nonfluorescent DHE is oxidized to fluorescent ethidium by superoxide anion ($O2^{\cdot -}$). The results were confirmed by standard immunohistochemical staining for heme oxygenase 1 (HO-1, an oxidative stress-inducible gene that confers cellular oxidative stress *in vivo*) [32, 36]. The percentage of apoptotic RPTCs was estimated semiquantitatively [22-24, 32] by the TUNEL assay (Roche Diagnostics, Laval, QC).

Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR) Assays for Gene Expression

Agt, Ace2, ACE, TGF-β1, collagen type IV and β-actin mRNA expression in RPTs was quantified by RT-qPCR with forward and reverse primers as described previously [24, 32].

***Western Blotting for Estimation of Protein* Expression**

Western blotting for Ace2, ACE and Agt was performed with RPT lysates [24, 32]. The membranes were first blotted with anti-Ace2, ACE or Agt antibodies and then re-blotted with anti-β-actin monoclonal antibodies and chemiluminescent developing reagent (Roche Biochemicals, Inc.). The relative densities of Ace2, ACE, Agt and β-actin bands were quantified by computerized laser densitometry (ImageQuant software (version 5.1), Molecular Dynamics, Sunnyvale, CA, USA).

Statistical Analysis

Statistical significance between experimental groups was analyzed by Student's t-test or 1-way ANOVA and the Bonferroni correction as appropriate. Data are expressed as mean ± SEM. $p<0.05$ was considered to be statistically significant.

2.4 Results

RPTC-specific Expression of the Cat Transgene in Akita and Tg Mouse Kidneys

We confirmed the presence of the mutated insulin2 gene in the RPTs of Akita and Akita Cat-Tg mice but not in wild-type (WT) non-Akita or Cat-Tg mice (Figure 1A, a). Likewise, the Cat-HA transgene was expressed only in RPTs of Cat-Tg and Akita Cat-Tg mice but not in RPTs of WT and Akita mice (Figure 1A, b). Cat levels were significantly higher in RPTCs in Cat-Tg mice (Figure 1B, b) and Akita Cat-Tg mice (Figure 1B, d) than in non-Akita WT mice

(Figure 1B, a) or in Akita mice (Figure 1B, c). The Cat expression was RPTC-specific and it co-localized to aquaporin-1-positive immunostained RPTCs (Figure 1C). HO-1 immunostaining and DHE staining demonstrated lower levels of oxidative stress in RPTCs of Cat-Tg (b in Figures 1D and 1E) than in non-Akita WT mice (a in Figures 1D and 1E). Akita mice (c in Figures 1D and 1E) exhibited significantly higher oxidative stress and normalized in Akita Cat-Tg mice (d in Figures 1D and 1E). Quantitation of DHE staining confirmed these findings (Figure 1F). These results confirm that the KAP gene promoter directs Cat transgene expression in the RPTCs of Cat-Tg and Akita Cat-Tg mice and that Cat expression effectively attenuates ROS production.

Physiological Parameters in Akita and Tg Mice

We detected significant differences in SBP between Akita and non-Akita WT mice as early as 8 weeks of age. These differences increased with age (from week 14 until week 20; **Figure 2A**). There were no significant differences in SBP between the Akita mouse and Akita Cat-Tg mouse until week 16 (**Figure 2A**). Overexpression of the Cat transgene protected the Akita Cat-Tg mouse against increased SBP as compared to Akita mice. SBP did not differ significantly in Cat-Tg mice and non-Akita WT mice (over time) and at 20 weeks (**Table I**).

Interestingly, at 20 weeks Akita mice had higher GFR (**Figure 2B**), urinary Agt (**Figure 2C**) and Ang II levels (**Figure 2D**) and lower Ang 1-7 levels (**Figure 2E**) than non-Akita mice or Cat-Tg mice. Overexpressing Cat attenuated elevated GFR, urinary Agt and Ang II levels and normalized Ang 1-7 levels in Akita Cat-Tg mice (**Figures 2B, 2C, 2D and 2E,** respectively). In contrast, serum Ang II levels did not differ significantly among the groups studied (**Figure 2F**).

Blood glucose level was significantly higher in Akita and Akita Cat-Tg mice at 20 weeks than in non-Akita WT or Cat-Tg mice, respectively (**Table I**). Akita mice also exhibited elevated kidney to body weight ratio and heart to body weight ratio at the end of the experiment as compared to non-Akita WT controls or Cat-Tg mice (**Table I**). Cat overexpression markedly attenuated, though not completely normalized, these ratios in Akita Cat-Tg mice. Urinary albumin/creatinine ratio (ACR) at 20 weeks was significantly higher in Akita mice than in non-Akita WT controls or Cat-Tg mice (**Table I**). Cat overexpression partially reduced the ACR in Akita Cat-Tg mice but did not completely normalize it to WT control levels. These findings indicate that Cat overexpression effectively attenuate kidney and heart hypertrophy and albuminuria without exerting an anti-hyperglycemic effect in the Akita Cat-Tg mice.

Agt , Ace2 and ACE Expression in Akita and Tg Kidneys

We noted higher Agt immunostaining in RPTCs of WT controls (**Figure 3A, a**) relative to Cat-Tg mice (**Figure 3A, b**). Agt immunostaining was significantly increased in Akita mice (**Figure 3A, c**) as compared to WT controls and Cat-Tg mice and normalized in Akita Cat-Tg mice (**Figure 3A, d**). Expression of Ace2 in the RPTCs of WT non-Akita controls (**Figure 3B, a**) or Cat-Tg mice (**Figure 3B, b**) was also significantly higher as compared to Akita mice (**Figure 3B, c**). Overexpression of Cat normalized Ace2 immunostaining in RPTCs of Akita Cat-Tg mice (**Figure 3B, d**). In contrast, the RPTCs of WT controls (**Figure 3C, a**) or Cat-Tg mice (**Figure 3C, b**) exhibited decreased staining for ACE relative to Akita mice (**Figure 3C,**

c). Overexpressing Cat decreased the level of immunostaining for ACE in the RPTCs of Akita Cat-Tg mice (**Figure 3C, d**) vs. Akita mice (**Figure 3C, c**).

We confirmed these findings by immunoblotting for Agt, Ace2 and ACE (**Figures 3 D-F**,) and by detecting Agt mRNA, Ace2 mRNA and ACE mRNA expression (**Figures 3G-I**,) in isolated RPTCs by RT-qPCR.

Histology at 20 Weeks

Unlike WT non-Akita mice and Cat-Tg mice, Akita mice exhibited renal structural damage (Figure 4A). Histologic findings included tubular luminal dilation and accumulation of cell debris in the tubular lumen. Some RPTCs were flattened. Remarkably, overexpression of Cat in the Akita Cat-Tg mice markedly suppressed, but did not completely prevent these abnormalities. We observed significantly enlarged tubular luminal area, increased glomerular tuft and RPTC volume in Akita mice compared to non-Akita WT or Cat-Tg mice **(Table I)**. Overexpression of the Cat transgene partially reduced tubular luminal area and glomerular tuft volume, and completely normalized RPTC volume in Akita Cat-Tg mice.

Tubulointerstitial Fibrosis and Profibrotic Gene Expression in Akita and Tg Kidneys

We assessed expression of collagenous components with Masson's trichrome staining, and by immunostaining for collagen type IV or TGF-β1. Kidneys from non-Akita WT (**a in Figures 4B, 4D and 4E**) or Cat-Tg mice (**b in Figures 4B, 4D and 4E**) exhibited significantly lower expression of collagenous components, collagen type IV and TGF-β1 relative to Akita mice (**c in respective Figure 4B, 4D and 4E**). Cat overexpression markedly reduced tubulointerstitial fibrosis (**d in Figures 4B, 4D and 4E**) as demonstrated by quantitative

analysis of Masson's trichrome staining (**Figure 4C**) and immunostaining for collagen IV (**Figure 4F**) and TGF-β1 (**Figure 4G**). Quantitation of collagen IV (**Figure 4H**) and TGF-β1 mRNA (**Figure 4I**) expression further confirmed these findings. Collectively, these data indicate that Cat overexpression effectively prevents tubulointerstitial fibrosis in Akita Cat-Tg mice.

Tubular Apoptosis in Akita and Tg Kidneys

Next we investigated the impact of Cat overexpression on tubular apoptosis in Akita mice by the TUNEL assay. The number of TUNEL-positive nuclei in RPTCs of non-Akita WT mice (**Figure 5A, a**) or Cat-Tg mice (**Figure 5A, b**) were significantly lower than in Akita mice (**Figure 5A, c**). Cat overexpression significantly reduced the number of TUNEL-positive cells in Akita Cat-Tg mice (**Figure 5A, d**) and this was confirmed by semi-quantitation (**Figure 5B**). Consistently, expression of active (cleaved) caspase-3 was also lower in non-Akita WT (**Figure 5C, a**) and Cat-Tg mice (**Figure 5C, b**) but was higher in RPTCs of Akita mice (**Figure 5C, c**). Cat overexpression attenuated expression of active caspase-3 in Akita Cat-Tg mice (**Figure 5C, d**). Caspase-3 activity assays in isolated RPTs confirmed these findings (**Figure 5D**).

Akita mice also exhibited increased expression of Bax and Bcl-2 mRNA (**Figure 5E**) and decreased expression of Bcl-xl mRNA (**Figure 5F**) as compared with non-Akita WT mice or Cat-Tg mice. mRNA expression was normalized in Akita Cat-Tg mice (**Figures 5E and 5F**).

Effect of Ang 1-7 Administration on SBP in Akita mice

Mean SBP was significantly higher in Akita mice at 13 weeks of age than in WT mice (**Figure 6A**) and remained higher for the duration of the study. Daily administration of Ang 1-7 for 4 weeks normalized SBP in Akita mice. Co-administration of Ang 1-7 with A-779 resulted in increases in SBP (**Figures 6A and 6B**).

2.5 Discussion

Our study demonstrates that Cat overexpression in RPTCs of Akita mice effectively attenuates oxidative stress, prevents systemic hypertension and renal injury, normalizes Ace2 expression in RPTCs, and suppresses Agt, pro-fibrotic and pro-apoptotic gene expression. Our observations indicate that ROS level in RPTC plays a critical role in regulating renal Agt gene expression and RAS activation and subsequently SBP and kidney injury *in vivo*.

Expanding our previous findings that overexpression of Cat in RPTCs prevents hypertension and attenuates tubulointerstitial fibrosis and RPTC apoptosis in non-diabetic Agt/Cat-Tg mice and type 2 diabetic db/db Cat-Tg mice [32, 37], here we demonstrate that overexpression of Cat also enhances renal Ace2 expression and Ang 1-7 formation. Administration of Ang 1-7 prevents systemic hypertension in Akita mice, indicating that intrarenal Ang 1-7 formation is critical in counteracting Ang II actions in Akita mice. These findings highlight an important role for intrarenal Ace2 expression and Ang 1-7 formation in preventing development of hypertension and nephropathy in diabetic mice.

The Akita mouse, an autosomal dominant model of spontaneous type 1 diabetes in which the insulin gene2 is mutated, have decreased numbers of β-cells of the pancreatic islets and develop hyperglycemia at age of 3-4 weeks [29]. By the age of 30 weeks, male Akita mice

manifest impaired renal function and increased oxidative stress markers in their RPTs [38] and elevated kidney/body weight and heart/body weight ratios, closely resembling those observed in patients with type 1 diabetes.

Our data indicate that mitigating oxidative stress via kidney-specific overexpression of Cat protects diabetes-prone Akita mice against hypertension. The mechanisms underlying elevated SBP in Akita mice are largely unknown. The possibility that down-regulation of Ace2 gene expression and consequently high Ang II/Ang 1-7 ratio facilitates the development of hypertension has received considerable attention [11, 12]. Indeed, our present findings demonstrate significantly lower Ace2 and urinary Ang 1-7 and higher ACE, urinary Agt and Ang II levels in Akita mice than in non-Akita WT or Cat-Tg mice. Cat overexpression in RPTCs normalized these changes. These observations are consistent with our previous findings of markedly elevated ACE and depressed Ace2 expression in the kidneys of Agt-Tg mice [32] as well as with studies on normotensive Lewis rats, which showed that RAS blockade increases cortical Ace2 activity and urinary Ang 1-7 excretion [39]. Normal human kidneys express low levels of ACE and high levels of Ace2, and this ratio is reversed in kidneys of hypertensive and diabetic patients [40-42]. Furthermore, Ang II was found to up-regulate ACE and down-regulate Ace2 expression in HK2 cells *in vitro* [40]. Taken together, these findings lend support to the concept that intrarenal RAS activation up-regulates ACE activity and down-regulates Ace2 expression via enhanced oxidative stress in RPTCs, ultimately contributing to development of hypertension.

As expected [24], we detected renal structural damage in Akita mice, including tubular luminal dilation, glomerular hypertrophy and increased RPTC volume. Increases in tubular lumen in Akita mice would indicate enhanced susceptibility of RPTCs to atrophy [43].

Furthermore, occurrence of RPTC injury in Akita mouse kidneys was supported by augmented immunostaining for KIM-1, a molecular marker of RPTC injury [44] (Unpublished results). Importantly, selective overexpression of Cat in RPTCs markedly attenuated these morphological changes. Thus, hyperglycemia and RAS activation induce kidney damage in diabetes, which could be attenuated by Cat overexpression-mediated inhibition of RAS activation and action.

Since glomerular hyperfiltration and microalbuminuria are early clinical markers of hypertension- or diabetes-induced nephropathy, we monitored GFR and urinary albuminuria and detected enhanced GFR and microalbuminuria in Akita mice at the age of 20 weeks. Cat overexpression significantly reduced, though not completely prevented these changes. These observations imply a link between intrarenal oxidative stress, RAS activation, GFR and albuminuria. However, the underlying molecular mechanism(s) remain large unknown. One possible mechanism is that increased intrarenal Ang 1-7 formation and decreased Ang II formation in Akita Cat-Tg mice would prevent or attenuate the actions of Ang II on efferent arterioles, thereby reducing the glomerular pressure (hyperfiltration), glomerular volume and eventually SBP. Increased urinary Ang 1-7 and reduced Ang II levels in Akita Cat-Tg mice lend support to this notion.

Treatment with Ang 1-7 prevented systemic hypertension in Akita mice, and this can be reversed by A-779. These findings are consistent with attenuation by recombinant human Ace2 of Ang II-dependent and pressure-overload-induced hypertension in Ace2 knockout mice [18-20], supporting an important counterregulatory role for Ang 1-7 in Ang II-mediated cardiac and renal abnormalities.

We observed modest thickening of the tubular basement membrane in Akita mice, which can be prevented by Cat overexpression. The mechanism by which oxidative stress leads to interstitial fibrosis in Akita mice remains unclear. One possibility is that augmented Agt and Ang II expression via ROS generation stimulates TGF-β1, subsequently enhancing the expression of extracellular matrix proteins, collagen type IV, profibrotic and pro-apoptotic proteins in RPTCs, with resultant apoptosis and, ultimately interstitial fibrosis [45]. Indeed, neutralizing TGF-β alleviates fibrosis and tubular cell apoptosis in animal models of diabetes [46]. Our present study shows higher Agt, TGF-β1 and collagen IV expression and lower LAP/TGF-β1 expression (a marker of the inactive TGF-β1 complex) [47] (Unpublished results) in RPTs of Akita mice than in non-Akita mice. These changes were mitigated in Akita Cat-Tg mice, linking intrarenal oxidative stress to interstitial fibrosis.

Confirming our previous findings in the kidneys of non-diabetic Agt-Tg, diabetic Agt-Tg and Akita mice [22-24, 32], we detected more apoptotic RPTCs in Akita mice, shown by increased percentage of TUNEL-positive RPTCs parallel with increased active caspase-3 immunostaining and Bax mRNA expression and decreased Bcl-xL mRNA expression. An elevated Bax/Bcl-xL ratio is consistent with induction of tubular apoptosis in Akita mice. Once again, these changes were mitigated in Akita Cat-Tg mice.

Our results may be clinically relevant for type 1 diabetes. Tubulointerstitial fibrosis and tubular apoptosis occur in human type 1 diabetic kidneys [48], and tubular atrophy appears to be a better indicator of disease progression than glomerular pathology [49]; we suggest that tubulointerstitial fibrosis and RPTC apoptosis may be initial events leading to tubular atrophy in diabetes. Oxidative stress-mediated decrease of Ace2 gene expression would further accelerate this process.

In summary, our data indicate a critical role for tubular oxidative stress in the development of hypertension, albuminuria, tubulointerstitial fibrosis and RPTC apoptosis in Akita mice.

ABBREVIATIONS

Ace2, angiotensin-converting enzyme-2; ACE, angiotensin-converting enzyme; ACR, albumin-creatinine ratio; Agt, angiotensinogen; Ang II, angiotensin II; Ang 1-7, angiotensin 1-7; Cat, catalase; HA-tag, a sequence encoding amino acid residues 98-106 (YPYDVPDYA) of human influenza virus hemagglutinin; KAP, kidney-specific androgen-regulated promoter; rAgt, rat angiotensinogen; RAS, renin-angiotensin system; RPTs, renal proximal tubules; RPTCs, renal proximal tubular cells; RT-qPCR, real time-quantitative polymerase chain reaction; SBP, systolic blood pressure; STZ, streptozotocin; Tg, transgenic; TGF-β1, transforming growth factor-beta 1; TUNEL, terminal transferase-mediated deoxyuridine triphosphate nick end-labeling assay; WT, wild-type.

2.6 Acknowledgments

This work was supported in part by grants from the Kidney Foundation of Canada to J.S.D.C, the Canadian Institutes of Health Research (MOP-84363, MOP-93650 and MOP-106688 to J.S.D.C., MOP-86450 to S.L.Z. and MOP-12573 to J.G.F.), the National Institutes of Health (NIH) of USA (HL-48455 to J.R.I.) and the Foundation of the CHUM.

No potential conflicts of interest relevant to this article were reported. This manuscript has no financial arrangement with commercial companies.

Y.S. and C-S. L. researched data, and contributed to discussion. I.C. researched data. H.M. researched data. J.G.F. contributed to discussion, and reviewed/edited manuscript. J.R.I. contributed to discussion, and reviewed/edited manuscript. S-L.Z. researched data, contributed to discussion and reviewed/edited manuscript. J.S.D.C. contributed to discussion, wrote manuscript, and reviewed/edited manuscript. J.S.D.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

This manuscript or any significant part of it is under consideration for publication elsewhere. The data, however, has been presented in part as free communication at the The 42nd Annual Meeting of the Am Soc of Nephrol. San Diego, CA, USA, Oct. 27 -Nov.1, 2009. Editorial assistance was provided by the CRCHUM's Research Support Office.

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2.8 Legends and Figures

Table 2-1. Physiological Measurements

	WT	CAT-Tg	Akita	Akita CAT-Tg
(A) Blood glucose (mmol/L)	10.84±0.65	11.16±0.68	34.50±0.72***	35.10±0.79***
(B) Systolic Blood Pressure (mmHg)	108.6±2.56	105.5±1.47	136.7±2.6***	113.25±5.48†††
(B) Body Weight (g)	34.46±0.80	34.80±0.39	26.71±0.63***	26.99±0.60***
(C) Kidney Weight (g)	0.40±0.011	0.41±0.011	0.63±0.022***	0.53±0.019***,††
(D) Heart Weight (g)	0.151±0.007	0.159±0.006	0.173±0.012	0.171±0.011
(E) Kidney/Body Weight ratio (g/g)	0.0118 ±0.000507	0.0119 ±0.000361	0.0234 ±0.000595***	0.0201 ±0.000543***,†††
(F) Heart/Body Weight ratio (g/g)	0.0044 ±0.000117	0.0046 ±0.000168	0.0071 ±0.000441***	0.0058 ±0.00023**,†
(G) Albumin/Creatinine ratio (μg/ml/mg/dL)	0.244 ±0.027	0.174 ±0.026	4.462 ±1.361***	2.15 ±0.553††
(H) Glomerular tuft volume (X 10^3 μm^3)	129.7 ±3.99	137.0 ±5.20	260.7 ±16.52***	180.3 ±9.57***,†††
(I) RPTC volume (X 10^3 μm^3)	5.44±0.11	4.82±0.05**	9.68±0.26***	5.86±0.14†††
(J) Tubular luminal Area (μm^2)	51.38±5.37	48.76±5.54	105.7±14.55**	66.27±9.11*,††

*P<0.05, **P<0.01 and ***P<0.005 vs WT

†P<0.05, ††P<0.01 and †††P<0.005 vs Akita

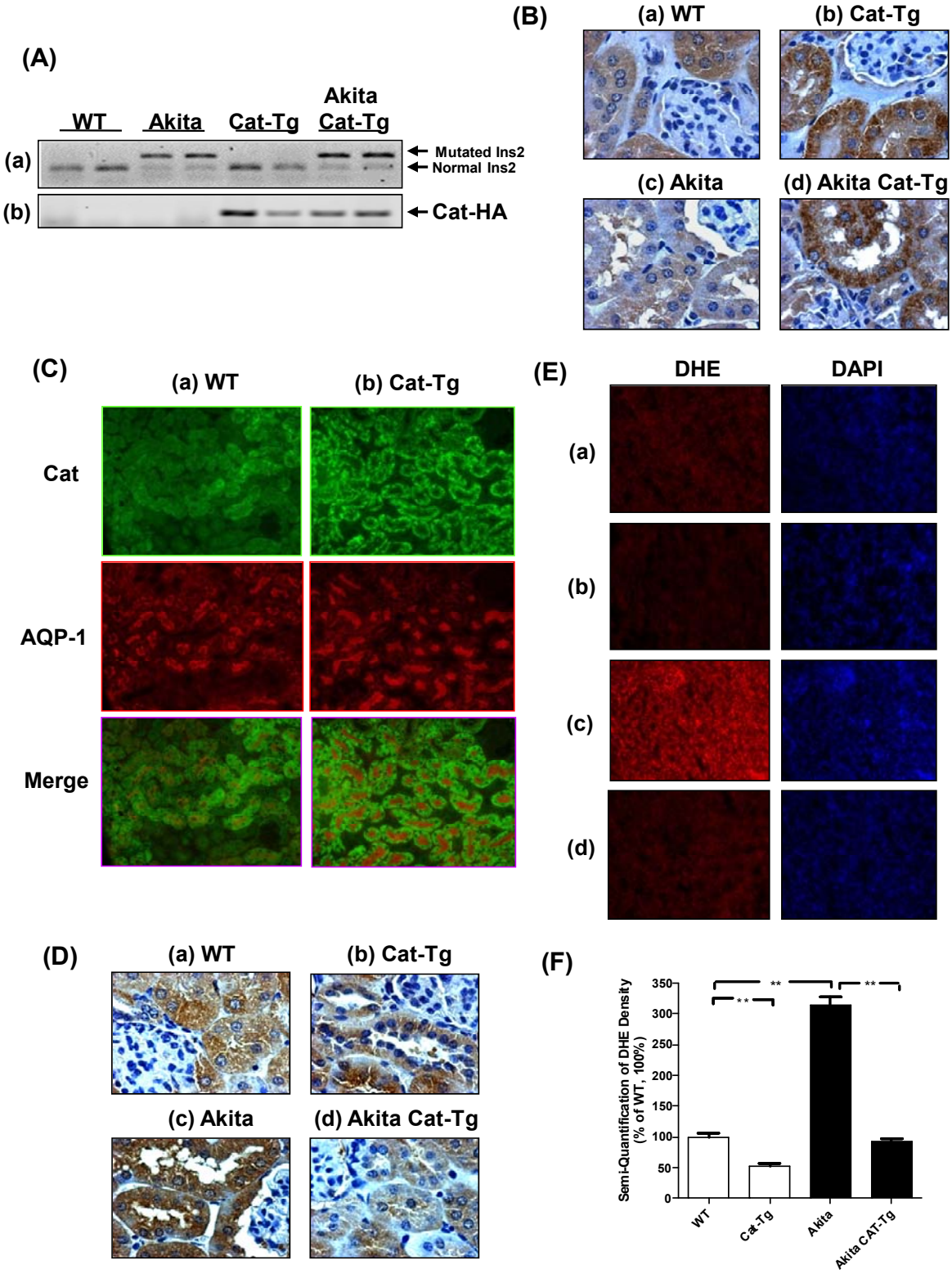

(A)
WT
Akita
Cat-Tg
Akita Cat-Tg
(a)
(b)
Mutated Ins2
Normal Ins2
Cat-HA
(B)
(a) WT
(b) Cat-Tg
(c) Akita
(d) Akita Cat-Tg
(C)
(a) WT
(b) Cat-Tg
Cat
AQP-1
Merge
(D)
(a) WT
(b) Cat-Tg
(c) Akita
(d) Akita Cat-Tg
(E)
DHE
DAPI
(a)
(b)
(c)
(d)
(F)
Semi-Quantification of DHE Density
(% of WT, 100%)
350
300
250
200
150
100
50
0
**
**
**
WT
Cat-Tg
Akita
Akita CAT-Tg

Figure 2-1: Generation of Akita Cat-Tg mice. (A) (a) Genotyping of the insulin2 mutation in non-Akita and Akita mice. (b) Cat-HA transgene expression. PCR analysis of Cat-HA transgene in offspring of Cat-Tg line 688 cross-bred with heterozygous Akita mice. Akita Cat-Tg mice displaying Cat-HA transgene were used in subsequent experiments. Immunohistochemical staining for Cat (B) in male non-Akita WT, Akita, Cat-Tg and Akita Cat-Tg mouse kidneys by employing rabbit anti-bovine Cat polyclonal antibody. Magnification X 600. (C) Co-localization of immunostaining of Cat and Aquaporine-1 (AQP-1) in male non-Akita WT and Cat-Tg mouse kidneys. Magnification X 200. (D) Immunostaining of HO-1 in male non-Akita WT, Akita, Cat-Tg and Akita Cat-Tg mouse kidney by employing HO-1 polyclonal antibody. Magnification X 600. (E) Dihydroethidium (DHE) (red) staining in mouse kidneys. Magnification X 200. (F) Semi-quantification of DHE fluorescence in mouse kidneys. Values are expressed as means ± SEM, N=8 per group. **$p<0.01$. (a) Non-Akita WT mouse. (b) Cat-Tg mouse. (c) Akita mouse. (d) Akita Cat-Tg mouse.

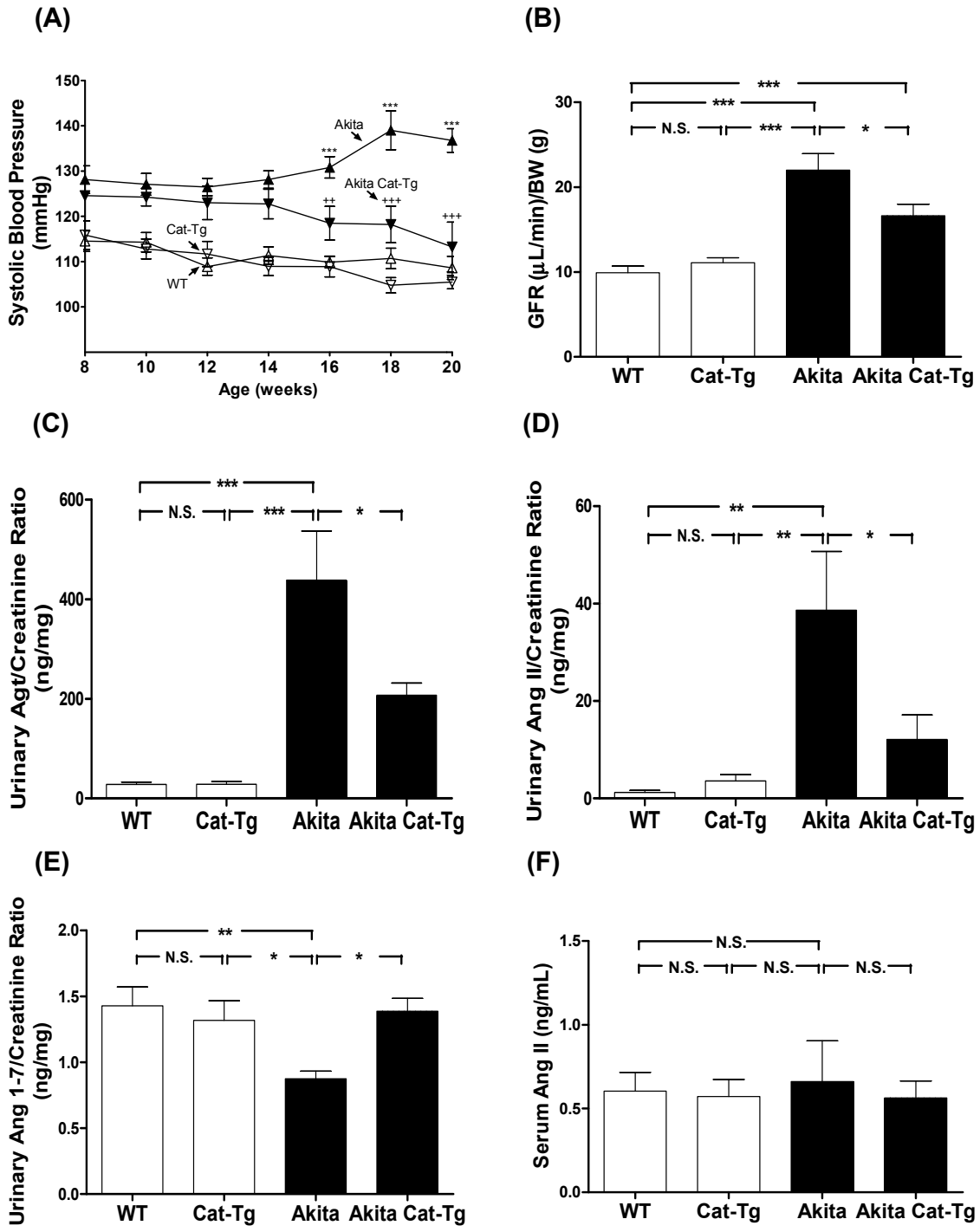

(A)
Systolic Blood Pressure (mmHg)
Age (weeks)
Akita
Akita Cat-Tg
Cat-Tg
WT
(B)
GFR (µL/min)/BW (g)
N.S.
WT
Cat-Tg
Akita
Akita Cat-Tg
(C)
Urinary Agt/Creatinine Ratio (ng/mg)
(D)
Urinary Ang II/Creatinine Ratio (ng/mg)
(E)
Urinary Ang 1-7/Creatinine Ratio (ng/mg)
(F)
Serum Ang II (ng/mL)

Figure 2-2: Effect of overexpression of Cat in RPTCs on systolic blood pressure (SBP), glomerular filtration rate (GFR), urinary Agt, Ang II and Ang 1-7 levels and serum Ang II level in Akita mice. (A) Longitudinal changes in mean SBP in male non-Akita WT (Δ), Cat-Tg (∇), Akita (▲),and Akita Cat-Tg (▼). Baseline SBP was measured daily over a 5-day period before initiation of measurement. (B) GFR, urinary levels of Agt (C), Ang II (d) and Ang 1-7 (E) and serum levels of Ang II (F) were measured at the age of week 20 in non-Akita WT, Cat-Tg, Akita and Akita Cat-Tg mice. Values are expressed as means ± SEM, N=8 per group. *$p<0.05$; **$p<0.01$; ***$p<0.005$; N.S., non-significant.

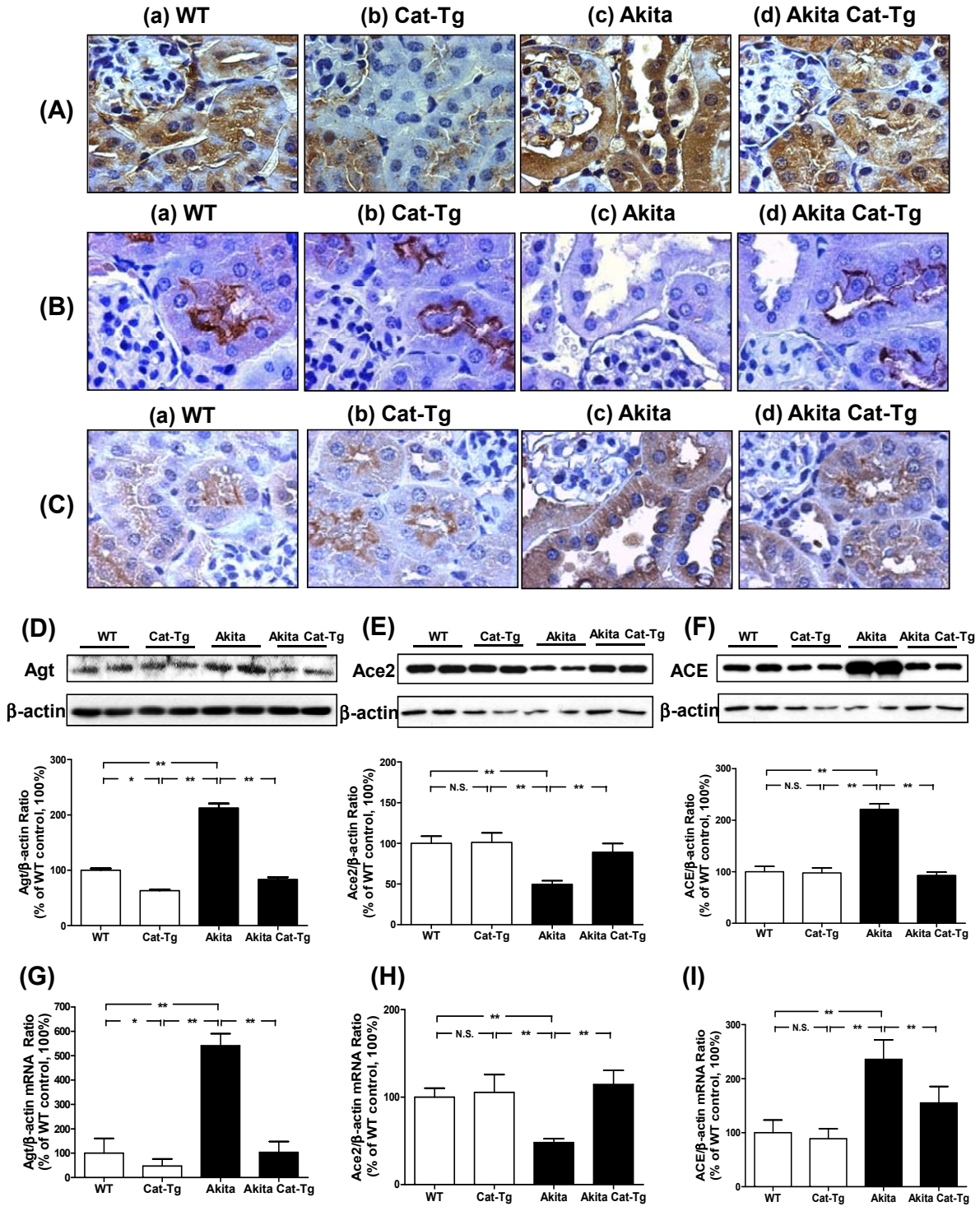

(a) WT
(b) Cat-Tg
(c) Akita
(d) Akita Cat-Tg
(A)
(B)
(C)
(D)
WT
Cat-Tg
Akita
Akita Cat-Tg
Agt
β-actin
(E)
Ace2
(F)
ACE
Agt/β-actin Ratio (% of WT control, 100%)
Ace2/β-actin Ratio (% of WT control, 100%)
ACE/β-actin Ratio (% of WT control, 100%)
N.S.
(G)
(H)
(I)
Agt/β-actin mRNA Ratio (% of WT control, 100%)
Ace2/β-actin mRNA Ratio (% of WT control, 100%)
ACE/β-actin mRNA Ratio (% of WT control, 100%)

Figure 2-3: Agt, Ace2 and ACE expression in mouse kidneys at the age of week 20. Agt (A), Ace2 (B) and ACE (C) immunostaining: a, Non-Akita WT control littermate; b, Cat-Tg mouse; c, Akita mouse; d, Akita Cat-Tg mouse. Magnification X 600. Western blotting of Agt (D), Ace2 (E) and ACE (F) in mouse RPTs. The membranes were re-blotted for β-actin. Agt, Ace2 and ACE levels were normalized by corresponding β-actin levels. Values are expressed as means ± SEM (n=8). *$p<0.05$; **$p<0.01$; N.S., non-significant. RT-qPCR of Agt (G), Ace2 (H) and ACE (I) mRNAs in mouse RPTs. Ace2, ACE and β-actin mRNAs were run simultaneously in the assays. Agt, Ace2 and ACE mRNA levels were normalized by corresponding β-actin mRNA levels. mRNA levels in non-Akita control littermates were considered as 100%. Values are expressed as means ± SEM, (n=8) *$p<0.05$; **$p<0.01$; N.S., non-significant.

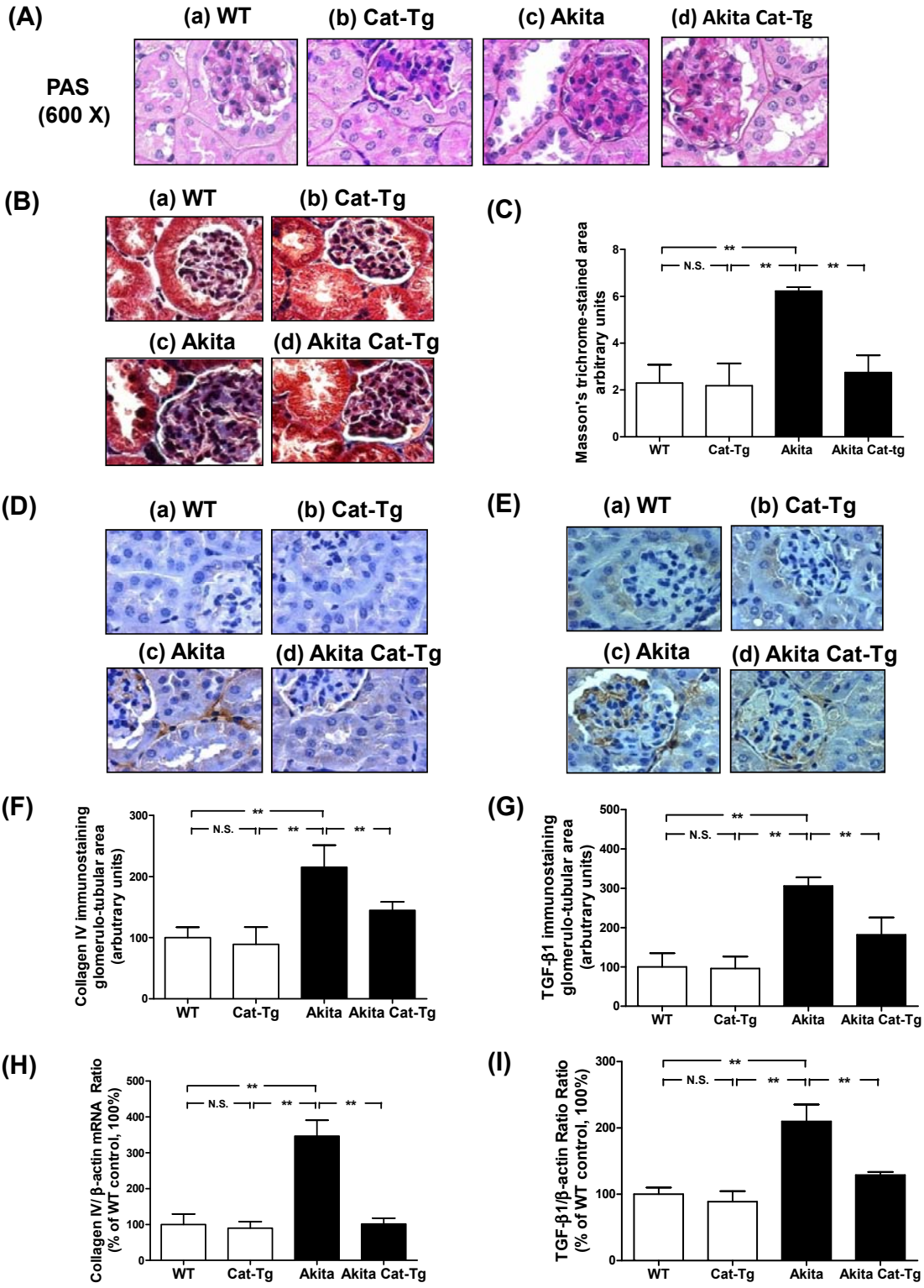

(A)
(a) WT
(b) Cat-Tg
(c) Akita
(d) Akita Cat-Tg
PAS
(600 X)
(B)
(a) WT
(b) Cat-Tg
(c) Akita
(d) Akita Cat-Tg
(C)
Masson's trichrome-stained area
arbitrary units
N.S.
**
WT
Cat-Tg
Akita
Akita Cat-tg
(D)
(a) WT
(b) Cat-Tg
(c) Akita
(d) Akita Cat-Tg
(E)
(a) WT
(b) Cat-Tg
(c) Akita
(d) Akita Cat-Tg
(F)
Collagen IV immunostaining
glomerulo-tubular area
(arbutrary units)
(G)
TGF-β1 immunostaining
glomerulo-tubular area
(arbutrary units)
(H)
Collagen IV/ β-actin mRNA Ratio
(% of WT control, 100%)
(I)
TGF-β1/β-actin Ratio Ratio
(% of WT control, 100%)

Figure 2-4: PAS staining, Masson's trichrome staining, collagen IV and TGF-β1 expression in mouse kidneys at the age of week 20. (A) PAS staining. (B) Masson's trichrome staining. (C) Quantification of extracellular matrix component accumulation (Masson's trichrome staining). (D) Immunostaining for collagen IV. (E) Immunostaining for TGF-β1. a, Non-Akita WT control littermate; b, Cat-Tg mouse; c, Akita mouse; d, Akita Cat-Tg mouse. Magnification X 600. (F) Quantitation of immunoreactive collagen IV deposition. (G) Quantitation of TGF-β1 immunostaining. RT-qPCR of collagen IV (H) and TGF-β1 (I) mRNA. Values are expressed as means ± SEM (n=8) **$p<0.01$; N.S., non-significant.

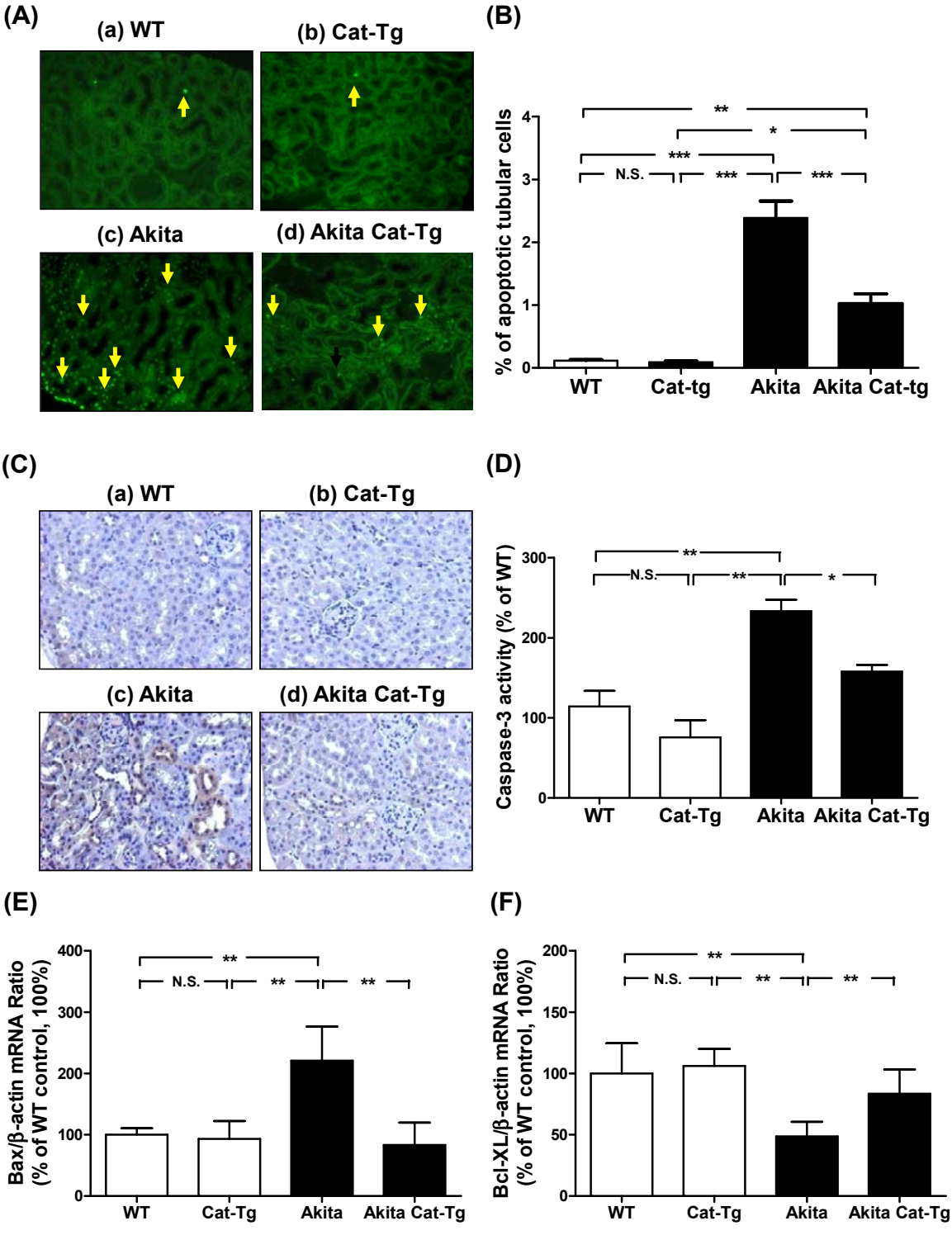
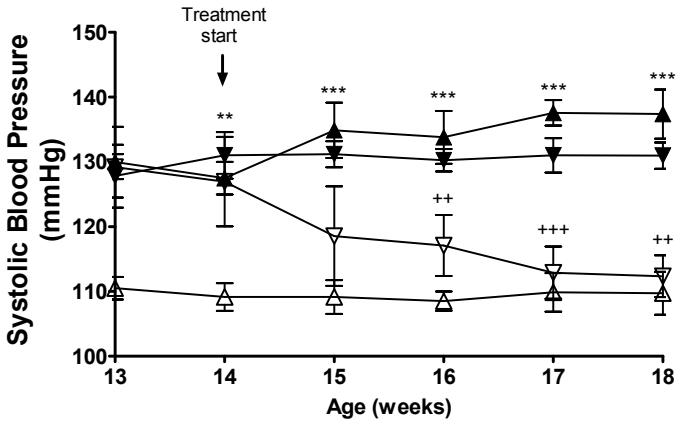

(A)
(a) WT
(b) Cat-Tg
(c) Akita
(d) Akita Cat-Tg
(B)
% of apoptotic tubular cells
N.S.
WT
Cat-tg
Akita
Akita Cat-tg
(C)
(a) WT
(b) Cat-Tg
(c) Akita
(d) Akita Cat-Tg
(D)
Caspase-3 activity (% of WT)
N.S.
WT
Cat-Tg
Akita
Akita Cat-Tg
(E)
Bax/β-actin mRNA Ratio (% of WT control, 100%)
N.S.
WT
Cat-Tg
Akita
Akita Cat-Tg
(F)
Bcl-XL/β-actin mRNA Ratio (% of WT control, 100%)
N.S.
WT
Cat-Tg
Akita
Akita Cat-Tg

Figure 2-5: Apoptosis in mouse kidneys at the age of week 20. (A) TUNEL (green) staining. Magnification X 200. Arrows indicate apoptotic cells in proximal tubules. (B) Semi-quantitation of apoptotic RPTCs in mouse kidneys. (C) Immunostaining for cleaved (active) caspase-3 and (D) Caspase-3 activity in isolated RPTs. a, non-Akita WT control littermate; b, Cat-Tg mice; c, Akita mouse; d, Akita Cat-Tg mouse. Magnification X 200. RT-qPCR for Bax (E) and Bcl-xL (F) mRNA. Bax, Bcl-xL and β-actin mRNAs were run simultaneously in the assays. Bax and Bcl-xL mRNA levels were normalized by corresponding β-actin mRNA levels. mRNA levels in non-Akita control littermates were considered as 100%. Values are expressed as means ± SEM, (n=8) *$p<0.05$; **$p<0.01$; ***$p<0.005$; N.S., non-significant.

(A)


Treatment start
Systolic Blood Pressure (mmHg)
150
140
130
120
110
100
13
14
15
16
17
18
Age (weeks)
**
***
***
***
***
++
+++
++

(B)

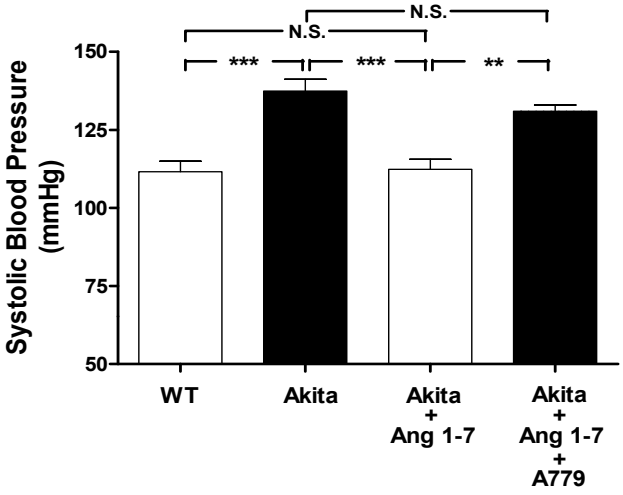

N.S.
N.S.
***
***
**
Systolic Blood Pressure (mmHg)
150
125
100
75
50
WT
Akita
Akita + Ang 1-7
Akita + Ang 1-7 + A779

Figure 2-6: Effect of Ang 1-7 with or without A-779 on SBP in Akita mice. (A) Longitudinal changes in mean SBP in male non-Akita WT mice (Δ), Akita mice (▲), Akita mice treated with Ang 1-7 (∇), and Akita mice treated with Ang 1-7 and A-779 (▼). Baseline SBP was measured daily over a 5-day period before initiation of treatment. (B) Cross-sectional analysis of SBP (measured 2 to 3 times per animal per week in the morning without fasting, at the age of week 18 in non-Akita WT mice, Akita mice, Akita mice treated with Ang 1-7 and Akita mice treated with Ang 1-7 and A-779. Values are expressed as means ± SEM, (n=6 per group). *$p<0.05$; **$p<0.01$; ***$p<0.005$; N.S., non-significant.

Chapter 3: Article 2

Ang 1-7 Prevents Systemic Hypertension, Attenuates Oxidative Stress and Tubulointerstitial Fibrosis, and Normalizes Renal Angiotensin-Converting Enzyme 2 and Mas Receptor Expression in Diabetic Mice

Clinical Science 2014 resubmitted: acceptance pending

Ang 1-7 Prevents Systemic Hypertension, Attenuates Oxidative Stress and Tubulointerstitial Fibrosis, and Normalizes Renal Angiotensin-Converting Enzyme 2 and Mas Receptor Expression in Diabetic Mice

Yixuan Shi[1], Chao-Sheng Lo[1], Ranjit Padda[1], Shaaban Abdo[1], Isabelle Chenier[1], Janos G. Filep[2], Julie R. Ingelfinger[3], Shao-Ling Zhang[1†], and John S D Chan[1†]

[1]Université de Montréal
Centre de recherche du Centre hospitalier de l'Université de Montréal (CRCHUM)
Tour Viger
900 Saint Denis Street
Montreal, Quebec
Canada H2X 0A9

[2]Université de Montréal
Centre de recherche
Maisonneuve-Rosemont Hospital
5415 boul. de l'Assomption
Montreal, Quebec
Canada H1T 2M4

[3]Harvard Medical School
Pediatric Nephrology Unit
Massachusetts General Hospital
15 Parkman Street, WAC 709
Boston, MA 02114-3117, USA

[1†] John S.D. Chan and Shao-Ling Zhang are joint senior authors
To whom correspondence should be addressed: John S. D Chan
Telephone: (514) 890-8000 ext. 15080; Fax: (514) 412-7204

Running Title: Ang 1-7 in Diabetic Kidneys
Keywords: Ang 1-7, Oxidative Stress, Kidney Injury, Diabetic Mice
Subject categories: Pathophysiology of renal disease and progression

Abstract: 212 words; Text: 3,896

3.1 Abstract

We investigated the relation between Ang 1-7 action, systolic hypertension (sHTN), oxidative stress, kidney injury, angiotensin converting enzyme-2 (Ace2) and angiotensin 1-7 receptor (MasR) expression in type 1 diabetic Akita mice**.** Ang 1-7 was administered daily (500 μg/kg body weight (BW)/day, subcutaneously) to male Akita mice from 14 weeks of age with or without co-administration of an antagonist of the MasR, A779 (10 mg/kg BW/day). The animals were euthanized at 20 weeks of age. Age-matched wild type (WT) mice served as controls. Ang 1-7 administration prevented sHTN and attenuated kidney injury (reduced urinary albumin/creatinine ratio, glomerular hyperfiltration, renal hypertrophy and fibrosis, and tubular apoptosis) without affecting blood glucose levels in Akita mice. Ang 1-7 also attenuated renal oxidative stress and the expression of oxidative stress-inducible proteins (NADPH oxidase 4, nuclear factor erythroid 2-related factor 2, heme oxygenase 1), pro-hypertensive proteins (angiotensinogen, angiotensin converting enzyme, sodium/hydrogen exchanger-3) and profibrotic proteins (transforming growth factor-beta 1 and collagen IV), and increased the expression of anti-hypertensive proteins (angiotensin-converting enzyme 2 (Ace2) and MasR) in Akita mouse kidneys. These effects were reversed by A779. Our data suggest that Ang 1-7 plays a protective role in sHTN and RPTC injury in diabetes, at least in part, through decreasing renal oxidative stress-mediated signaling and normalizing Ace2 and MasR expression.

3.2 Introduction

Angiotensin-converting enzyme 2 (Ace2), an enzyme homologue of angiotensin-converting enzyme (ACE), was identified by 2 independent groups in 2000 [1, 2]. Ace2 is 42% homologous to ACE in the catalytic domain and specifically cleaves angiotensin I (Ang I) and Ang II into Ang 1-9 and Ang 1-7, respectively. However, the catalytic efficiency of Ace2 on Ang II is 400-fold greater than on Ang I, resulting in direct Ang 1-7 formation [3, 4]. Evidence now supports a counter-regulatory role for Ang 1-7 via its own receptor, the Ang 1-7 receptor or MasR, which oppose many Ang II subtype 1 receptor (AT_1-R)-mediated outcomes (for recent reviews, see [5-7]).

Earlier studies on the effects of Ang 1-7 on hypertension, cardiovascular and renal function in hypertensive rats with or without diabetes yielded contradictory results. For instance, Giani et al. [8] reported Ang 1-7 attenuation of systolic blood pressure (SBP) and reduction in renal oxidative stress and inflammatory markers in Zucker rats. Benter's group [9, 10] reported decreased SBP by Ang 1-7 in spontaneously hypertensive rats (SHR) but not in streptozotocin-induced diabetic SHR despite amelioration of albuminuria and renal vascular dysfunction. In contrast, Tan et al. [11] found no significant reductions in SBP in response to Ang 1-7 in SHR and Wistar-Kyoto rats.

We have established that high glucose induces reactive oxygen species (ROS) generation and stimulates angiotensinogen (Agt, the sole precursor of angiotensins) gene expression in renal proximal tubular cells (RPTCs) *in vitro* [12, 13]. Transgenic (Tg) mice specifically overexpressing rat Agt in their RPTCs develop hypertension, albuminuria and kidney injury [14, 15]. Hyperglycemia and Agt overexpression act in concert to elicit systolic hypertension (sHTN) and RPTC apoptosis in diabetic Agt-Tg mice [16]. More recently, we

documented that renin-angiotensin system (RAS) blockade and catalase (Cat) overexpression in RPTCs prevent sHTN and tubular ROS generation, suppress RPTC apoptosis and normalize Ace2 expression in RPTCs of diabetic Akita Agt-Tg [17] and Akita Cat-Tg mice [18], supporting the view that enhanced ROS generation and RAS activation are pivotal in down-regulation of Ace2 expression and renal injury in diabetes.

In the present study, we have investigated the impact of Ang 1-7 on sHTN, oxidative stress and kidney injury in diabetic Akita mice. Here, we report that Ang 1-7 administration prevented sHTN and renal oxidative stress, attenuated glomerular hyperfiltration, albuminuria, renal hypertrophy, tubulointerstitial fibrosis and tubular apoptosis, and inhibited profibrotic as well as pro-apoptotic gene expression. Furthermore, Ang 1-7 through MasR suppressed Agt and ACE expression and normalized renal Ace2 and MasR expression in Akita mice. Finally, Ang 1-7 suppressed the expression of renal TNF-alpha converting enzyme (TACE) and sodium/hydrogen exchanger-3 (NHE-3) in Akita mice.

3.3 RESEARCH DESIGN AND METHODS

Chemicals and Constructs

Ang 1-7 and A-779 (d-Ala^{-7}-Ang I/II/1-7) were purchased from Bachem Americas, Inc. (Torrence, CA, USA). A rabbit polyclonal antibody against rat Agt was generated in our lab (by J.S.D.C.) [19]. It is specific to intact rat and mouse Agt (55-62 kDa) and does not cross-react with pituitary hormone preparations or other rat or mouse plasma proteins. Monoclonal anti-NHE-3 antibody was a gift from Dr. Orson Moe (UT Southwestern Medical Center,

Dallas, TX). The sources of other antibodies were: polyclonal anti-bovine Cat antibody and β-actin monoclonal antibody (Sigma-Aldrich Canada Ltd.,Oakville, ON, Canada), polyclonal anti-heme-oxygenase-1 (HO-1) antibody (Assay Designs, Ann Arbor, MI, USA), monoclonal anti-collagen type IV antibody (Chemicon International, Temecula, CA, USA), polyclonal anti-transforming growth factor-beta 1 (TGF-β1), anti-NADPH oxidase 4 (Nox4) and anti-ACE (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-MasR (Novus Canada, Oakville, ON, Canada), and anti-TACE (ENZO Life Sciences Inc., Farmingdale, N.Y., USA). Polyclonal anti-nuclear factor erythroid 2-related factor 2 (Nrf2) antibody was obtained from BD Biosciences (Mississauga, ON, Canada). Oligonucleotides were synthesized by Invitrogen (Burlington, ON, Canada). Restriction and modifying enzymes were purchased from Invitrogen, Roche Biochemicals, Inc. (Dorval, QC, Canada) and GE Healthcare Life Sciences (Baie d'Urfé, QC, Canada).

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol, animal care and experimental procedures were approved by the CRCHUM Animal Care Committee. All surgery was performed under sodium pentobarbital anesthesia. Mice were euthanized by sodium pentobarbital overdose (75 mg/kg BW), and efforts were made to minimize suffering.

Physiological Studies

Male heterozygous Akita mice (C57BL/6-Ins2^{Akita}/J) were obtained from Jackson Laboratories, Bar Harbor, ME, USA (http://jaxmice.jax.org). Akita mice, an autosomal dominant model of spontaneous type 1 diabetes in which insulin gene2 is mutated, have decreased numbers of pancreatic islet β-cells and develop hyperglycemia at age 3-4 weeks, manifesting impaired renal function with increased oxidative stress markers in their renal proximal tubules (RPTs) by age 30 weeks [20, 21] and closely resembling those observed in type 1 diabetes patients. Adult Akita mice (age 14 weeks) were treated subcutaneously with Ang 1-7 (500 μg/kg^{-1}.day^{-1}) ± A-779 (10 mg/kg^{-1}.day^{-1}) and euthanized at age 20 weeks (8 mice per group), as described previously [18]. Untreated non-Akita wild type (WT) mice served as controls. All animals were given *ad libitum* access to standard mouse chow and tap water.

Systolic blood pressure (SBP) was monitored in the morning with a BP-2000 tail-cuff pressure monitor (Visitech Systems, Apex, NC, USA) at least 2-3 times per week per animal, for 10 weeks [14-18, 22-26]. The mice were habituated to the procedure for at least 15-20 min per day for 5-7 days before the first SBP measurements. SBP values were expressed as means±SEM. All animals were housed individually in metabolic cages for 24 h prior to euthanasia at age 20 weeks. Body weight was recorded. Urines were collected and assayed for albumin and creatinine by enzyme-linked immunosorbent assays (ELISAs, Albuwell and Creatinine Companion (Exocell, Inc., Philadelphia, PA, USA) [14-18, 22-26]. Immediately following euthanasia, the kidneys were removed, decapsulated and weighed. The left kidneys were processed for histology and immunostaining, and the right kidneys were harvested for isolation of RPTs by Percoll gradient [14-18, 22-26].

The glomerular filtration rate (GFR) was estimated as described by Qi et al. [27], as recommended by the Animal Models of Diabetic Complications Consortium (http://www.diacomp.org/) with slight modifications [18, 22, 28].

Urinary Ang 1-7 and Ang II Measurement

Mouse urinary Ang II and Ang 1-7 levels were quantified by specific ELISAs after extraction in accordance with the manufacturer`s protocol (Bachem Americas Inc.) and were normalized by urinary creatinine levels [17, 18, 22, 25, 26].

Histology

Four to 5 kidney sections (3-4 μm thick) per kidney from 8 animals per group were stained with Periodic acid-Schiff (PAS) or Masson's trichrome, and assessed by 2 independent, blinded observers under light microscopy. The collected images were analyzed and quantified by the NIH ImageJ software (http://rsb.info.nih.gov/ij/) [14-18, 22-26].

Mean glomerular volume on 30 random glomerular sections per mouse was assessed by Weibel's method [29] with Motic Images Plus 2.0 analysis software (Motic Instruments, Inc., Richmond, BC, Canada) [17, 18, 22, 26]. Tubular luminal areas were measured on renal sections (6 animals/group, 4 to 5 sections per kidney, 4 random fields per section, 10 tubules around the glomerulus per field) with Motic Images Plus 2.0 analysis software [17, 18, 22, 26]. Outer cortical RPTs with similar cross-sectional views and clear nuclear structures were selected. Mean cell volume was estimated by nucleation [30], as described elsewhere [17, 18, 22, 26].

Immunohistochemical staining was performed according to the standard avidin-biotin-peroxidase complex method (ABC Staining System, Santa Cruz Biotechnology) [14-18, 22-26]. Immunostaining with non-immune normal rabbit serum in non-Akita mouse kidneys served as control, with no immunostaining being observed (photographs not included). Oxidative stress in RPTs *in vivo* was assessed by staining of frozen kidney sections with dihydroethidium (DHE; Sigma-Aldrich Canada Ltd.) and 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA; Life Technologies, Burlington, ON, Canada) [18, 31]. In these assays, non-fluorescent DHE is oxidized to fluorescent ethidium by superoxide anion whereas non-fluorescent carboxy-H2DCFDA is oxidized to fluorescent carboxydichlorofluorescein by intracellular ROS (all species). The results were confirmed by standard immunohistochemical staining for HO-1, an oxidative stress-inducible gene that confers cellular oxidative stress *in vivo* [18, 25, 32], Nox4 (a constitutively-expressed and predominant form of Nox in diabetic kidneys) [33, 34] and Nrf2 (a master regulator of redox balance in cellular cytoprotective responses) [35]. The percentage of apoptotic RPTCs was estimated semiquantitatively by terminal transfer-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) assay (Roche Biochemicals, Inc., Laval, QC, Canada) [15-18, 22-26].

Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR) Assays for Gene Expression

Agt, ACE, Ace2, MasR, collagen IV, TGF-β1, Nox1, Nox2, Nox4, HO-1, Nrf2, Bax, Bcl-xL, TACE, NHE-3 and β-actin mRNA expression in RPTs was quantified by RT-qPCR with forward and reverse primers as described [15-18, 22-26] (**Supplemental Table I**).

Western Blotting for Estimation of Protein Expression

Western blotting for Nox4, Nrf2, HO-1, Agt, Ace2, ACE, MasR, TACE and NHE-3 performed on RPT lysates [15-18, 22-26]. The membranes were first blotted with anti-Nox4, Nrf2, HO-1, Agt, Ace2, ACE, MasR, TACE and NHE-3 antibodies and then re-blotted with anti-β-actin monoclonal antibodies and developed with chemiluminescent developing reagent (Roche Biochemicals, Inc.). The relative densities of Nox4, Nrf2, HO-1, Agt, Ace2, ACE, MasR, TACE, NHE-3 and β-actin bands were quantified by computerized laser densitometry (ImageQuant software (version 5.1), Molecular Dynamics, Sunnyvale, CA, USA).

Statistical Analysis

Statistically significant differences between the experimental groups were analyzed by Student's t-test or one-way ANOVA and Bonferroni correction as appropriate. Data are expressed as means ± SEM. $P<0.05$ was considered to be statistically significant.

3.4 RESULTS

Physiological Parameters in Akita Mice With or Without Ang 1-7 Administration

We have previously reported significant differences in SBP between Akita and non-Akita WT mice as early as 8 weeks of age [17, 18, 22, 26]. These differences increased with age (from week 14 until week 20). Ang 1-7 administration protected Akita mice against SBP elevation compared to non-Akita mice. This protective effect of Ang 1-7 on SBP in Akita mice was reversed by A779 co-administration (**Table I**).

At 20 weeks, Akita mice exhibited elevated kidney weight to body weight and heart weight to body weight ratios, higher urinary albumin/creatinine ratio (ACR) and GFR than non-Akita mice **(Table I)**. Ang 1-7 markedly attenuated these parameters in Akita mice, and its effects (except for urinary ACR and GFR) were reversed by A779 co-administration (**Table I**). In contrast, blood glucose levels did not differ significantly in Akita mice with or without Ang 1-7 treatment (**Table I**).

Cat Expression and Oxidative Stress in Akita Mouse Kidneys

We have reported that Cat expression was RPTC-specific and co-localized to aquaporin-1-positive RPTCs [18]. We detected lower Cat levels in RPTCs from Akita (Figure 1A, b) than in non-Akita WT mice (Figure 1A, a). Ang 1-7 normalized Cat expression in Akita mice (Figure 1A, c), and its effect was reversed by A779 (Figure 1A, d). DHE and carboxy-H2DCFDA staining demonstrated higher levels of oxidative stress in RPTCs from Akita mice (Figures 1B and 1C, panels b) than in non-Akita WT mice (Figures 1B and 1C, panels a). Ang 1-7 decreased oxidative stress in Akita mice (Figures 1B and 1C, panel c), which was reversed by A779 (Figures 1B and 1C, panels d). Quantitation of Cat activity, DHE and carboxy-H2DCFDA staining confirmed these findings (Figures 1D, 1E and 1F, respectively). These results confirmed down-regulation of Cat expression and activity in diabetic Akita mice. Ang 1-7 enhanced Cat expression and activity and effectively attenuated ROS production and its effects were reversed by A779 co-administration

ROS generation (Figure 2A), NADPH oxidase activity (Figure 2B), Nox4 mRNA and protein expression (Figure 2C-E) were significantly elevated in the kidneys of Akita than in non-Akita mice. Administration of Ang 1-7 normalized these changes and these actions were

reversed by A779. Interestingly, Nox1 and Nox2 mRNA expression did not differ significantly among different groups (Figure 2C).

Consistently, immunostaining for the oxidative stress-inducible proteins Nrf2 and HO-1 was significantly higher in the kidneys of Akita (Figures 3A and 3B, panels b) than in non-Akita WT mice (Figures 3A and 3B, panels a). Once again, Ang 1-7 normalized Nrf2 and HO-1 immunostaining (Figures 3A and 3B, panels c) and these actions were reversed by A779 (Figures 3A and 3B, panel d). Western blotting for Nrf2 (Figure 3C) and HO-1 (Figure 3D) and quantifying Nrf2 mRNA (Figure 3E) and HO-1 mRNA expression (Figure 3F) confirmed these findings. Collectively, these results document Ang 1-7 attenuation of enhanced oxidative stress in diabetic Akita mice.

Effect of Ang 1-7 on Agt, Ace2, ACE, MasR, TACE and NHE-3 Expression in Akita Kidneys

Consistent with previous observations [17, 18, 22, 26], Agt immunostaining was increased significantly in Akita mice (Figure 4A, b) compared to WT controls (Figure 4A, a). Ang I-7 attenuated Agt expression in Akita mice (Figure 4A, c), and its effect was reversed by A779 (Figure 4A, d). Quantitation of Agt protein and Agt mRNA expression by respective WB and qPCR confirmed these findings (Figure 4B and 4C, respectively). Furthermore, urinary Ang II levels were significantly higher in Akita than in non-Akita WT mice (Figure 4D). Ang 1-7 normalized urinary Ang II levels in Akita mice with reversal by A779. In contrast, urinary Ang 1-7 levels were significantly lower in Akita mice than in non-Akita WT mice (Figure 4E). Ang 1-7 administration normalized urinary Ang 1-7 levels in Akita mice with partial, statistically not significant reversal by A779.

The RPTCs of WT controls (Figure 5A, a) exhibited decreased ACE staining relative to Akita mice (Figure 5A, b). Ang 1-7 normalized ACE immunostaining with reversal by A779 co-administration in RPTCs from Akita mice (Figure 5A, c and d, respectively). In contrast, Ace2 and MasR expression in the RPTCs of non-Akita WT controls (Figures 5B and 5C, panels a respectively) was significantly higher than in Akita mice (Figure 5B and 5C, panels b, respectively). Ang 1-7 normalized Ace2 and MasR immunostaining in RPTCs of Akita mice Figures 5B and 5C, c respectively), and its effects were reversed by A779 co-administration (Figures 5B and 5C, panels d, respectively). We confirmed these findings by immunoblotting and qPCR for ACE, Ace2 and MasR protein (Figure 5D) and their respective mRNA expression in isolated RPTs (Figure 5E)

In contrast to Ace2 and MasR expression, TACE and NHE-3 expression (Figures 6A and 6B, b) were reduced in RPTCs of Akita mice as compared to WT mice (Figures 6A and 6B, a). Ang 1-7 administration normalized TACE and NHE-3 expression in Akita mice (Figures 6A and 6B, c). These actions were reversed by A779 (Figures 6A and 6B, d). These findings were confirmed by immunoblotting for TACE and NHE-3 protein (Figures 6C and 6D, respectively) in freshly isolated RPTs. TACE and NHE-3 mRNA levels, however, did not differ among different groups (Figures 6E and 6F, respectively), as quantified by qPCR.

Effect of Ang 1-7 on Renal Fibrosis and Profibrotic Gene Expression in Kidneys of Akita Mice

Unlike WT non-Akita mice (Figure 7A, a), Akita mice exhibited renal structural damage (Figure 7A, b). Histological findings included tubular luminal dilatation and accumulation of cell debris in the tubular lumen. Some RPTCs were flattened. Remarkably, Ang 1-7

administration in Akita mice markedly suppressed, but never completely prevented these abnormalities (Figure 7A, c). A779 co-administration partially reversed Ang 1-7's effect (Figure 7A, d). We observed significantly-increased glomerular tuft volume, tubular luminal area and RPTC volume in Akita compared to non-Akita WT mice **(Table I)**. Ang 1-7 partially reduced tubular luminal area and glomerular tuft volume, and completely normalized RPTC volume in Akita mice. Again, co-A779 administration partially reversed the effect of Ang 1-7 on these parameters (**Table I**).

We assessed the expression of collagenous components with Masson's trichrome staining (Figure 7B) and immunostaining for collagen type IV (Figure 7C) and TGF-β1 (Figure 7D). Kidneys from non-Akita WT exhibited significantly lower collagenous contents, collagen type IV and TGF-β1 (Figures 7B, 7C and 7D, panels a, respectively) relative to Akita mice (Figures 7B, 7C and 7D, panels b, respectively). Ang 1-7 markedly reduced glomerulotubular fibrosis (Figures 7B, 7C and 7D, panels c, respectively), and its effect was reversed by A779 co-administration Figures 7B, 7C and 7D, panels d, respectively). Quantitative analysis of Masson's trichrome staining (Figure 7E) and collagen immunostaining (Figure 7F), collagen IV mRNA (Figure 7G) and TGF-β1 mRNA (Figure 7H) expression confirmed these findings. Collectively, the data indicated that Ang 1-7 effectively prevented renal fibrosis in Akita mice.

Effect of Ang 1-7 on Tubular Apoptosis in Akita Kidneys

Next, we investigated the impact of Ang 1-7 administration on tubular apoptosis in Akita mice by TUNEL assay. The number of TUNEL-positive nuclei in RPTCs from non-Akita WT mice (Figure 8A, a) were significantly lower than in Akita mice (Figure 8A, b).

Ang 1-7 significantly reduced the number of TUNEL-positive cells in Akita mice (Figure 8A, c) and its effect was reversed by A779 (Figure 8A, d). Consistently, active (cleaved) caspase-3 expression was lower in RPTCs from non-Akita WT controls (Figure 8B, a) but higher in Akita mice (Figure 8B, b). Ang 1-7 attenuated active caspase-3 expression in Akita mice (Figure 8B, c) with its reversal by A779 (Figure 8B, d). Quantitation of TUNEL-positive cell numbers (Figure 8C) and caspase-3 activity assays in isolated RPTs (Figure 8D) confirmed these findings.

Akita mice exhibited increased Bax mRNA expression (Figure 8E) and decreased Bcl-xL mRNA expression (Figure 8F) compared to non-Akita WT mice. These changes were normalized in Akita mice by Ang 1-7 and the actions of Ang 1-7 were reversed by A779 (Figures 8E and 8F).

3.5 DISCUSSION

Our study demonstrates that Ang 1-7 treatment in Akita mice effectively attenuates oxidative stress, normalizes Ace2 and MasR expression in RPTCs, and suppresses expression of pro-hypertensive, pro-fibrotic and pro-apoptotic proteins. These findings indicate an important role for Ang 1-7 in regulating renal oxidative stress and subsequently modulating sHTN and kidney injury *in vivo*.

Expanding our previous findings that Cat overexpression in RPTCs normalizes Ace2 expression and prevents hypertension, reduces tubulointerstitial fibrosis and RPTC apoptosis in Akita Cat-Tg mice [18], here, we show that Ang 1-7 attenuates sHTN, indicating that intrarenal Ang 1-7 formation is critical for counteracting Ang II's impact in Akita mice. These

observations highlight an important role of intrarenal Ace2 expression and Ang 1-7 formation in thwarting hypertension and nephropathy development in diabetic mice.

The mechanisms underlying SBP elevation in Akita mice are largely unknown. The possibility that down-regulation of Ace2 and Mas receptor gene expression and consequently high Ang II/Ang 1-7 ratios facilitate the development of hypertension has received considerable attention [5-7]. Indeed, our present findings demonstrate significantly lower RPTC Ace2, MasR and urinary Ang 1-7 and higher RPTC ACE and urinary Ang II levels in Akita mice than in non-Akita WT. Ang 1-7 normalized these changes and A779 co-administration reversed the effects of Ang 1-7. These observations are consistent with our previous findings of markedly-elevated ACE and depressed Ace2 expression in the kidneys of Akita Agt-Tg mice [17] as well as with studies on normotensive Lewis rats, which showed that RAS blockade increases cortical Ace2 activity and urinary Ang 1-7 excretion [36]. Normal human kidneys express low ACE and high Ace2 levels, and this ratio is reversed in the kidneys of hypertensive and diabetic patients [37-39]. Furthermore, Ang II has been observed to up-regulate ACE and down-regulate Ace2 expression in HK2 cells *in vitro* [37]. Taken together, our current data lend support to the concept that intrarenal RAS activation up-regulates Agt and ACE expression and down-regulates Ace2 and Mas receptor expression via enhanced oxidative stress in RPTCs, ultimately contributing to hypertension development.

The mechanisms underlying the antihypertensive action of Ang 1-7 are not well-defined. One possibilty is that Ang 1-7 prevents or attenuates the influence of Ang II on glomerular arterioles, thereby lowering glomerular pressure (hyperfiltration), glomerular tuft volume and, eventually, SBP. This possibility is supported by the studies by Benter et al [9, 10], which showed that Ang 1-7 reduces renal vascular resistance in diabetic hypertensive rats.

Furthermore, other studies have shown that recombinant human Ace2 attenuates Ang II-dependent and pressure-overload-induced hypertension in Ace2 knockout mice [40-42], supporting an important counterregulatory role for Ang 1-7 in Ang II-mediated sHTN and renal abnormalities.

Another possibility is that Ang 1-7 could affect TACE (an enzyme responsible for Ace2 shedding) and NHE-3 (a key transporter mediating sodium reabsorption in the RPTs). Indeed, our data show increased renal TACE and NHE-3 expression in the RPTCs of Akita mice as compared to WT mice. Ang 1-7 administration normalized these changes and the effects of Ang 1-7 were reversed by A779. These findings suggest that the SBP lowering action of Ang 1-7 is mediated, at least in part, via down-regulation of TACE and NHE-3 expression in the RPTs in Akita mice.

Since glomerular hyperfiltration and microalbuminuria are early clinical markers of hypertension- or diabetes-induced nephropathy, we monitored the GFR and urinary albuminuria, and detected enhanced GFR and microalbuminuria in Akita mice at age 20 weeks. Ang 1-7 significantly reduced, but never completely prevented these changes. Surprisingly, A779 co-administration could not completely overturn Ang 1-7's effect on the GFR and albuminuria despite complete reversal of SBP in Akita mice. The exact reasons for such discordant inhibitory actions remain largely unknown. One possibility is that different subtype(s) of Ang 1-7 receptor might be present in podocytes that are less sensitive to A779 inhibition. This notion is further supported by the studies of Silva et al [43], which showed that Ang 1-7 receptor is sensitive to D-pro^{7}-Ang 1-7 but not to A779 in the aorta of Sprague-Dawley rats. Clearly, additional studies are needed to address this issue in podocytes.

The mechanism by which oxidative stress leads to interstitial fibrosis in Akita mice is far from being fully understood. It is possible that augmented ROS generation via enhanced Agt/Ang II expression would stimulate TGF-β1, subsequently heightening the expression of extracellular matrix proteins, collagen type IV, profibrotic and pro-apoptotic proteins in RPTCs, with resultant apoptosis and, ultimately, interstitial fibrosis [44]. Indeed, neutralizing TGF-β alleviates fibrosis and tubular cell apoptosis in animal models of diabetes [45]. Our present study shows higher TGF-β1 and collagen IV expression in RPTs from Akita than non-Akita mice. Ang 1-7 mitigated these changes, thus linking intrarenal oxidative stress to interstitial fibrosis.

To further investigate the role of oxidative stress in mediating the underlying mechanism(s) of Ang 1-7 action on SBP regulation, we treated Akita mice with the ROS scavenger tempol (4-hydroxy-tempol) or with tempol and Ang 1-7. Confirming the observations of Fujita et al [46], tempol had no effect on SBP, whereas it attenuated renal oxidative stress in Akita mice (Supplemental Figure 1). In contrast, co-administration of tempol and Ang 1-7 effectively lowered SBP and reduced renal oxidative stress in Akita mice. The reasons why tempol failed to lower SBP in Akita mice are not known at the present.

Confirming our previous findings in Akita kidneys [17, 18, 22, 26], we detected higher number of apoptotic RPTCs in Akita mice, evidenced by higher percentages of TUNEL-positive RPTCs in parallel with increased active caspase-3 immunostaining and Bax mRNA expression and decreased Bcl-xL mRNA expression. Elevated Bax/Bcl-xL ratios are consistent with tubular apoptosis in Akita mice. Once again, these changes were mitigated in Akita mice by Ang 1-7 and the actions of Ang 1-7 were reversed by A779.

Our results may have clinical implications for the assessment of progression in type 1 diabetes. Tubulointerstitial fibrosis and tubular apoptosis occur in the kidney in human type 1 diabetes [47], and tubular atrophy appears to be a better indicator of disease progression than glomerular pathology [48]. We postulate that tubulointerstitial fibrosis and RPTC apoptosis may be the initial events leading to tubular atrophy in diabetes. Oxidative stress-mediated decreases of Cat, Ace2 and MasR expression could further accelerate this process.

In summary, our data indicate an important role of Ang 1-7 in inhibiting intrarenal oxidative stress and subsequent prevention of the development of hypertension and renal injury in Akita mice.

3.6 CLINICAL PERSPECTIVES

1. Enhanced intrarenal Agt gene expression/RAS activation induces systemic hypertension and kidney injury in diabetes and its effects can be countered by Ang 1-7; However, the molecular mechanism(s) underlying the beneficial actions of Ang 1-7 on systemic hypertension and kidney injury are not fully understood.
2. In the present study in diabetic Akita mice, Ang 1-7 administration normalized systemic hypertension and attenuated glomerular injury and tubulointerstitial fibrosis. Ang 1-7 decreased oxidative stress and expression of pro-hypertensive genes, and normalized the expression of Ace2 and MasR in the kidneys. Co-administration of A779, an antagonist of MasR effectively reversed most of the effects of Ang 1-7.

3. Our results indicate the potential of Ang 1-7 as a therapeutic agent for treatment of systemic hypertension and kidney injury in diabetes.

AUTHOR CONTRIBUTION

Y.S. performed research data, and contributed to discussion. C-S. L. performed research data, and contributed to discussion. R.P. performed research data. S.A. performed research data. I.C. performed research data. J.G.F. contributed to discussion, and reviewed/edited manuscript. J.R.I. contributed to discussion, and reviewed/edited manuscript. S-L.Z. performed research data, contributed to discussion and reviewed/edited manuscript. J.S.D.C. contributed to discussion, wrote manuscript, and reviewed/edited manuscript. J.S.D.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

FUNDING

This work was supported in part by grants from the Kidney Foundation of Canada to J.S.D.C. (KFOC120008), the Canadian Institutes of Health Research (MOP-93650 and MOP-106688 to J.S.D.C., MOP-86450 to S.L.Z. and MOP-97742 to J.G.F.), the National Institutes of Health (NIH) of USA (HL-48455 to J.R.I.) and the Foundation of the CHUM.

ABBREVIATIONS

ACE, angiotensin-converting enzyme; Ace2, angiotensin-converting enzyme-2; ACR, albumin-creatinine ratio; Agt, angiotensinogen; Ang II, angiotensin II; Ang 1-7, angiotensin 1-7; carboxy-H2DCFDA, 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate; Cat, catalase; DHE, dihydroethidium; ELISA, enzyme-linked immunosorbent assay; GFR, glomerular filtration rate; HO-1, heme-oxygenase-1; Nox4, NADPH oxidase 4; Nrf2, Nuclear factor erythroid 2-related factor 2; RAS, renin-angiotensin system; ROS, reactive oxygen species; RPTs, renal proximal tubules; RPTCs, renal proximal tubular cells; RT-qPCR, real time-quantitative polymerase chain reaction; SBP, systolic blood pressure; SHR, spontaneously hypertensive rats; sHTN, systolic hypertension; Tg, transgenic; TGF-β1, transforming growth factor-beta 1; TUNEL, terminal transferase-mediated deoxyuridine triphosphate nick end-labeling assay; WT, wild type

3.7 Acknowledgments

Our manuscript or any significant part of it is not under consideration for publication elsewhere. Parts of the data ha been presented as a poster communicationat the 43rd Annual Meeting of the American Society of Nephrology, Atlanta, GA, USA, November 5-10, 2013. Editorial assistance was provided by the CRCHUM Research Support Office and Ovid Da Silva.

Disclosures

None

3.8 Reference List

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3.9 Legends and Figures

Table 3-1. Physiological Measurements

	WT	Akita	Akita + Ang 1-7	Akita + Ang 1-7 + A779
(A) Systolic blood pressure (SBP)	107.00±0.79	136.50±2.83	117.6±1.09	133.9±2.47
(B) Blood Glucose (mmol/L)	9.51±0.75	35.47±0.72***	35.19±0.52***	35.28±0.65***
(C) Body Weight (BW) (g)	30.62±0.70	24.4±0.45***	23.5±0.40***	24.43±0.44***
(D) Kidney Weight (KW) (mg)	366.2±8.14	547.6±18.42***	415.6±13.03†††	429±12.15†††
(E) KW/BW ratio (mg/g)	11.85±0.29	20.28±0.37***	18.2±0.64***,††	17.26±0.24***,†††
(F) GFR (uL/min)/ BW (g)	10.20±0.79	22.00±1.96 ***	16.03±0.92 **,†	19.68±1.17 ***
(G) ACR (μg/ml/mg/dL)	0.28 ±0.02	3.14 ±0.87***	0.67 ±0.16†††	0.70 ±0.13†††
(H) Glomerular tuft volume (X 10^3 μm^3)	110.3 ±12.17	178.2 ±15.36**	125.2 ±11†	148.7 ±8.31
(I) RPTC volume (X 10^3 μm^3)	6.12 ±0.26	10.77 ±0.39***	6.76 ±0.22†††	10.92 ±0.30***
(J) Tubular laminar Area (μm^2)	86.51±2.28	184.00±5.32	96.42±6.23†††	177.00±3.42***

*P<0.05 **P<0.01 and ***P<0.005 vs WT; †P<0.05 ††P<0.01 and †††P<0.005 vs Akita mice

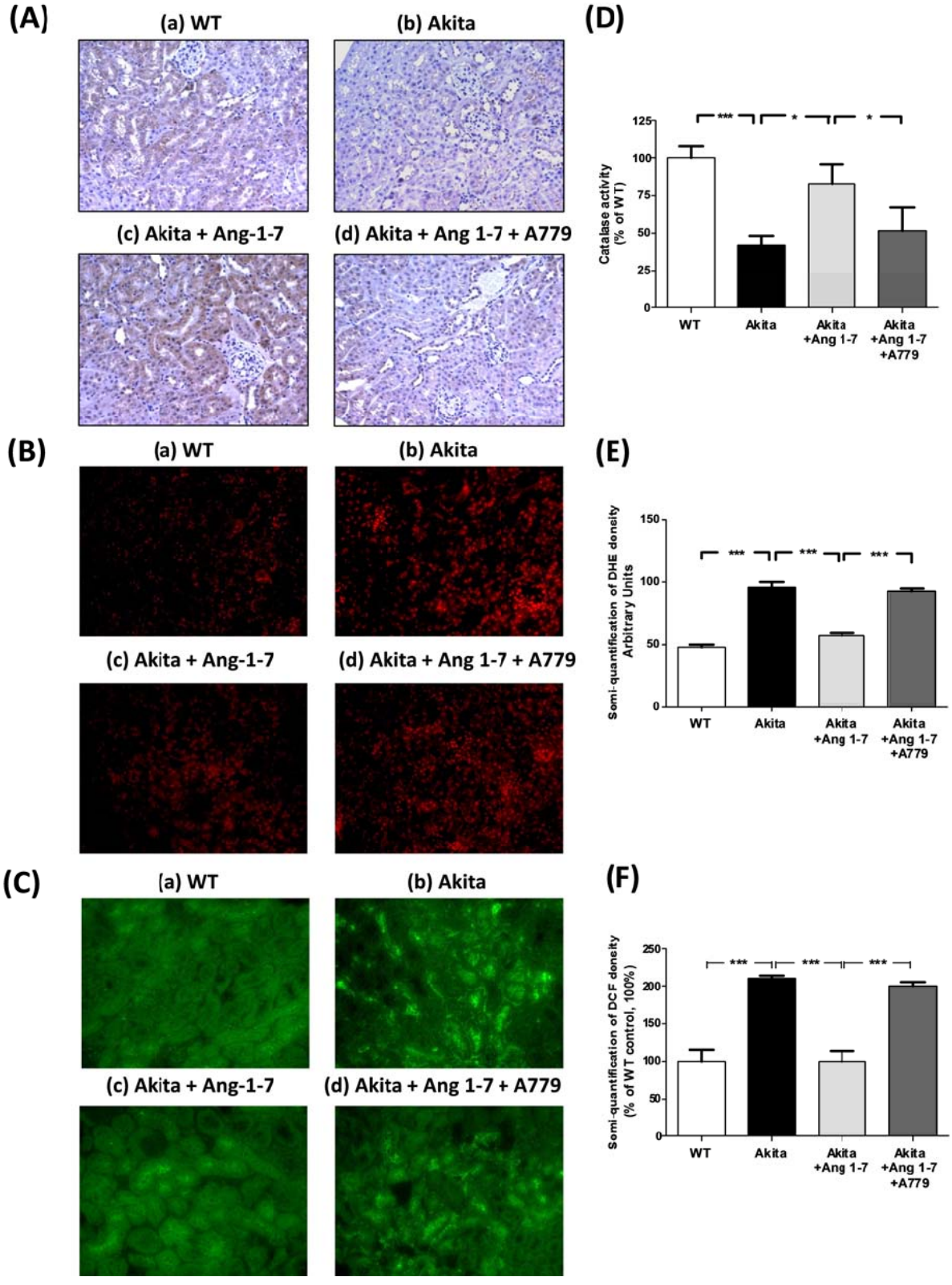

(A)
(a) WT
(b) Akita
(c) Akita + Ang-1-7
(d) Akita + Ang 1-7 + A779
(B)
(a) WT
(b) Akita
(c) Akita + Ang-1-7
(d) Akita + Ang 1-7 + A779
(C)
(a) WT
(b) Akita
(c) Akita + Ang-1-7
(d) Akita + Ang 1-7 + A779
(D)
Catalase activity (% of WT)
125
100
75
50
25
0
***
*
*
WT
Akita
Akita +Ang 1-7
Akita +Ang 1-7 +A779
(E)
Semi-quantification of DHE density Arbitrary Units
150
100
50
0
***
***
***
WT
Akita
Akita +Ang 1-7
Akita +Ang 1-7 +A779
(F)
Semi-quantification of DCF density (% of WT control, 100%)
250
200
150
100
50
0
***
***
***
WT
Akita
Akita +Ang 1-7
Akita +Ang 1-7 +A779

Figure 3-1: Immunohistochemical staining for Cat (A), dihydroethidium (DHE) (red) (B) and 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) (green) (C) in male mouse kidneys at age 20 weeks. Magnification X200. Quantitation of Cat activity (D) and semi-quantitation of DHE (E) and carboxy-H2DCFDA (F) fluorescence in mouse kidneys. Values are expressed as means ± SE (n=8 per group). *P<0.05, ***P<0.005, N.S., non-significant.

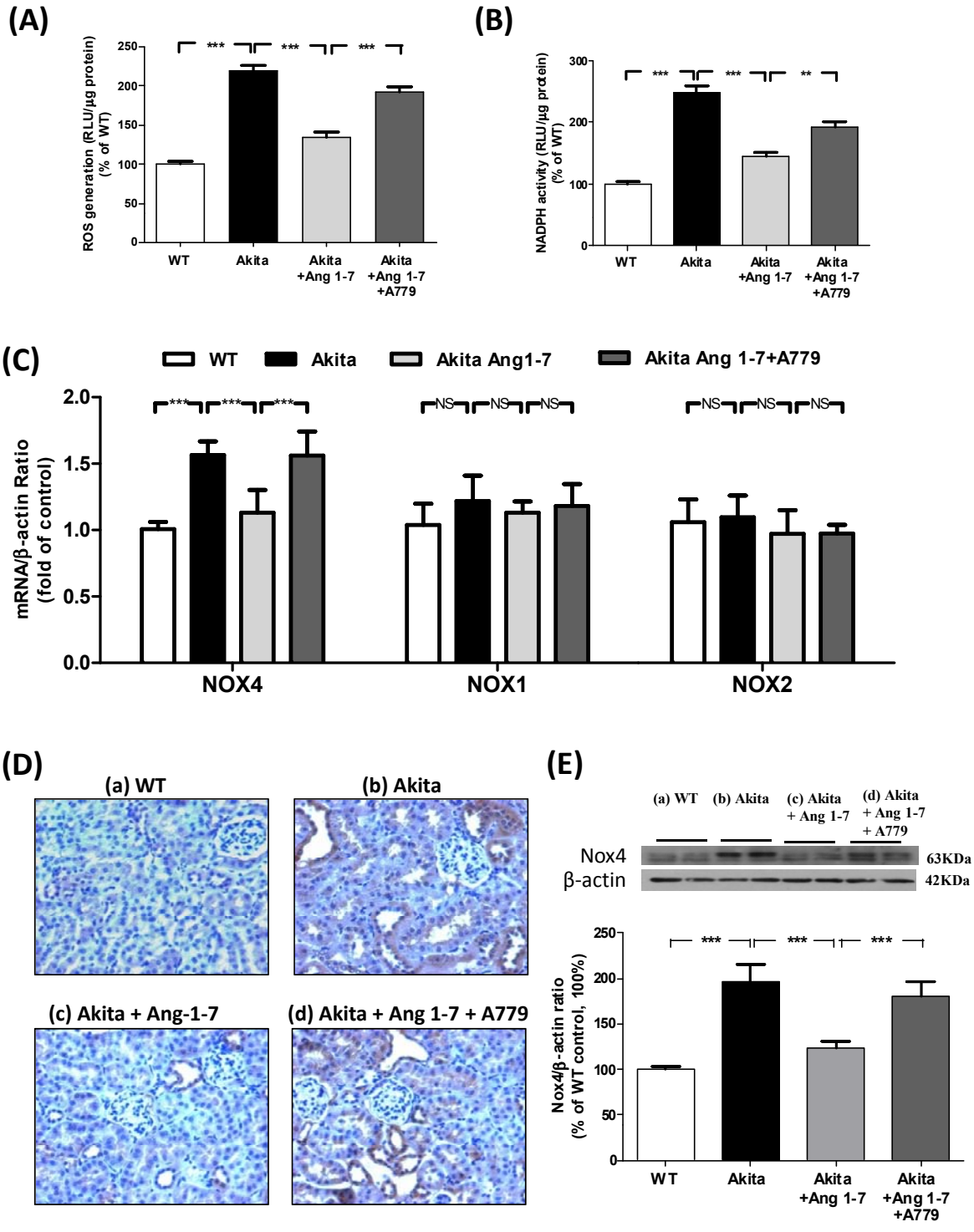

(A)
ROS generation (RLU/μg protein) (% of WT)
250
200
150
100
50
0
***
***
***
WT
Akita
Akita +Ang 1-7
Akita +Ang 1-7 +A779
(B)
NADPH activity (RLU/μg protein) (% of WT)
300
200
100
0
***
***
**
WT
Akita
Akita +Ang 1-7
Akita +Ang 1-7 +A779
(C)
WT
Akita
Akita Ang1-7
Akita Ang 1-7+A779
mRNA/β-actin Ratio (fold of control)
2.0
1.5
1.0
0.5
0.0
***
***
***
NS
NS
NS
NS
NS
NS
NOX4
NOX1
NOX2
(D)
(a) WT
(b) Akita
(c) Akita + Ang-1-7
(d) Akita + Ang 1-7 + A779
(E)
(a) WT
(b) Akita
(c) Akita + Ang 1-7
(d) Akita + Ang 1-7 + A779
Nox4
β-actin
63KDa
42KDa
Nox4/β-actin ratio (% of WT control, 100%)
250
200
150
100
50
0
***
***
***
WT
Akita
Akita +Ang 1-7
Akita +Ang 1-7 +A779

Figure 3-2: ROS generation, NADPH oxidase activity, Nox 1, 2 and 4 expression in mouse RPTs at age 20 weeks. ROS generation (A) and NADPH oxidase activity (B) were quantified by lucigenin assays. Nox1, Nox2 and Nox 4 mRNA expression in mouse RPTs were quantified by qPCR (C). Immunostaining (D) and Western blotting (E) for Nox4 in mouse RPTs. Membranes were blotted for Nox4, then re-blotted for β-actin. Nox4 levels were normalized by corresponding β-actin levels. Corresponding values in non-Akita control littermates were considered as 100%. Values are expressed as means±SEM (n=8). **P*<0.05, ***P*<0.01, ****P*<0.005, N.S., non-significant.

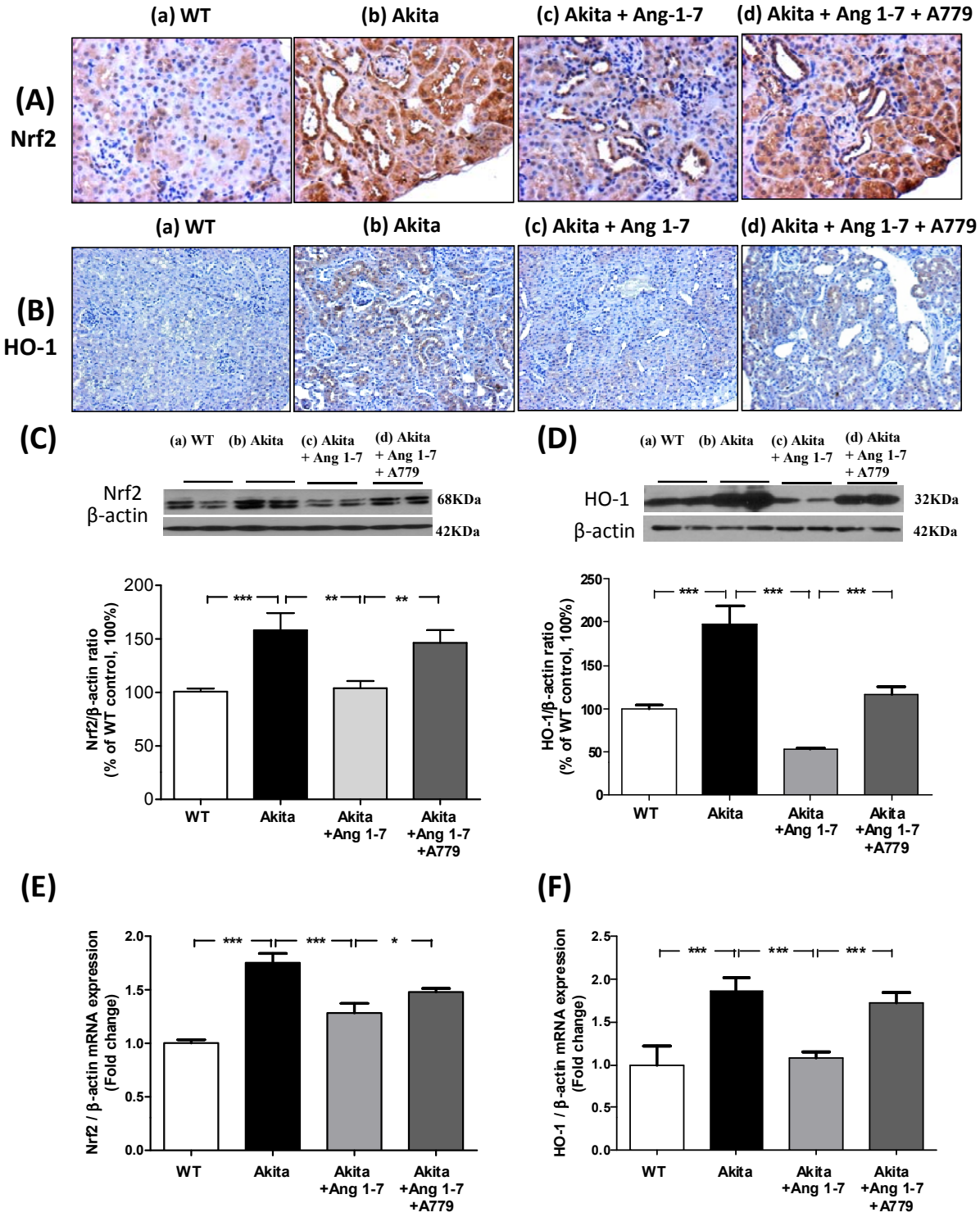

(a) WT
(b) Akita
(c) Akita + Ang-1-7
(d) Akita + Ang 1-7 + A779
(A)
Nrf2
(a) WT
(b) Akita
(c) Akita + Ang 1-7
(d) Akita + Ang 1-7 + A779
(B)
HO-1
(C)
(a) WT
(b) Akita
(c) Akita + Ang 1-7
(d) Akita + Ang 1-7 + A779
Nrf2
β-actin
68KDa
42KDa
(D)
HO-1
β-actin
32KDa
42KDa
Nrf2/β-actin ratio (% of WT control, 100%)
HO-1/β-actin ratio (% of WT control, 100%)
WT
Akita
Akita +Ang 1-7
Akita +Ang 1-7 +A779
(E)
(F)
Nrf2 / β-actin mRNA expression (Fold change)
HO-1 / β-actin mRNA expression (Fold change)

Figure 3-3: Nrf2 and HO-1 expression in mouse kidneys at age 20 weeks. Nrf2 (A) and HO-1 (B) immunostaining. Magnification X200. Semi-quantitation of WB of Nrf2 (C) and HO-1 (D) in mouse kidneys. Values in non-Akita control littermates were considered as 100%. Quantitation of Nrf2 (E) and HO-1 (F) mRNA level by qPCR in mouse kidneys. Values in non-Akita control littermates were considered as arbitrary unit 1. Values are expressed as means ± SE (n=6). *P<0.05, **P<0.01, ***P<0.005, N.S., non-significant.

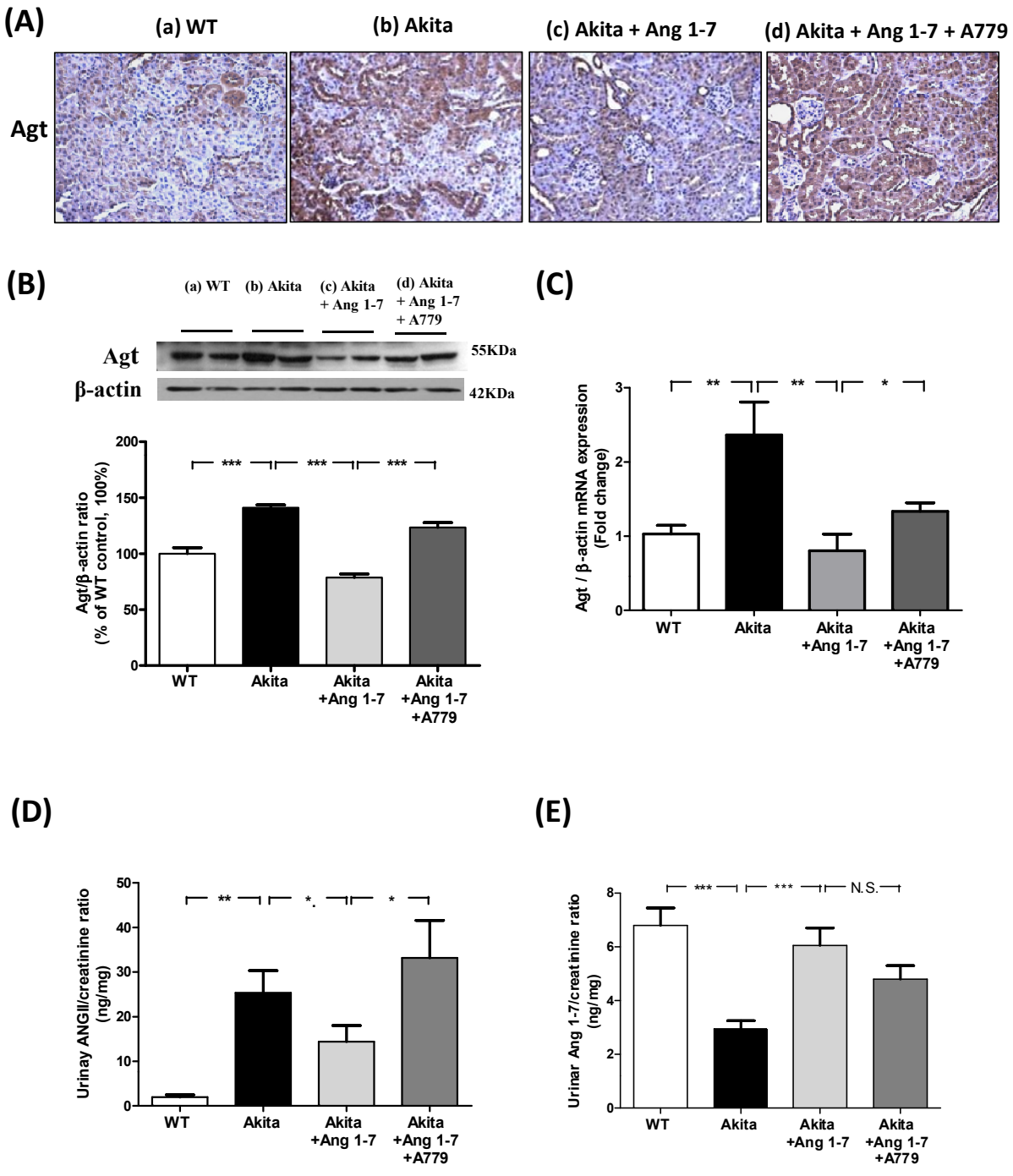

(A)
(a) WT
(b) Akita
(c) Akita + Ang 1-7
(d) Akita + Ang 1-7 + A779
Agt
(B)
(a) WT
(b) Akita
(c) Akita + Ang 1-7
(d) Akita + Ang 1-7 + A779
Agt
55KDa
β-actin
42KDa
Agt/β-actin ratio (% of WT control, 100%)
WT
Akita
Akita +Ang 1-7
Akita +Ang 1-7 +A779
(C)
Agt / β-actin mRNA expression (Fold change)
WT
Akita
Akita +Ang 1-7
Akita +Ang 1-7 +A779
(D)
Urinay ANGII/creatinine ratio (ng/mg)
WT
Akita
Akita +Ang 1-7
Akita +Ang 1-7 +A779
(E)
Urinar Ang 1-7/creatinine ratio (ng/mg)
N.S.
WT
Akita
Akita +Ang 1-7
Akita +Ang 1-7 +A779

Figure 3-4: Agt expression in mouse kidneys at age 20 weeks. Agt (A) immunostaining. Magnification X200. Western blotting of Agt protein (B) and qPCR of its mRNA level (D) in mouse RPTs. Values in non-Akita control littermates were considered as 100% control or arbitrary unit 1 . Values are expressed as means ± SE (n=8). $^{**}P<0.01$, $^{***}P<0.005$, N.S., non-significant. Urinary Ang II (D) and Ang 1-7 (E) levels. Urinary Ang II and Ang 1-7 levels in non-Akita control littermates were expressed as controls. Values are expressed as means ± SE (n=8). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$, N.S., non-significant.

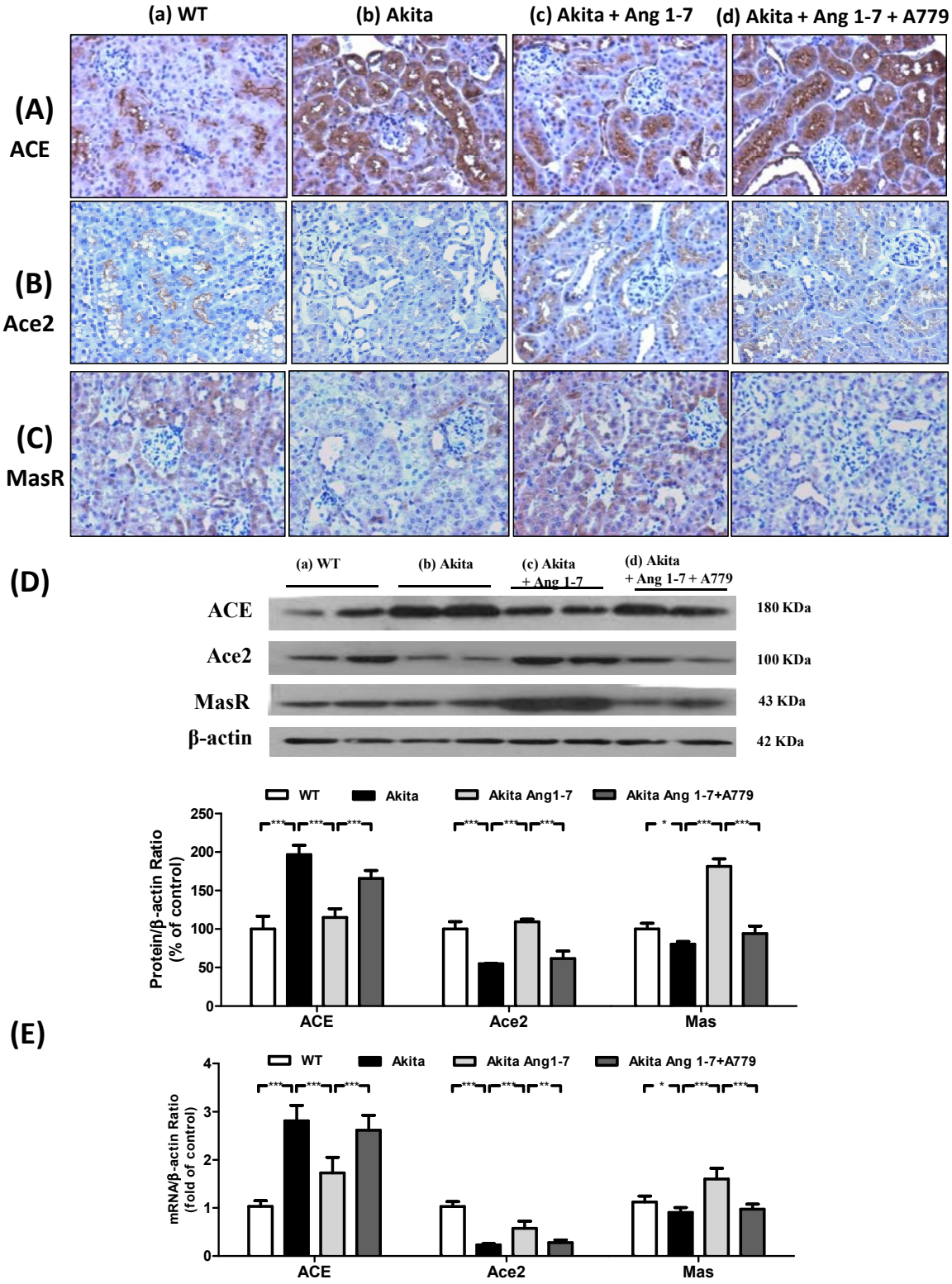

(a) WT
(b) Akita
(c) Akita + Ang 1-7
(d) Akita + Ang 1-7 + A779
(A)
ACE
(B)
Ace2
(C)
MasR
(D)
(a) WT
(b) Akita
(c) Akita + Ang 1-7
(d) Akita + Ang 1-7 + A779
ACE
Ace2
MasR
β-actin
180 KDa
100 KDa
43 KDa
42 KDa
(E)
WT
Akita
Akita Ang1-7
Akita Ang 1-7+A779
Protein/β-actin Ratio (% of control)
mRNA/β-actin Ratio (fold of control)
ACE
Ace2
Mas

Figure 3-5: Immunostaining for ACE (A), Ace2 (B) and MasR (C) expression in mouse kidneys at age 20 weeks. Magnification X200. Western blotting of ACE, Ace2 and MasR (D) in mouse RPTs. Membranes were re-blotted for β-actin. ACE, Ace2 and MasR levels were normalized by corresponding β-actin levels. Values in non-Akita control littermates were considered as 100% control. q-PCR of ACE, Ace2 and MasR mRNA levels in mouse kidneys (E). Values in non-Akita control littermates were considered as arbitrary unit 1. Values are expressed as means ± SE (n=8). *P<0.05, ***P<0.005, N.S., non-significant.

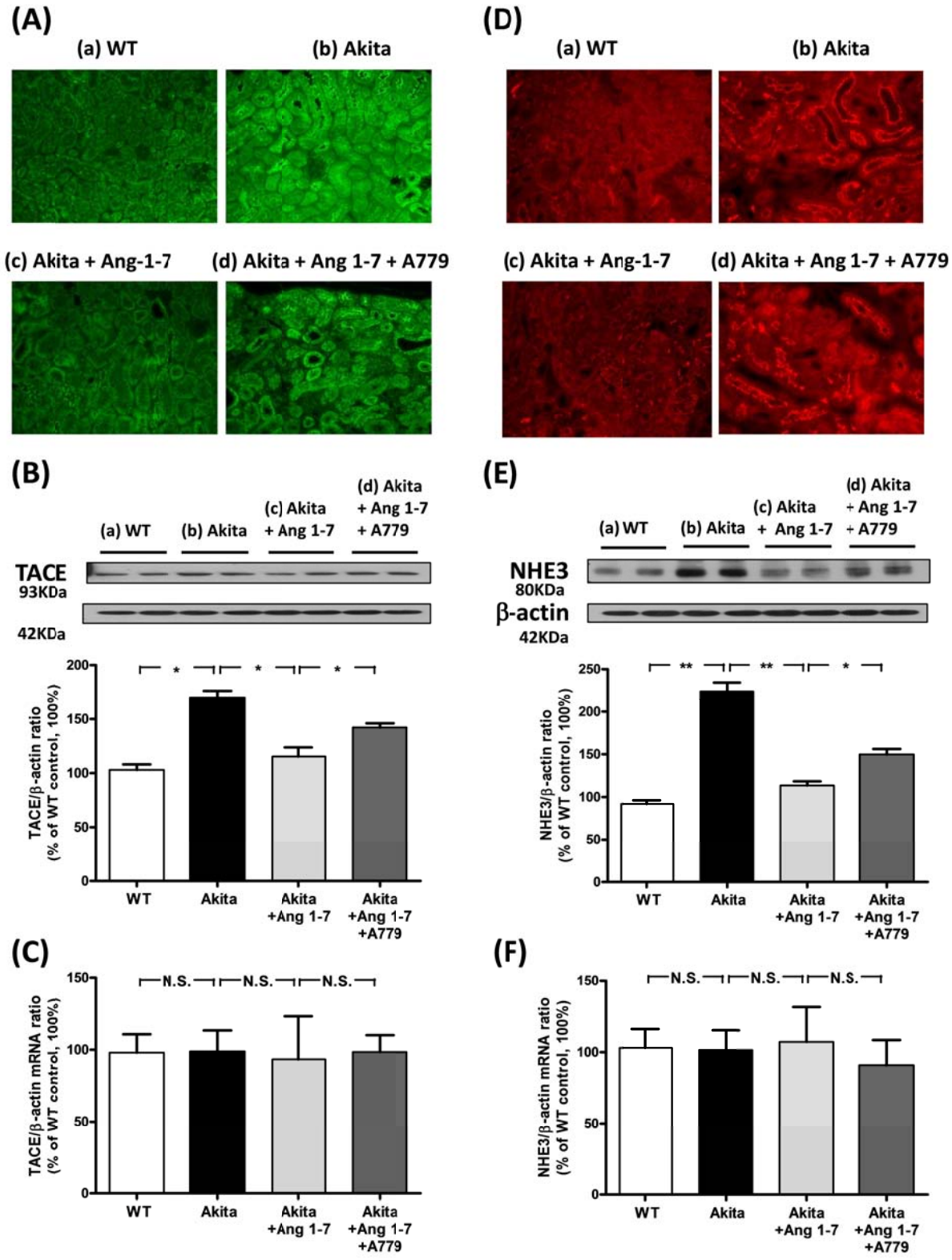

(A)
(a) WT
(b) Akita
(c) Akita + Ang-1-7
(d) Akita + Ang 1-7 + A779
(D)
(a) WT
(b) Akita
(c) Akita + Ang-1-7
(d) Akita + Ang 1-7 + A779
(B)
(a) WT
(b) Akita
(c) Akita + Ang 1-7
(d) Akita + Ang 1-7 + A779
TACE
93KDa
42KDa
TACE/β-actin ratio (% of WT control, 100%)
WT
Akita
Akita +Ang 1-7
Akita +Ang 1-7 +A779
(E)
(a) WT
(b) Akita
(c) Akita + Ang 1-7
(d) Akita + Ang 1-7 + A779
NHE3
80KDa
β-actin
42KDa
NHE3/β-actin ratio (% of WT control, 100%)
WT
Akita
Akita +Ang 1-7
Akita +Ang 1-7 +A779
(C)
TACE/β-actin mRNA ratio (% of WT control, 100%)
N.S.
WT
Akita
Akita +Ang 1-7
Akita +Ang 1-7 +A779
(F)
NHE3/β-actin mRNA ratio (% of WT control, 100%)
N.S.
WT
Akita
Akita +Ang 1-7
Akita +Ang 1-7 +A779

Figure 3-6: Immunostaining for TACE (A) and NHE-3 (B) in mouse kidneys at age 20 weeks. Magnification X200. Western blotting of TACE (C) and NHE-3 (D) in mouse RPTs. Membranes were re-blotted for β-actin. TACE and NHE-3 levels were normalized by corresponding β-actin levels. Values in non-Akita control littermates were considered as 100% (control). q-PCR of TACE (E) and NHE-3 (F) mRNA levels in mouse RPTs. Values in non-Akita control littermates were considered as arbitrary unit 100%. Values are expressed as means ± SE (n=8). **P*<0.05, ***P*<0.01, N.S., non-significant.

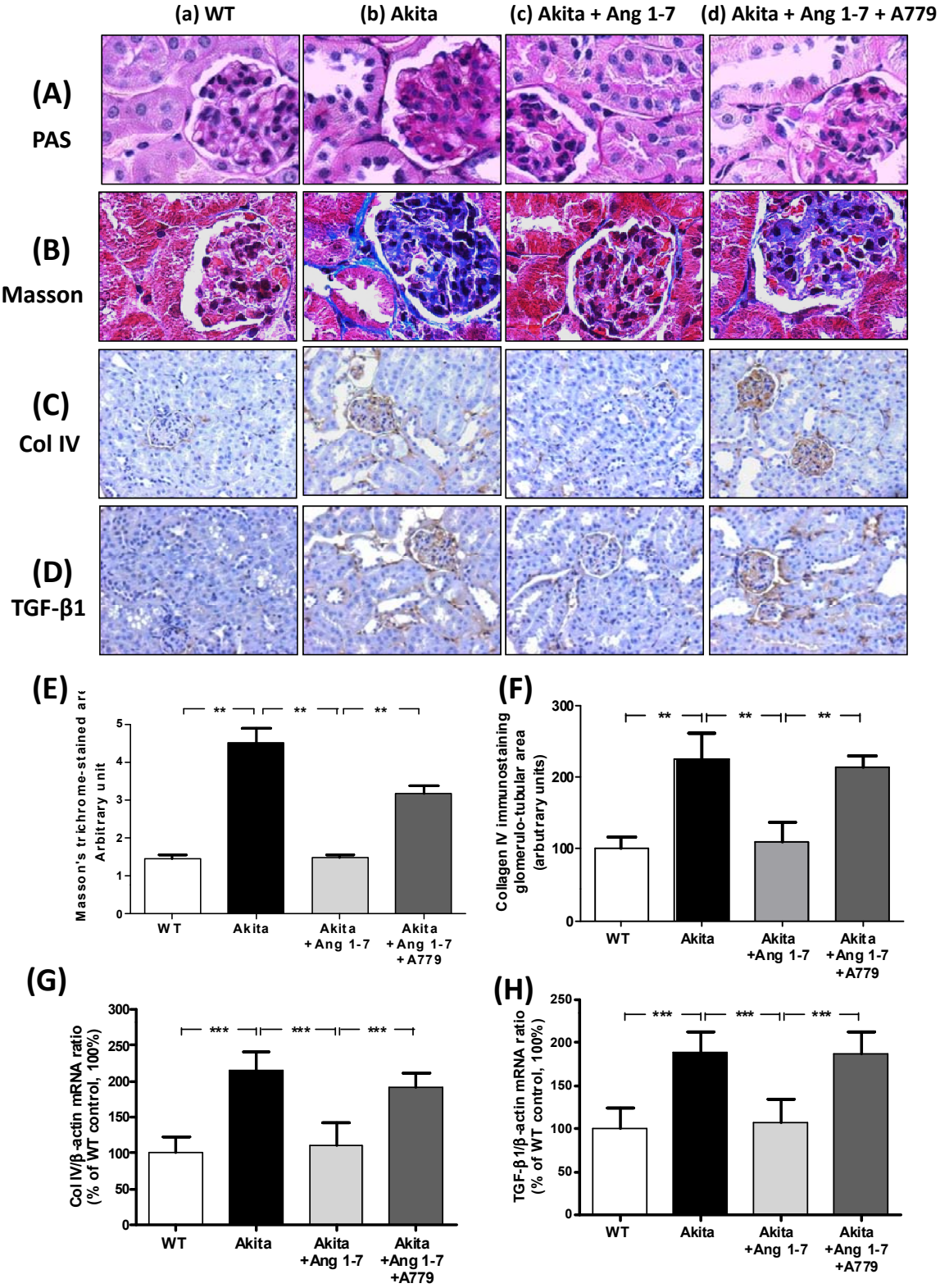
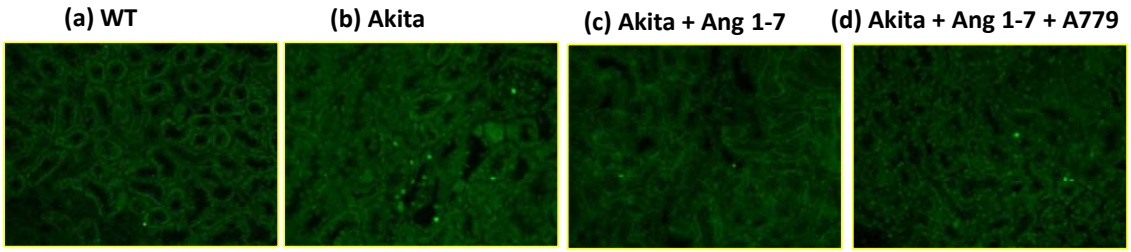
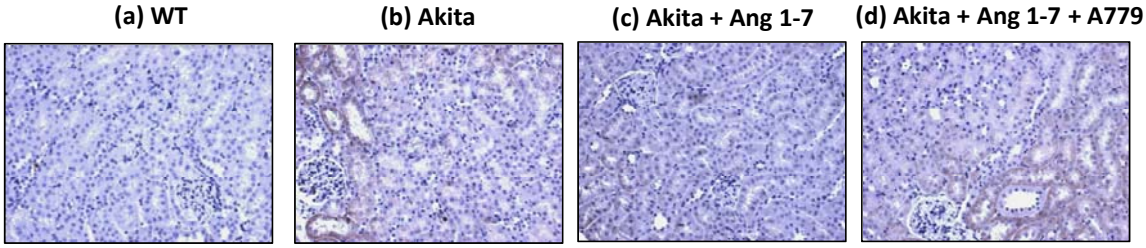

(a) WT
(b) Akita
(c) Akita + Ang 1-7
(d) Akita + Ang 1-7 + A779
(A)
PAS
(B)
Masson
(C)
Col IV
(D)
TGF-β1
(E)
Masson's trichrome-stained area
Arbitrary unit
(F)
Collagen IV immunostaining
glomerulo-tubular area
(arbutrary units)
(G)
Col IV/β-actin mRNA ratio
(% of WT control, 100%)
(H)
TGF-β1/β-actin mRNA ratio
(% of WT control, 100%)
WT
Akita
Akita +Ang 1-7
Akita +Ang 1-7 +A779

Figure 3-7: PAS staining (A) and Masson's trichrome staining (B) (magnification X600) and immunostaining for collagen IV (C) and TGF-β1 (D) (magnification X200) expression in mouse kidneys at age 20 weeks. (E) Quantification of extracellular matrix component accumulation (Masson's trichrome staining). (F) Quantitation of immunoreactive collagen IV deposition. (G) qPCR of collagen IV (G) and TGF-β1 (H) mRNA level in mouse kidneys. Values in non-Akita control littermates were considered as 100% control. Values are expressed as means ± SE (n=8). $^{**}P<0.01$, $^{***}P<0.005$, N.S., non-significant.

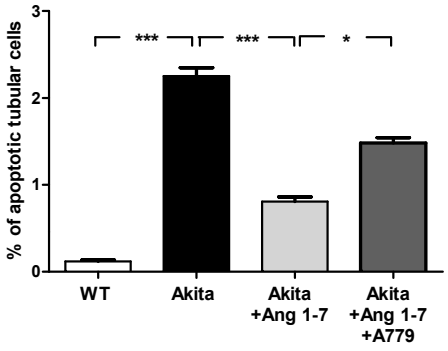
(A) TUNEL Assay



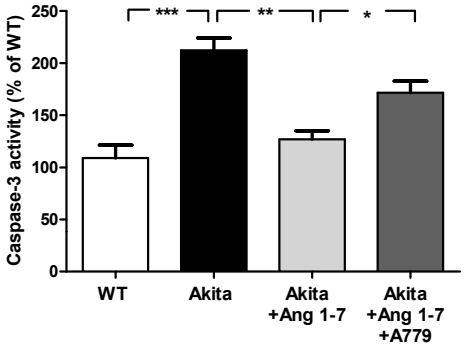
(B) Active Caspase-3



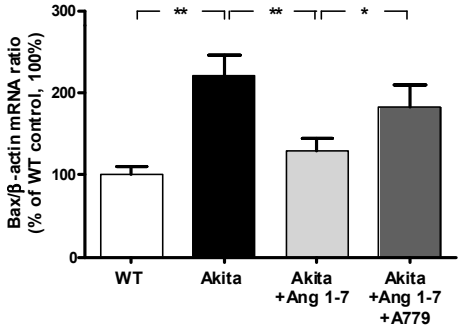
(C)



(D)



(E)



(F)

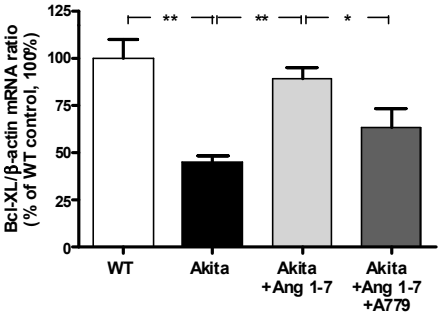


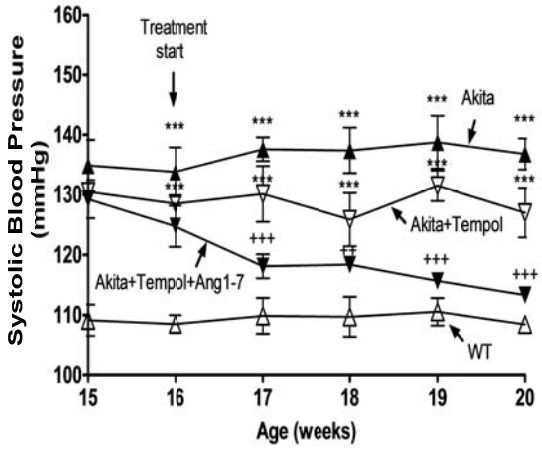
Figure 3-8: Apoptosis in mouse kidneys at age 20 weeks. (A) TUNEL staining (green) and (B) Immunostaining for cleaved (active) caspase-3. Magnification X200. Semi-quantitation of apoptotic RPTCs (C) and (D) caspase-3 activity in isolated RPTs. RT-qPCR for Bax (E) and Bcl-xL (F) mRNA. Assays were run simultaneously for Bax, Bcl-xL and β-actin mRNAs. Bax and Bcl-xL mRNA levels were normalized by corresponding β-actin mRNA levels. mRNA levels in non-Akita control littermates were considered as 100%. Values are expressed as means ± SE (n=8). **P*<0.05, ***P*<0.01, ****P*<0.005, N.S., non-significant.

Supplemental Table 1 (Primers)

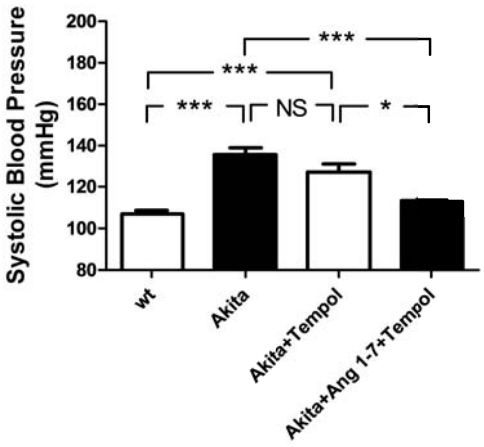
Gene	Primer sequences	Species	Reference Sequence
NOX1	S: GGTCACTCCCTTTGCTTCCA AS: GGCAAAGGCACCTGTCTCTCT	Mouse	NM_172203.2
NOX2	S: CCCTTTGGTACAGCCAGTGAAGAT AS : CAATCCCGGCTCCCACTAACATCA	Mouse	NM_007807.5
NOX4	S: TGGCCAACGAAGGGGTTAAA AS : GATGAGGCTGCAGTTGAGGT	Mouse	NM_001285835.1
HO-1	S: CACCAAGTTCAAACAGCTCT AS: CAGGAAACTGAGTGTGAGGA	Mouse	NM_010442.2
Nrf2	S: CGCCGCCTCACCTCTGCTGCCAGTAG AS: AGCTCATAATCCTTCTGTCG	Mouse	NM_010902.3
Agt	S: ACAGACACCGAGATGCTGTT AS: CCACGCTCTCTGGATTTATC	Mouse	NM_007428.3
ACE	S: GACCGGACAGCCCAAGTG AS: AGCTTCTTTATGATCCGCTTGATG	Mouse	NM_207624.5
Ace2	S: ATATGACTCAAGGATTCTGGG AS: GCTGCAGAAAGTGACATGATT	Mouse	NM_001130513.1
MasR	S: GCATTCGTCTGTGCCCTTCT AS: TTCCGTATCTTCACCACCAAGA	Mouse	NM_008552.4
TACE	S: AGAGAGCCATCTGAAGAGTTTGT AS: TTATCCCACGACGTGTTCCG	Mouse	NM_001277266.1
NHE3	S: AGGTGGTCACCTTCAAATGGC AS: GGGACAGGTGAAAGACGATTT	Mouse	NM_001081060.1
Col IV	S: CATGTCCATGGCACCCATCT AS: ATGGCCGGTGCTTCACAAAC	Mouse	NM_009931.2
TGF-β1	S: CCAAACTAAGGCTCGCCAGTC AS: GGCACTGCTTCCCGAATGTC	Mouse	NM_011577.1
Bax	S: TCATGAAGACAGGGGCCTTTT AS: CAATCATCCTCTGCAGCTCCA	Mouse	NM_007527.3
Bcl-xL	S: TACCGGAGAGCGTTCAGTGATCTA AS: CTGCATCTCCTTGTCTACGCTTTC	Mouse	NM_009743
β-Actin	S: CGTGCGTGACATCAAAGAGAA AS: GCTCGTTGCCAATAGTGATGA	Mouse	NM_007393

Supplemental Figure 1

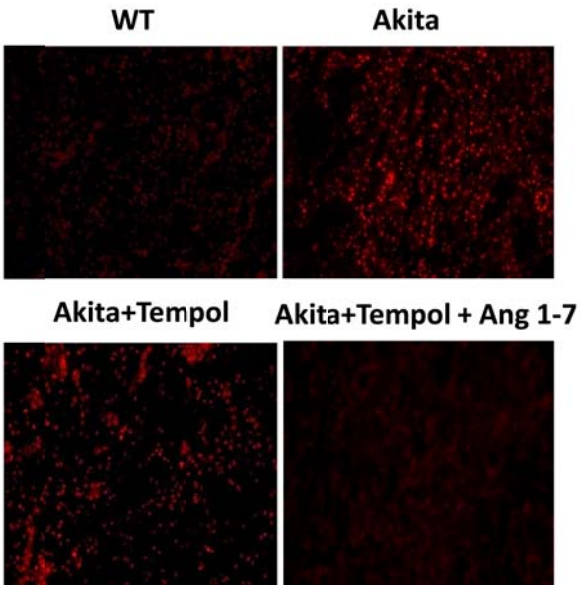
(A)Longitudinal analysis of SBP



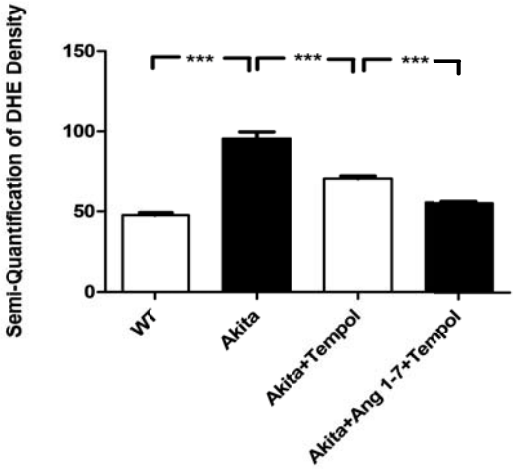
(B) Cross-sectional analysis of SBP



(C) DHE staining of mouse kidneys



(D) Quantitation of DHE staining



Chapter 4: Discussion

In our studies, Tg mice overexpressing Cat were created to investigate whether the oxidative stress is involved in the development of hypertension and nephropathy in Akita mice via down-regulation of renal ACE2 expression. We found that overexpression of Cat attenuated renal oxidative stress; prevented hypertension; ameliorated glomerular filtration rate, albuminuria, kidney hypertrophy, tubulointerstitial fibrosis, and tubular apoptosis; and suppressed profibrotic and proapoptotic gene expression in RPTCs of Akita Cat-Tg mice compared with Akita mice. Furthermore, overexpression of Cat in RPTCs of Akita mice normalized renal ACE2 expression and urinary Ang 1–7 levels. These findings confirmed our hypothesis.

Next, we investigated the impact of Ang 1-7 on hypertension, oxidative stress and kidney injury in diabetic Akita mice by injecting Akita mice with Ang 1-7. The administration of Ang 1-7 also attenuated oxidative stress and the expression of NADPH oxidase 4, Agt, ACE, TGF-β1 and collagen IV, and increased the expression of ACE2 and MasR in Akita mouse kidneys. These effects were reversed by co-administration of A779. Our data suggest that Ang 1-7 plays a protective role in hypertension and RPTC injury in diabetes, predominantly through decreasing renal oxidative stress-mediated signaling and normalizing ACE2 and Mas receptor expression.

4.1 Intrarenal RAS and ACE2 in DM

In the kidney, all major components of the RAS, including the precursor Agt, the rate-limiting enzyme renin and ACE, and the receptors for Ang II (AT1R and AT2R), ACE2, Ang 1-7 and its receptor (MasR) have been demonstrated [251]. Indeed, human and murine RPTCs express all components of the RAS [136, 252]

The main effector of classic RAS system, Ang II, has the effect of vasopressor, inflammation, hypertrophy, pro-fibrotic and pro-apoptotic. On the opposite, Ang 1-7 exhibits anti-hypertensive, anti-inflammatory, anti-hypertrophic, anti-fibrotic and anti-apoptotic properties. Therefore, ACE2/ Ang 1-7/ MAS axis can counteract ACE/ Ang II /AT1R axis.

It is well accepted that in the hyperglycemic milieu, RAS system is activated. An increase in intrarenal Agt mRNA has been reported in diabetic rats, implying increased Agt synthesis by the proximal tubule [182, 253]. *In vitro* studies has shown that high extracellular glucose stimulates the synthesis of Agt in a concentration-dependent manner, via PKC [254], or via ROS in the rat proximal tubule [255]. These findings are confirmed by our observation in Akita TID mice (Fig. 3Aa, 3d, 3g. page 92).

On the other hand, ACE2 expression is suppressed in DM. Tubular ACE2 mRNA and protein expression were reduced in male Sprague-Dawley rats with STZ -induced diabetes after 24 weeks [193], and in male C57Bl/6 STZ-diabetic mice, which was associated with a reduction in kidney cortical levels of Ang 1–7 [256]. Within the glomerulus, ACE2 protein expression in podocyte from T2D db/db mice was decreased, whereas ACE protein expression, by contrast, was increased [187]. Similarly, glomerular ACE2 mRNA and protein was reduced in STZ-induced C57BL/6J mice [257]. Consistent with the results from experimental diabetes, renal biopsy studies from humans with type II diabetic nephropathy showed that decreased ACE2 and increased ACE expression in both the tubulointerstitium and glomeruli resulted in a significant increase in ACE/ACE2 ratio in patients with diabetes with overt nephropathy compared with controls [191]. Similarly, Reich et al. [192] found a significant decrease in ACE2 mRNA expression in laser captured glomeruli and proximal tubules from patients with T2D and diabetic nephropathy, accompanied by enhanced expression of ACE mRNA in both

compartments. ACE2 protein expression was significantly reduced in proximal tubules from diabetic subjects as well by immunohistochemistry. Our studies confirmed that ACE2 expression was downregulated at both mRNA and protein levels in Akita mice (Fig. 3B, 3E and 3H, page 92), compared with WT mice.

4.2 Tg mouse model overexpressing Cat in the RPTCs

To create Tg mice overexpressing Cat in the RTPCs, we inserted rat Cat cDNA fused with an HA tag (a sequence encoding amino acid residues 98 to 106 (YPYDVPDYA) of human influenza virus hemagglutinin) at the 3' terminal into a construct containing the kidney androgen regulated promoter, pKAP2. The KAP promoter can drive the targeted gene to express in the RPTCs in response to testosterone stimulation. We chose male Tg mice, which had enough endogenous testosterone to induce Tg gene expression. Then we crossbred male heterozygous Akita mice with female homozygous Cat Tg mice to obtain the Akita Cat-Tg mice.

We confirmed the presence of Cat in RPTCs by immune-staining. In the kidney, Cat is localized predominantly in the cytoplasm of proximal tubules of the superficial cortex. Cat was not detected in the glomeruli, distal tubules, loop of Henle, and collecting ducts [52]. To clearly show the location of RPTC, a biomarker, aquaporin 1, was chosen. Aquaporins (AQPs) are a class of membrane water channels whose primary function is to facilitate the passive transport of water across the plasma membrane of the cell in response to osmotic gradients that are created by the active transport of solutes [258]. There are at least 11 AQPs, of which, AQP1 is expressed in the RPT and the thin descending limb of the loop of Henle, but not in the distal tubule or collecting duct [258]. The co-localization of Cat and AQP1 confirmed the

presence of Cat in RPTC (Fig. 1C, page 88). Furthermore, Cat levels were significantly higher in RPTCs in Cat-Tg mice (Fig. 1Bb) and Akita Cat-Tg mice (Fig. 1Bd) than in non-Akita WT mice (Fig. 1Ba) or in Akita mice (Fig. 1Bc). These results confirmed that the KAP gene promoter directs Cat Tg expression in the RPTCs of Cat-Tg and Akita Cat-Tg mice.

To assess the oxidative stress, different methods were employed. Heme oxygenase-1 (HO-1) is an enzyme that catalyzes the degradation of heme and is inducible in response to oxidative stress [259]. Thus, increased cellular expression of HO-1 has been considered to be a marker of oxidative stress [260]. Dihydroethidium (DHE) has been extensively used to evaluate ROS production *in vivo*. It is freely permeable and after entry it is oxidized to fluorescent ethidium bromide (EB) in the presence of O_2^-. Ethidium bromide is a potent mutagen and it is trapped intracellularly by intercalation into the DNA. In our experiment, both HO-1 immunostaining and DHE staining showed significantly higher oxidative stress in Akita mice (Fig. 1, Dc and Ec, page 88) than in non-Akita WT mice (Fig. 1, Da and Ea, page 88). This is consistent with the finding that high glucose can induce the ROS generation [124]. Cat is an antioxidant enzyme and can convert H_2O_2 to water and oxygen, thus mitigating the toxic effect of H_2O_2. Cat overexpression in Akita Cat-Tg could effectively attenuate oxidative stress, as shown in Fig. 1, Dd and Ed, page 88.

4.3 ACE2, Ang 1-7 and hypertension in diabetic mice

Activation of intrarenal RAS can lead to systemic hypertension in diabetic condition. Within the kidney, AT1 receptors are expressed on epithelial cells throughout the nephron, in the glomerulus, and on the renal vasculature [261, 262]. First, renal Ang II might exert a vaso-constrictive effect on glomerular arterioles via AT1R. Second, Ang II may elicit hypertension

through changes in sodium or fluid homeostasis, possibly via alterations in transport mechanisms in the kidneys. In the proximal tubule, Ang II promotes sodium reabsorption by co-ordinately stimulating the sodium-hydrogen exchanger on the luminal membrane along with the Na^+/K^+-ATPase on the basolateral surface [263, 264]. In the collecting duct, Ang II directly stimulates ENaC activity via AT1 receptors [265]. Renal vasoconstriction caused by Ang II reduces medullary blood flow and blunts the kidney's excretory capacity for sodium [266, 267].

ACE2 have beneficial effects on the regulation of blood pressure, because it degrades vasoconstrictor Ang II as well as produces vasodilator Ang 1-7. ACE2-deficient C57BL/6 mice have raised basal blood pressure, with notable amplifications of Ang II-induced hypertension, which was associated with exaggerated accumulation of Ang II in the kidney after Ang II infusion [198]. On the opposite, overexpression of ACE2 in the rostral ventrolateral medulla [268], the paraventricular nucleus [269] or the whole brain [270] have attenuated high blood pressure and improved baroreflex dysfunction induced by Ang II [270]. Tg rats with ACE2 overexpression in blood vessels of SHRSP rats reduced blood pressure and improved endothelial function [271]. Pharmacological intervention studies have revealed similar beneficial effects of ACE2 on high blood pressure. Systemic administration of human recombinant ACE2 counteracted pressor effects induced by Ang II and resulted in a decrease in blood pressure [247, 272].

The effect of ACE2 on regulation of blood pressure might be the result of less Ang II, more Ang 1-7 or both. In contrast with the reported beneficial effects of ACE2 on hypertension, studies with Ang 1–7 have yielded rather contradictory results. For instance, Giani et al. [273] reported Ang 1-7 attenuation of systolic blood pressure in Zucker rats. A transient decrease in

BP has also been observed with intravenous infusion of Ang 1–7 in SHR [207]. Iyer et al [274] revealed an important contribution of Ang 1–7 in mediating the antihypertensive effects caused by combined inhibition of ACE and AT1 receptors in SHR. This is because plasma levels of Ang 1-7 were elevated after combined inhibition and treatment with an antibody against Ang 1–7 induced a significant elevation in systolic BP. In contrast, Tan et al. [275] found no significant reductions in SBP in response to Ang 1-7 in SHR and Wistar-Kyoto rats.

In our studies, we found that Ang 1-7 administration indeed prevented systemic hypertension and A-779 could reverse the BP (Fig. 6, page 98). However the mechanisms underlying the antihypertensive action of Ang 1-7 are not well-defined. One possibility is that Ang 1-7 exerts a vaso-dilatory effect that involves release of endothelial derived nitric oxide, production of vasodilator prostaglandins, and potentiation of the hypotensive effects of bradykinin [201]. Furthermore, Ang 1-7 possesses natriuretic/diuretic properties *in vivo*. In anesthetized rats, administration of Ang 1-7 increases urinary flow rate and sodium excretion, an effect abolished by A-779 [211]. *In vitro* studies also showed that Ang 1-7 could limit trans-cellular sodium transport. For instance, in cultured rabbit proximal tubular cells, Ang 1-7 inhibits sodium flux through the activation of phospholipase A_2 [210]. In isolated rat proximal tubules, Ang 1-7 inhibits the ouabain-sensitive Na^+/K^+-ATPase [211]. Our studies showed that the RPTCs of Akita mice exhibited increased renal NHE-3 expression as compared to WT mice. Ang 1-7 administration normalized NHE-3 expression in Akita mice and reversed by A779 co-administration. These findings suggest that Ang 1-7 lowering SBP in Akita mice might be mediated, at least in part, via down-regulation of NHE-3 expression in the RPTs in Akita mice.

4.4 Antioxidants and hypertension

The antioxidants vitamins C and E and other antioxidants have been considered as possible therapy for decreasing oxidative stress and thereby lowering blood pressure.

In experimental models of hypertension, vitamin C, alone or in combination with vitamin E, reduces blood pressure [276, 277]. In spontaneously hypertensive rats (SHR), dietary supplementation of α-tocopherol for 3 months prevented development of increased blood pressure and reduced lipid peroxides in plasma and vessels [278]. However, long-term clinical trials have failed to consistently support the antihypertensive effects of antioxidants. For instance, one of the largest trials, performed by the Heart Protection Collaborative Group, found no improvement in BP after treatment with a combination of ascorbic acid, synthetic vitamin E, and β-carotene versus placebo after 5 years in 20 536 UK adults (aged 40–80) with coronary disease, other occlusive arterial disease, or diabetes [279]. Possible reasons for these disappointing outcomes relate to 1) type of antioxidants used, 2) patient cohorts included in trials, and 3) the trial design itself. With regard to antioxidants, it is possible that agents examined were ineffective and non-specific and that dosing regimens and duration of therapy were insufficient [280]. Another possibility is that orally administered antioxidants may be inaccessible to the source of free radicals, especially if ROS are generated in intracellular compartments and organelles. Moreover, antioxidant vitamins do not scavenge H_2O_2, which may be more important than $\bullet O_2^-$ in cardiovascular disease [280]. In our Akita Cat-Tg mice, Cat expressed in RPTCs can eliminate intracellular H_2O_2 and therefore more effectively ameliorates oxidative stress. If we specifically deliver Cat gene to the kidney or using specific molecule to activate the expression of Cat, we might obtain a good therapeutic way to reduce BP and ameliorate kidney injury in human condition.

Another factor of importance is that antioxidants do not inhibit ROS production [280]. In our experiment, infusion of Ang 1-7 could inhibit the ROS generation, especially that from NADPH oxidase. Therefore Ang 1-7 might be a potential therapeutic agent for treatment of systemic hypertension.

4.5 ROS, Cat and ACE2/Ang 1-7 in DM

4.5.1 ROS generation in DM

Accumulating evidence suggests that the overproduction of ROS is one major factor in the onset and progression of diabetic complications as well as diabetes per se [281]. High glucose (25 mm D-glucose) induces the generation of ROS [124]. At the same time, Ang II, which is highly activated in DM, can potently stimulate ROS generation by heightened NADPH oxidase in mesangial cells and RPTCs [265, 282].

In addition of mitochondrial respiratory chain, another important source of ROS production is NADPH oxidase. The classic NADPH oxidase consists of membrane-bound subunits (gp91phox and p22phox) and cytosolic subunits (p47phox, p40phox, p67phox, and Rac). Griendling et al first reported NADPH oxidase regulation by Ang II in vascular smooth muscle cells [283]. Onozato et al confirmed in the kidney that NADPH oxidase activation is dependent on Ang II action on AT1 receptor and the blockade of AT1 receptor reduced p47phox expression and O_2^- production in the kidney [284]. Stimulation of AT1 receptor will lead to the activation of phospholipase D, protein kinase C (PKC), p38 mitogen activated protein kinase (MAPK) and c-Src tyrosine kinase stimulating the phosphorylation of p47phox. Once phosphorylated, p47phox translocates to the membrane where it will bind to the membrane component, cytochrome b558, through its two Src-homology 3 (SH3) domains.

Activation of c-Src also promotes the transactivation of epidermal growth factor receptor which leads to activation of phosphatidylinositol 3-kinase (PI3K) and Rac-1. Rac-1 then translocates to the membrane and keeps the enzyme complex activated [285].

Other homologues of gp91phox (Nox-2) in the phagocyte, including Nox-1, Nox-3, Nox-4, Nox-5, Duox-1 and Duox-2, have been described. At least three different NOX isoforms are expressed in the kidney cortex: NOX4, NOX2 and NOX1 [37]. NOX4 was originally identified as an NADPH oxidase homolog highly expressed in the kidney [40] and is the most abundant isoform. The predominant NOX4 localization within the kidney cortex is renal tubular cells, but at lower levels it is found in other cell types as well, including glomerular mesangial cells [37].

Ang II has also been shown to employ Nox4 as a mediator of injurious effects in tubular epithelial cells. Chronic Ang II treatment upregulates Nox4 expression and induces EMT in cultured renal epithelial cells through Nox4-dependent ROS production [286]. Furthermore, it was reported recently that Ang II upregulates Nox4 expression in the mitochondrial and membrane fractions and is required for Ang II-mediated mitochondrial and intracellular ROS production in cultured renal tubular cells [287].

In our studies, we found in Akita diabetic mice, NOX4 mRNA was increased, whereas NOX1 and NOX2 mRNA expression did not change, compared to WT mice (Figure 2C, page 127). This is consistent with the *in vitro* findings that exposure of cultured renal proximal tubular epithelial cells to high glucose leads to the up-regulation of Nox4 protein expression, but has no effect on Nox2 or Nox1 expression [80]. Furthermore, You et al [81] investigated the role of NOX2 and Nox4 in diabetic kidney disease by using NOX2 knockout mice. Their study demonstrated that lack of Nox2 did not protect against diabetic kidney disease in T1D, despite

a reduction in macrophage infiltration. The lack of renoprotection may be due to upregulation of renal Nox4 [81]. We demonstrated that Ang II increases the expression of NOX4 both at mRNA and protein levels as shown by qRT-PCR, western blotting and immunostaining (Fig. 2C-E, page 127).

4.5.2 Ang 1-7 and ROS

In our studies, Ang 1-7 administration normalized the expression of NOX4 in Akita mice, whereas A-779 reversed this change (Fig. 2A-E, page 127). These results are consistent with earlier observations. In the diabetic SHR kidney, Ang 1-7 decreased the elevated levels of renal NADPH oxidase (NOX) activity and attenuated the activation of NOX-4 gene expression [288]. In primary cultures of mouse mesangial cells, Moon et al. [289] showed Ang 1–7 prevented the Ang II-induced expression of NOX subunits, including p47*phox*, p67*phox*, and NOX-4.

4.5.3 Cat overexpression upregulates ACE2

An important finding in our studies is that Cat overexpression could upregulate ACE2. But the underlying mechanism is still unknown. One possibility is that Cat interrupts the negative feedback of Ang II on the ACE2 expression. In our previous study, we observed that RAS blockade could normalize ACE2 expression, which is decreased in diabetic condition [250].

Koka et al demonstrated that Ang II upregulates ACE and downregulates ACE2 expression in HK2 cells *in vitro* via the AT1R-ERK/p38 MAP kinase pathway [190]. Ang II can enhance the ROS generation by NADPH oxidase. The ROS, in turn, activates ERK1/2 and p38 MAPK in proximal tubular cells [290]. Therefore ROS mediates the negative feedback of Ang II on

the ACE2 expression. Cat could mitigate ROS generation and thus block the feedback, leading to the up-regulation of ACE2.

We noted Cat overexpression could decrease the Agt expression. Ang II induces intrarenal NF-κB activity [291]. Activation of NF-κB plays an important role in the stimulation of Agt expression in cultured proximal tubule cells [292]. Furthermore, ROS could mediate the activation of NF-κB by Ang II. Therefore, ROS scavenger, Cat could decrease Agt expression (Fig. 4- 1).

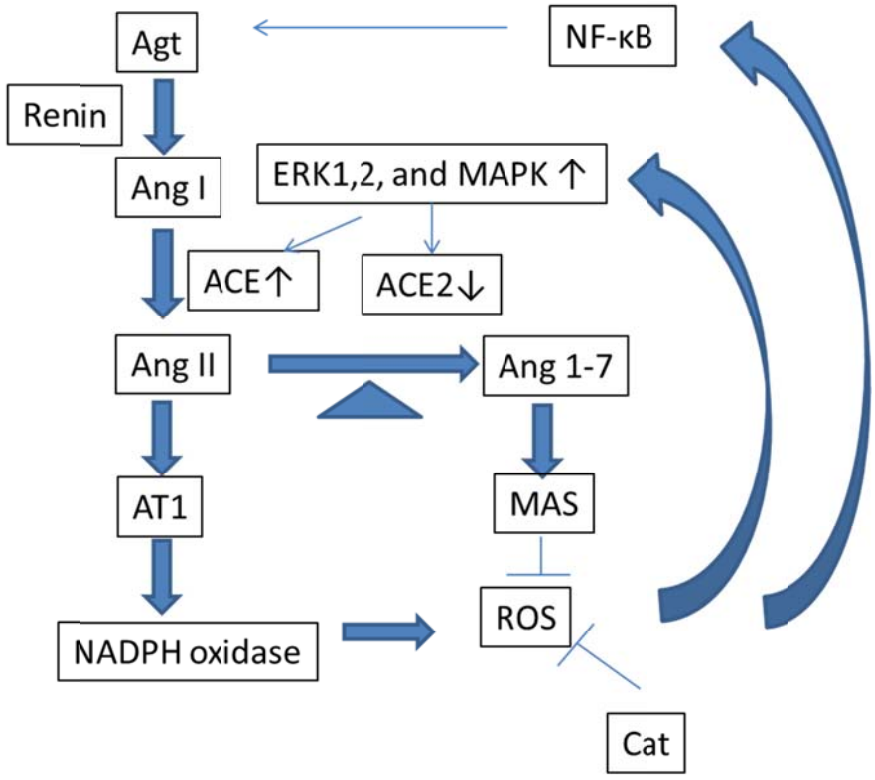


Figure 4-1. Schematic diagram of regulation of ACE2 by Cat and Ang 1-7

4.5.4 Ang 1-7 upregulates ACE2 and Mas receptor

Another important finding in our study is that Ang 1-7 could upregulate ACE2 and Mas receptor. Ang 1-7 mitigates ROS generation by reducing NOX4 expression. Thus, similar to Cat, Ang 1-7 could block the negative feedback of Ang II on the ACE2 expression, resulting in the upregulation of ACE2. Little is known on the regulation of Mas receptor. Lakshmanan et al [293] reported that Mas receptor and ACE2 were downregulated in STZ-diabetic mice and Telmisartan treatment could upregulate Mas receptor and ACE2. However, the mechanism whereby Ang 1-7 upregulates MasR still needs further investigation.

4.6 Cat, ACE2/Ang 1-7 and kidney injury in diabetic mice

4.6.1 Proteinuria in diabetic mice

Microalbuminuria is an important clinical marker for the early detection of hypertension- or diabetes-induced nephropathy. Albuminuria/proteinuria results from defects in the glomerular filtration barrier, however, abnormalities in tubular albumin reabsorption may also contribute to proteinuria. The glomerular filtration barrier comprises a series of layers separating the blood side (glomerular capillary) from the urinary space. These include the innermost fenestrated endothelium, the middle GBM, and the outermost podocyte (also called the glomerular visceral epithelial cell), forming a size, charge, and conformational glomerular barrier. Low molecular weight proteins (mw<40 kDa) are essentially freely filtered, whereas, high molecular weight proteins (mw>100 kDa) are almost completely restricted [294]. Although the glomerular endothelium is highly fenestrated, it can still hinder protein permeability [295]. Widespread endothelial dysfunction is believed to result in proteinuria

[296]. GBM heparan sulfate proteoglycans (HSPGs) contributes to the charge selectivity of the glomerular capillary wall, since administration of a monoclonal anti-heparan sulfate antibody to rats induced massive proteinuria [297]. *In vitro*, decreased podocyte synthesis of the core protein agrin may be caused by both a high ambient glucose [254] and Ang II [253]. ACE inhibition prevents HSPG loss in diabetic rats, thus decreasing albuminuria [256]. Nephrin protein has been located to the slit diaphragm of glomerular podocytes, suggesting a role for nephrin in the renal ultrafilter barrier function. Mutations in the nephrin gene, NPHS1, leads to a severe congenital nephrotic syndrome [9]. Diabetic spontaneously hypertensive rats developed albuminuria and had a reduction in both gene and protein expression of nephrin when compared with control rats. Treatment of Irbesartan, an AT1 receptor antagonist, prevented the development of albuminuria and completely abrogated the down regulation of nephrin in diabetic rats [181].

Interestingly, an alternative model suggests interruption of the protein reabsorption process in the proximal tubule may cause nephrotic range proteinuria. Using fluorescently labelled albumin, together with the powerful technique of intravital 2-photon microscopy, Russo et al [298] showed that renal albumin filtration in non-proteinuric rats was ~50 times greater than previously measured and was followed by rapid endocytosis into RPTCs. In nephrotic rats, the rate of uptake of albumin by the proximal tubule is decreased. Thus, dysfunction of this retrieval pathway leads to albuminuria. In fact, 4–5 g of albumin are filtered at the normal glomerulus each day [299]. In diabetes, proximal tubular reuptake of protein may be impaired by high glucose [300], TGF-β [301], or Ang II [302].

Cat overexpression upregulates ACE2 and thus counteracts the effect of Ang II. Chronic blockade of ACE2 with the enzyme inhibitor, MLN-4760, in control or diabetic mice

produced albuminuria [257]. In addition, Cat attenuates ROS, resulting in mitigating damage to proximal tubule. Taken together, Cat could reduce the degree of albuminuria.

Ang 1-7 has been documented to decrease protein excretion. For instance, Benter et al [288] treated STZ-diabetic SHR with Ang 1–7 and found a significant reduction in urinary protein excretion compared with control diabetic rats. Ang 1-7 also induced a reduction in proteinuria, and systolic BP together with a restoration of creatinine clearance in Zucker diabetic fatty rats [273]. On the contrary, genetic deletion of the Ang 1–7 receptor Mas leads to glomerular hyperfiltration and microalbuminuria [164].

4.6.2 Tubulointerstitial injury in DN

Although there is no question that there are changes seen in the glomerulus in patients with DN, it is also well known that tubulointerstitial changes are a prominent component of the disease [237]. The tubulointerstitium accounts for more than 90% of the kidney volume [303]. The progression of DN clearly is predicted by the extent of tubular atrophy and interstitial fibrosis [304]. The finding that cultured tubular cells respond to glucose suggests that these cells contribute directly to the pathological changes of DN rather than as a consequence of glomerular injury [305]. In T1D, tubulointerstitial disease has been seen to occur independent of glomerular disease [67]. Therefore Bonventre [237] proposed that the kidney tubule plays a critical role in the genesis of DN. Rather than the canonical view that the glomerular injury in DN is primary with the tubular injury secondary, he took the view that in fact the tubular injury is primary. Furthermore, the well-accepted data that disease progression is correlated best with the degree of tubulointerstitial disease is also consistent with a primary role for the

tubule, particularly the proximal tubule [237]. Therefore, our following studies focused on the tubular injury in DN.

4.6.3 RAS, Cat and tubular hypertrophy

Renal enlargement occurs in the early stage of DN. Since tubulointerstitial parenchyma accounts for 90% of the kidney volume, renal enlargement in diabetes predominantly reflects tubulointerstitial changes. In the STZ-induced diabetic rat, the first 7 days following the induction of experimental diabetes are accompanied by a 37% increase in proximal tubular length, a doubling of luminal diameter and wall volume, and an increase in cell height [36], reflecting both hypertrophy and hyperplasia [37]. However, the growth pattern switches early from hyperplasia to hypertrophy growth at around day 4 in the model of STZ diabetes [306]. This renal enlargement is associated with an increase in GFR.

Akita mice exhibited elevated kidney-to-body weight ratio at the end of the experiment compared with non-Akita WT controls (Table 2-1, page 88). In agreement with this, a significantly enlarged tubular luminal area and increased glomerular tuft and RPTC volume were observed in Akita mice compared with non-Akita WT (Table 2-1, page 88). Cat overexpression markedly attenuated these ratios, partially reduced tubular luminal area and glomerular tuft volume and completely normalized RPTC volume in Akita Cat-Tg mice.

The mechanism by which tubular hypertrophy occurs in DM remains to be elucidated. Ang II and TGF-β1 might be important mediators. Ang II could increase TGF- β1 gene expression via AT1R in RPTCs. Zhang et al [307] have observed this effect *in vitro* where Ang II stimulation of immortalized rat proximal tubular cells induces cell hypertrophy. TGF-β can induce a G1 phase cell cycle arrest by induction of the cyclin-dependent kinase (CDK)

inhibitor p27KIP1 (p27) [308], which can also be induced in diabetes by PKC [309]. Diabetes also increases the renal expression of the CDK p21 and loss of p21 increases tubular cell proliferation [310]. The role of ROS is indicated by the fact that the antioxidants N-acetylcysteine and taurine attenuated high-glucose-induced activation of the JAK/STAT signalling pathways, p21 and p27 expression, and hypertrophic growth in renal tubular epithelial cells [311]. Similar to these antioxidants, Cat could effectively attenuate ROS, leading to mitigating tubular hypertrophy.

4.6.4 Cat, RAS and tubulointerstitial fibrosis

Although the glomerulus has been the focus of intense investigation in diabetes, tubulointerstitial injury, characterized by tubulointerstitial fibrosis and tubular atrophy, is also a major feature of diabetic nephropathy and an important predicator of renal dysfunction [238]. The renal tubule in diabetes is subject to pathogenetic influences as a consequence of its position in the nephron and its reabsorptive function.

To assess tubulointerstitial fibrosis, we performed Masson's trichrome staining and immunostaining for collagen type IV. Kidneys from Akita mice (Fig. 4, *Bc* and *Dc,* page 94) exhibited significantly higher expression of collagenous components and collagen type IV relative to non-Akita WT (Fig. 4, *Ba* and *Da*, page 94). Cat overexpression markedly reduced tubulointerstitial fibrosis (Fig. 4, *Bd* and *Dd*) as demonstrated by quantitative analysis of Masson's trichrome staining (Fig. 4*C*, page 94) and immunostaining for collagen IV (Fig. 4*F*, page 94). Quantitation of collagen IV (Fig. 4*H*, page 94) expression further confirmed these findings. Collectively, these data indicate that Cat overexpression effectively prevents tubulointerstitial fibrosis in Akita Cat-Tg mice.

The precise mechanism by which oxidative stress leads to interstitial fibrosis in Akita mice remains unclear. One possibility is that augmented Ang II expression via ROS generation stimulates TGF-β1 and subsequently enhances the expression of extracellular matrix proteins. TGF-β1 is known to have powerful fibrogenic actions resulting from both stimulation of matrix synthesis and inhibition of matrix degradation [312]. Ang II stimulates mesangial matrix synthesis, an effect mediated by TGF-β [178]. The Ang II competitive inhibitor saralasin prevented the effects of Ang II [178]. Similar effects of Ang II on TGF- β synthesis also occur in proximal tubule cells [313]. Cat overexpression significantly attenuated the ROS generation and normalized ACE2 and ACE expression, resulting in the decrease of Ang II. Thus, Cat overexpression could decrease TGF-β expression (Fig. 4C, 4E, 4G and 4I, page 94), subsequently ameliorating fibrosis. Similarly, Ang 1-7 could attenuate ROS generation and upregulate ACE2 expression, reducing tubular fibrosis.

Recently Mori et al [314] studied the mechanism of Ang 1-7-induced beneficial effects on DN in db/db mouse. The genetic background of the mice is C57BL/6J and therefore no hypertension was observed. These effects were associated with reduction of oxidative stress, fibrosis, inflammation and lipotoxicity. Ang 1-7 attenuated renal ROS production via reduced NADPH oxidase activity, as we observed on our experiment. In T2D, insulin resistance and renal lipid accumulation are involved in the pathogenesis of diabetic nephropathy [315]. Ang 1-7 can ameliorate lipid metabolism related pathway, such as sirtuin- FOXO1- ATGL (adipose triglyceride lipase), as well as inflammation in perirenal adipose tissue, which plays an important role in the development of insulin resistance [314].

In our experiment, we found that Ang 1-7 could normalize the expression of ACE2 in proximal tubule. An explanation for this is that Ang 1-7 regulates the shedding of ACE2. As

showed in Figure 3-6 (page 135), TACE expression was increased in Akita mice. Administration of Ang 1-7 could attenuate the TACE expression and increase intrarenal ACE2, leading to the improvement of hypertension and DN.

4.6.5 Cat, Ang 1-7 and tubular apoptosis

Atubular glomeruli, glomeruli that not connected to proximal tubules, have been reported in both type 1 and type 2 diabetes [316, 317]. From T1D patients, atubular glomeruli may occur in 8–17% of nephrons [317]. Tubular atrophy results in the glomerulus losing its connection to a functional tubule [237]. Furthermore, tubular atrophy appears to be a better predictor of renal disease progression than glomerular pathology because of its close association with loss of renal function [318].

The mechanisms underlying tubular atrophy remain unclear. One possible mechanism is apoptosis. In our studies, we detected higher number of apoptotic RPTCs in Akita mice. Overexpression of Cat in the kidney could mitigate the apoptosis in RPTC. The mechanism(s) by which RPTC apoptosis increases with DM remains undefined. One possibility is oxidative stress, directly from hyperglycemia or from activation of NADPH oxidase by Ang II. Increased ROS generation activates p38 MAPK signalling, in turn stimulating p53 phosphorylation [319]. Phosphorylated p53 then translocates to the nucleus and induces the expression of Bax gene [320]. Bax translocates to the mitochondria and competes with the anti-apoptotic proteins Bcl-2 and Bcl-xL, leading to mitochondrial dysfunction, mitochondrial outer membrane permeabilization and ultimately, caspase-3 activation. Our data on increased Bax and caspase-3 expression, together with decreased Bcl-xL expression in RPTs of diabetic mice, lend support to this notion. Furthermore, enhanced intrarenal RAS activation in the DM

stimulates Bax mRNA and protein expression per se [182]. Another explanation is role of TGF-β, which was reported to promote apoptosis after renal injury in both *in vivo* and *in vitro* models [321, 322]. Cat overexpression could effectively attenuate ROS production and therefore block the above signal pathways. Furthermore, Cat overexpression could upregulate ACE2 expression, resulting in attenuated Ang II. Taken together, Cat overexpression leads to the decreased Bax/Bcl-xL ratio and finally attenuated caspase-3 activation.

4.7 Comparison between Ang 1-7 and RAS blocker

Interestingly, Zhang et al compared the effect of Ang 1-7 with AT1R antagonist on the DN. They reported that Ang 1–7 ameliorated STZ-induced diabetic renal injury in a dose-dependent manner, and the renoprotective effect of large-dose Ang 1–7 treatment was superior to valsartan treatment [323]. The combined treatment of Ang 1–7 and valsartan had no additive effects. The possible mechanisms of Ang 1–7-mediated effects involved reduced renal oxidative stress regulated by NOXs and PKCs, and inhibited TGF-β1/Smad3 and VEGF signalling [323]. Another study used a recombinant adenoviral-mediated *ACE2* gene transfer (Ad-ACE2) and/or ACEi in a rat model of DN to compare the effects of the combined therapies (Ad-ACE2 + ACEi) and isolated therapy (Ad-ACE2 or ACEi) on glomerular morphology and function and to explore the signaling pathways mediating these therapeutic effects [195]. The study demonstrated that Ad-ACE2 and ACEI had similar effects, whereas combination of Ad-ACE2 and ACEi offered no additional benefits. To date, no comparison has been performed between the effect of Ang 1-7 and that of ACEi. However, several guidelines suggest that ACEi and ARB are equivalent on the DN [324], thus Ang 1-7 treatment might be superior to ACEi treatment.

4.8 ACE2 and non-diabetic kidney disease

Inhibition of the RAS by ACEi and/or ARB can reduce albuminuria/proteinuria independent of the baseline degree of albuminuria/proteinuria and underlying disease and has become standard practice for delaying the progression of kidney disease both in patients with diabetic nephropathy and in non-diabetic albuminuric patients [325, 326]. ACE2 seems to have the same effect as ACEi or ARB; however, much less investigations on ACE2 have been performed in non-diabetic kidney disease than in DN. In patients with IgA nephropathy, increased glomerular ACE expression and decreased ACE2 expression were observed in both the tubulointerstitium and glomeruli, as in DN [327]. Roberts et al. [328] showed that among patients with CKD plasma ACE2 activity is lower in those undergoing hemodialysis for ESRD when compared with predialysis patients with CKD or renal transplant patients. These findings suggest that decreased ACE2 activity may be involved in the pathogenesis of kidney disease [329]. Zhang et al demonstrated that a 5-day infusion of Ang 1–7 reduced proteinuria and improved glomerulosclerosis experimental glomerulonephritis [330]. Treatment with the Mas receptor agonist AVE 0991 improved renal function parameters, reduced proteinuria and attenuated histological changes in a murine model of adriamycin-induced nephropathy [331]. Dilauro et al. demonstrated that kidney ACE2 is downregulated in early CKD in the 5/6 nephrectomy in FVB mice. Moreover, inhibition of ACE2 increases kidney Ang II levels and albuminuria via an AT1 receptor-dependent mechanism. Their data also indicated that administration of Ang 1–7 to 5/6 nephrectomy mice normalizes kidney ACE2 expression and levels of Ang II in kidney and plasma, yet does not affect BP, albuminuria, or FITC-inulin clearance [332]. Therefore endogenous ACE2 is renoprotective in CKD; however, the beneficial effect of ACE2 therapy on the non-diabetic CKD still need further study.

4.9 Limitations of the present study

We used a Tg mouse model overexpressing rCat in their RPTCs to study the role of ACE2 in the development of hypertension and DN. Although we demonstrated that the beneficial effect of Cat on the hypertension and DN was because of up-regulation of ACE2, the study is not as direct as the one using ACE2 Tg or ACE2 knockout mice. In fact, we have attempted to create Tg mice overexpressing ACE2 in RPTCs. We placed human ACE2 cDNA fuse with a HA tag at the 5' terminal into pKAP2. Southern blot analysis revealed the presence of the transgene in heterozygote and homozygote animals. RT-PCR also showed Tg specifically expressed in kidney not in other tissue. Unfortunately, however, we could not detect more ACE2 protein expression in the RPTCs by both western blot and IHC, compared to WT. Neither did we detect increased ACE2 activity in Tg mice. Why the Tg did not express in the protein level is still unknown.

Another limitation of the present study is the method we used to administer Ang 1-7. Previously we implanted mini-pump with Ang 1-7 into the back of Akita mice. This method could provide continuous drug delivery and were supposed to more conform to pharmacological rule. However, the experiment failed because all the implanted mini-pump came out. Lack of subcutaneous fat and dehydration of skin in diabetic condition prevent the cut from healing. In the end, we employed one-shot subcutaneous injection to treat the animal every day.

Chapter 5: Unpublished Results and Perspectives of Research

5.1 Generation of ACE2 Transgenic mice

To directly study the role of ACE2 in the development of hypertension and DN, we attempted to create the Tg mice overexpressing ACE2 in the RPTCs.

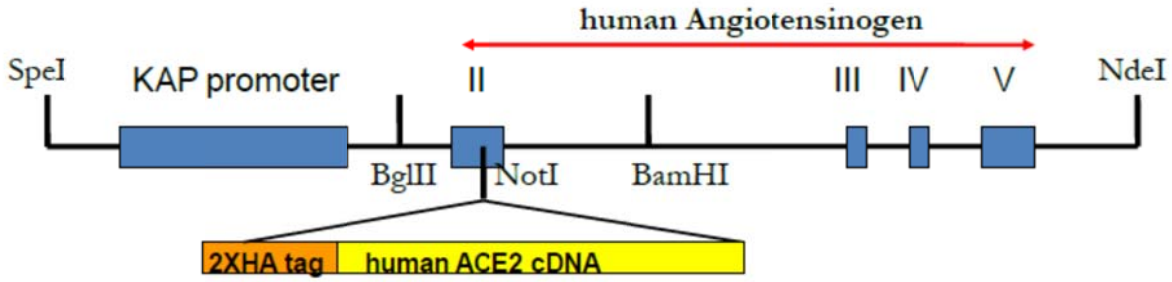


Figure 5-1. Schematic map of the KAP2hACE2 construct.

The cDNA encoding full-length human ACE2 fused with HA-tag 5' end (NotI site flanked at both 5'- and 3'-terminali) (obtained from Dr. Kevin Burns of the University of Ottawa) was inserted into pKAP2 plasmid at the NotI site of exon II of human Agt gene (Figure 5-1). Since the exon II contains most of the Agt-coding area including the start codon, the signal peptide, and the Ang I and Ang II peptides, its removal disrupts the Agt expression. However, it has been demonstrated that a transcriptional enhancer in exon 5 and the 3'-untranslated region of the hAgt gene could cooperate with the KAP promoter to drive the transgene expression in the proximal tubules and in response to androgen [333]. With this KAP2 construct, our lab has created transgenic mice overexpressing rAgt [182], rCat [334] and hnRNP F [335] in RPTCs successfully. The isolated KAP2-hACE2 transgene was then microinjected into one-cell fertilized mouse embryos using a standard procedure (performed by Dr. Zhou at the Clinical Research Institute of Montreal). The positive Tg founders were then crossed with WT C57Bl/6

mice for F1 generation. Breeding was continued until homozygous F3 and F4 Tg mice were found.

We used southern blotting to screen the positive founders and obtained 13 male and 5 female positive clones (Figure 5-2). Then we used RT-PCR to examine the mRNA expression in different tissues, including brain, heart, liver, lung, spleen, testis and kidney. We found 2 lines (line 17 and line 56) where Tg was only expressed in the kidney (Figure 5-3). At the same time, we determined the ACE2 protein level by western blotting and immunostaining. Unfortunately we could not detect more ACE2 protein in Tg mice than in control mice. (Figure 5-4, Figure 5-5).

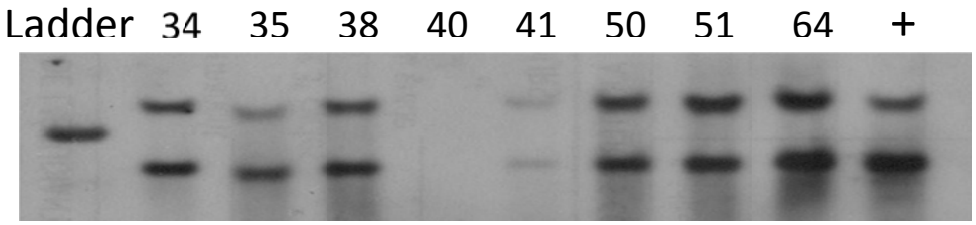


Figure 5-2. Analysis of clonality by Southern blotting

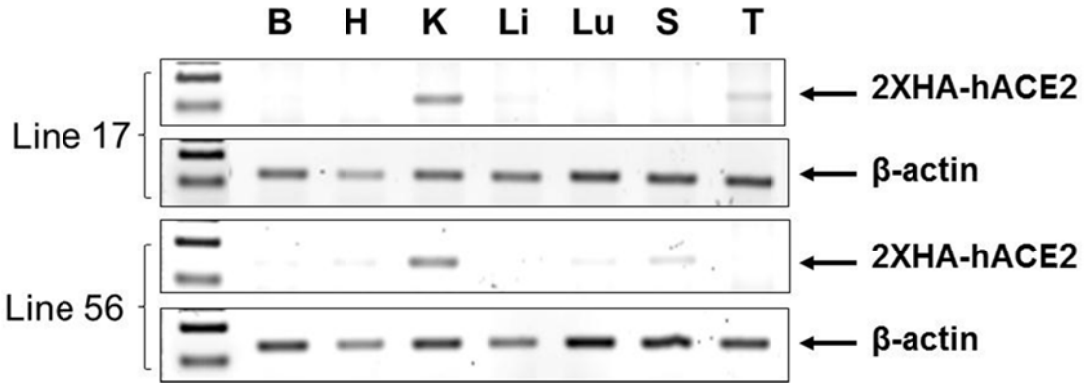
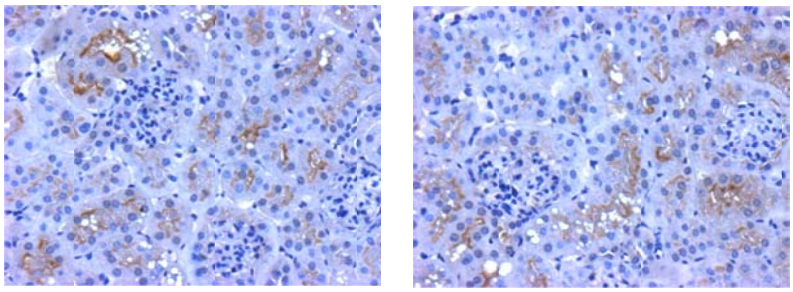


Figure 5-3. Tissue specific expression of hACE2 Tg.

The reason why in Tg mice we could not detect the ACE2 protein or more protein expression than in WT is still puzzling us. First, we have thought that HA tag at the 5' end of hACE2 might affect its expression and sorting. Then we created another construct with Myc tag at the 3' end but without 2X HA and generated another Tg monse. Unfortunately, however, we still could not detect more ACE2 protein in Tg mice despite the kidney-specific mRNA expression of transgene. Therefore, fused tag seems not to affect the expression and sorting. Another explanation is the abundance of endogenous ACE2 protein. Under normal condition, RPTCs expresses a large quantity of ACE2 protein so that the increased level in Tg cannot change the final level significantly.



Control 20W ACE2-Tg 20W

Figure 5-4. Immunostaining for ACE2

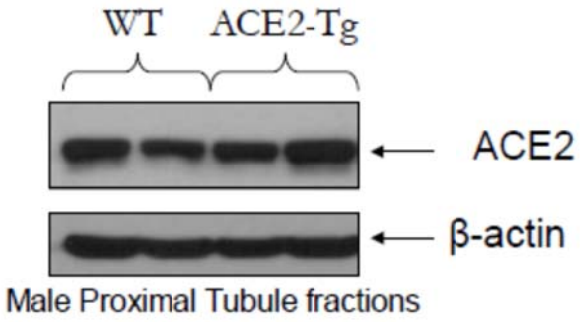


Figure 5-5. Western blotting of ACE2

Future Experiments

5.2 Kidney-specific ACE2 knock-out mice and DN

Since generation of Tg mice failed, we propose to create kidney-specific ACE2 knock-out mice. Cre-Lox recombination should be employed as described below.

Cre recombinase is an enzyme that catalyzes recombination between two loxP sites and LoxP sites are specific 34-base pair sequences consisting of an 8-bp core sequence, where recombination occurs, and two flanking 13-bp inverted repeats. If the loxP sites are oriented in the same direction on a chromosome segment, Cre recombinase results in a deletion of the floxed segment (Figure 5-6).

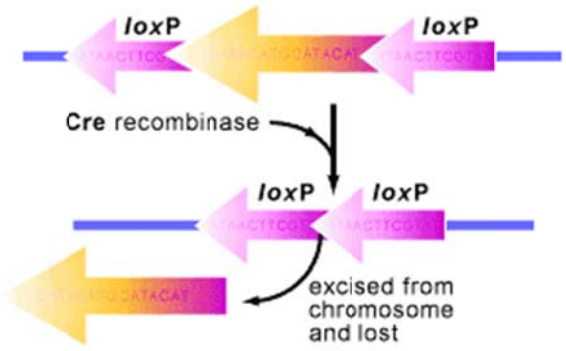


Figure 5-6. A model of *Cre* Function [336]

If *Cre* is placed under the control of KAP promotor (KAP-*Cre*), it will only express in the RPTCs. Another mouse model will be generated in which ACE2 gene is flanked with loxP sites. Crossing KAP- *Cre* mice with LoxP-ACE2-LoxP mice will result in deletion of ACE2 in RPTCs and generation of RPTCs-specific KO mice (Figure 5-7).

After RPTCs-specific mice have been generated, they will be crossbred with Akita mice to create Akita ACE2 KO mice. We will check if kidney-specific ACE2 KO would exacerbate

the DN and hypertension. We will treat Akita ACE2 KO with Ang 1-7 or combination of Ang 1-7 and A-779 as well.

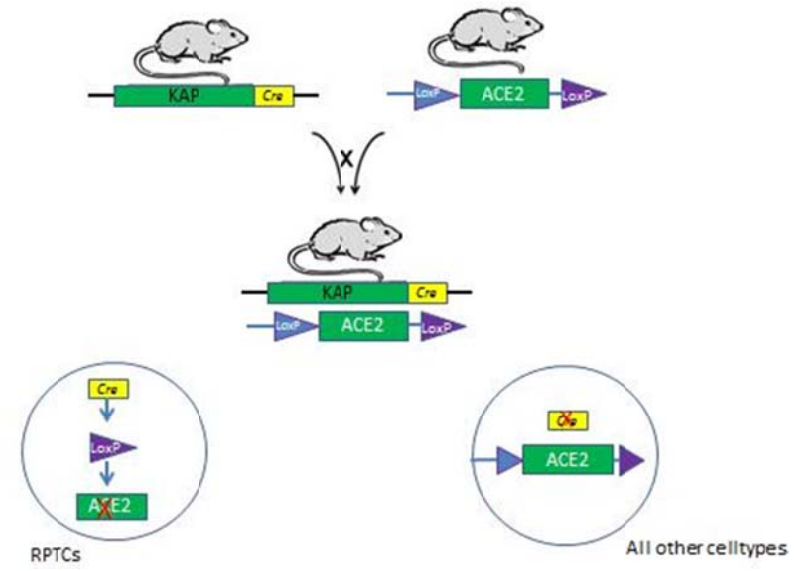


Figure 5-7.Generation of RPTCs-specific KO mice (revised from Ref.[337])

BP will be measured from age 8 or 9 weeks until 16 weeks, 2-3 times per week. Urine will be collected for albuminuria analysis by the ACR. The GFR will be done on the day of animal sacrifice. After sacrifice, half of the kidneys will be fixed for histology, with hematoxylin-eosin (HE) and Masson's trichrome staining, immunohistochemistry, TUNEL assay, etc. Another half will be placed in a vinyl mold with OCT for frozen section. RPTCs will be isolated from the remaining kidneys for RNA and protein extraction to detect the relative gene expression of ACE, ACE2, Agt, TGFβ1, collagen IV, Nox, Bcl-Xl, Bax, etc., by RT-qPCR and Western blotting. It is anticipated that Ang 1-7 treatment will improve the renal histology, attenuate ROS generation and NOX isoforms expression, and decrease the expression of pro-

apoptotic genes, pro-fibrogenic genes. These findings will confirm whether Ang 1-7 alone contributes to the renal protective role of ACE2.

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