Studies on Angiotensin Converting Enzyme 2, Angiotensin-(1-7), and p47phox-Dependent NADPH Oxidase and Their Roles in Diabetic Nephropathy

by

George Chu Liu

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Medical Science
University of Toronto

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Doctor of Philosophy
Institute of Medical Science
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2012

Abstract

Diabetic nephropathy is the leading cause of end-stage renal disease, yet the mechanisms responsible for hyperglycemia-induced kidney injury have not been fully elucidated. Activation of the renin-angiotensin system and NADPH oxidase-dependent generation of reactive oxygen species are important mediators of chronic kidney disease. I first studied the effect of ACE2, an important enzyme in the renin-angiotensin system, in diabetic kidney injury in the Akita mouse and related the effect to angiotensin peptide and NADPH oxidase. I then demonstrated the interaction between Angiotensin II, the main substrate, and angiotensin-(1-7), the main product of ACE2, respectively, on cell signaling in mesangial cells to better understand the in vitro effect of ACE2. Finally I studied the effect of deletion of p47phox, a regulatory subunit of the NADPH oxidase, on initiation and progression of diabetic nephropathy in the Akita mouse and mesangial cell.

Administration of human recombinant ACE2 decreased angiotensin II levels, increased angiotensin-(1-7) levels, normalized NADPH oxidase activity in the Akita mice, and ameliorated
diabetes-induced kidney injury. In vitro, hrACE2 attenuated both high glucose and ANG II–induced oxidative stress and NADPH oxidase activity in mesangial cells.

Ang-(1–7)-induced ERK1/2 phosphorylation in mesangial cells in a mas receptor-cAMP-PKA-dependent manner. This effect of ang-(1-7) on ERK1/2 phosphorylation is not mediated by AT1R, AT2R, epidermal growth factor or NADPH oxidase. Pre-treatment with Ang-(1-7) attenuated Ang II-induced NADPH oxidase activity and ERK1/2 activation also in a cAMP-PKA-dependent manner.

Deletion of p47phox not only reduced diabetes-induced kidney injury but also reduced hyperglycemia by increasing pancreatic and circulating insulin concentrations. p47phox−/− mice exhibited improved glucose tolerance but modestly decreased insulin sensitivity. Deletion of p47phox attenuated high glucose-induced activation of NADPH oxidase and pro-fibrotic gene expression in mesangial cells. There was a positive correlation between p47phox and collagen Ia1 mRNA levels in renal biopsy samples from control subjects and subjects with diabetic nephropathy.

The data generated in this thesis strongly suggest a protective role of ACE2, via Ang-(1-7), and a deleterious role of p47phox in diabetic nephropathy. Future therapeutic strategies should include enhancing ACE2 activity in the kidney and inhibiting p47phox-dependent activation of NADPH oxidase in both the kidney and the pancreas.
Acknowledgments

I would like to take this opportunity to extend my gratitude to my supervisor Dr. James Scholey, who has always been knowledgeable, compassionate, and encouraging to my research ideas. He provided me with endless guidance and support during my undergraduate summer studentship and six years of graduate studies.

I would also like to thank my graduate committee members, Dr. Adria Giacca, Dr. Richard Gilbert, and Dr. Sue Quaggin for their continuous support to my graduate studies. All of them provided valuable input to the work in this thesis and helped me to develop important skills to become an independent researcher and thinker.

A very special thank you to Dr. Gavin Oudit, my collaborator, and to his laboratory team in University of Alberta. I had a lot fun working with Gavin at the bench. Doing research is tough, and experiments often involve repetitive and tedious labour, but working with Gavin certainly made it less boring.

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Unfortunately, my former committee member Dr. Wolfgang Vogel and collaborator Dr. Andrew Herzenberg passed away during my graduate studies and this thesis is dedicated to them.

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Author Contribution and Copyright Acknowledgements

Chapter 2: Human Recombinant ACE2 Reduces the Progression of Diabetic Nephropathy

A) Contributions:

Liu GC contributed to conception and design, acquisition, analysis and interpretation of data, drafting and revising the entire chapter. Oudit GY contributed to conception and design, obtaining of the human recombinant ACE2, interpretation of data, drafting and revising the chapter. Zhong J, Basu R, Chow FL, Loibner H, Janzek E, and Schuster M assisted in acquisition, analysis and interpretation of data for Figures 2.1, 2.3, and 2.4. Zhou J assisted in acquisition and analysis of data for Figure 2.6. Penninger JM contributed to supplying of the human recombinant ACE2. Herzenberg AM assisted in interpretation of data for Figures 2.2 and 2.3. Kassiri Z and Scholey JW contributed to conception and design, interpretation of data, drafting and revising the chapter.

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Chapter 3: Angiotensin-(1-7)-Induced Activation of ERK1/2 is cAMP/Protein Kinase A-dependent in Glomerular Mesangial Cells

A) Contributions:

Liu GC generated all the data, and completed all data analysis and writing for this chapter. Oudit GY and Scholey JW contributed to the experimental design and revising the chapter. Fang F and Zhou J contributed to acquisition, analysis and interpretation of data.

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Chapter 4: Deletion of $p47^{{phox}}$ Attenuates the Progression of Diabetic Nephropathy and Reduces the Severity of Diabetes in the Akita Mouse

A) Contributions:

Liu GC generated all the data, and completed all data analysis and writing for this chapter. Fang F and Zhou J contributed to acquisition, analysis and interpretation of data. Koulajian K, Kan K, Giacca A contributed to acquisition of data for Figure 4.8. Yang S, Reich HN, John R contributed to acquisition of data for Figure 4.13. Herzenberg AM assisted in interpretation of data for Figure 4.3. Oudit GY and Scholey JW contributed to the experimental design and revising the chapter.

B) Publications:

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<th>Description</th>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>ACE2</td>
<td>Angiotensin converting enzyme 2</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation end-product</td>
</tr>
<tr>
<td>ANG</td>
<td>Angiotensin I</td>
</tr>
<tr>
<td>ANG II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ANG-(1-7)</td>
<td>Angiotensin-(1-7)</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AT₁R</td>
<td>Angiotensin II type 1 receptor</td>
</tr>
<tr>
<td>AT₂R</td>
<td>Angiotensin II type 2 receptor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BTBR</td>
<td>Black and Tan Brachyuric mice</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic 3’, 5’-adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic-AMP response element binding protein</td>
</tr>
<tr>
<td>C-terminal</td>
<td>Carboxy-terminal</td>
</tr>
<tr>
<td>db/db</td>
<td>diabetic:diabetic mice (homozygous for leptin receptor deficiency)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dubelcco’s modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>Epac</td>
<td>Exchange proteins directly activated by cAMP</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>G protein</td>
<td>Guanine nucleotide-binding protein</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine-nucleotide exchange factor</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
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<tr>
<td>GLUT-1</td>
<td>Glucose transporter-1</td>
</tr>
<tr>
<td>GLUT-4</td>
<td>Glucose transporter-4</td>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GTT</td>
<td>Glucose tolerance test</td>
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xx
<table>
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<tr>
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<tbody>
<tr>
<td>HbA1c</td>
<td>Glycated haemoglobin A1c</td>
</tr>
<tr>
<td>Hepes</td>
<td>4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>Ins2</td>
<td>Insulin 2 gene</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobases</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out (null)</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>N-terminal</td>
<td>amino-terminal</td>
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<tr>
<td>ob/ob</td>
<td>obese:obese mice (homozygous for leptin deficiency)</td>
</tr>
<tr>
<td>P</td>
<td>Observed significance level</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B (also known as Akt)</td>
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<td>Abbreviation</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end-product</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
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<td>Reverse-transcriptase polymerase chain reaction</td>
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<tr>
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<td>Standard deviation</td>
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<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SGLT-1</td>
<td>Sodium-dependent glucose transporter-1</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes mellitus</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>vs</td>
<td>versus</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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Nucleic Acid Abbreviations

A  adenine or deoxyadenosine
C  cytosine or deoxycytidine
G  guanine or deoxyguanosine
T  thymine or deoxythymidine

Amino Acid Abbreviations

Ala  A  alanine
Arg  R  arginine
Asn  N  asparagine
Asp  D  aspartic acid
Cys  C  cysteine
Gln  Q  glutamine
Glu  E  glutamic acid
Gly  G  glycine
His  H  histidine
Ile  I  isoleucine
Leu  L  leucine
Lys  K  lysine
Met  M  methionine
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<td>Val</td>
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**Methodological Abbreviations**

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<tr>
<td>U</td>
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wk  week
wt  weight
vol volume

**Prefixes**

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<td>kilo-</td>
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<tr>
<td>c</td>
<td>centi-</td>
<td>(x 10⁻²)</td>
</tr>
<tr>
<td>m</td>
<td>milli-</td>
<td>(x 10⁻³)</td>
</tr>
<tr>
<td>μ</td>
<td>micro-</td>
<td>(x 10⁻⁶)</td>
</tr>
<tr>
<td>n</td>
<td>nano-</td>
<td>(x 10⁻⁹)</td>
</tr>
<tr>
<td>p</td>
<td>pico-</td>
<td>(x 10⁻¹²)</td>
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Chapter 1

Introduction
1.1 DIABETIC NEPHROPATHY

1.1.1 Introduction to diabetic nephropathy

Diabetes mellitus and its complications are important causes of mortality and morbidity worldwide. With devastating effects on life expectancy and health care cost, diabetes is considered the epidemic of the 21st century. Currently there are 171 million people affected by diabetes and this number is expected to reach 366 million in 2030. Therefore it becomes increasingly important to understand the mechanisms responsible for diabetes and its clinical complications so that strategies can be designed to limit the disease burden.

There are two major types of diabetes mellitus: type 1 (T1DM) and type 2 (T2DM). T1DM accounts for approximately 10% of all cases of clinical diabetes. It is characterized by a progressive defect of insulin secretion in the β-cell of the pancreas. T2DM is associated with reduced insulin sensitivity and loss of insulin action on the body. Both T1DM and T2DM lead to chronic hyperglycemia which damages kidney, heart, eye, nervous system and blood vessels.

One of the most important microvascular complications of diabetes is hyperglycemia-induced kidney injury, and diabetic nephropathy is the leading cause of end-stage renal disease (ESRD). The basic pathophysiological mechanisms eventually leading to nephropathy are similar in type 1 and 2 diabetes. However, other risk factors such as hypertension, obesity, dyslipidaemia and ischaemic renal disease caused by arteriosclerosis that are associated with type 2 diabetes, could increase kidney damage and contribute to nephropathy.

Genetic factors are important determinants of diabetic nephropathy. Zheng and colleagues demonstrated that phenotypic changes in bone marrow-derived mesangial cell progenitors transmit diabetic nephropathy from donors with type 2 diabetes to naive, normoglycaemic recipients. They may directly affect the initiation and progression of diabetic nephropathy indirectly through other cardiovascular diseases. Up to 30% of diabetic patients develop nephropathy. Ongoing research is focused on genomic screening and candidate gene approaches to identify genetic loci for diabetic nephropathy susceptibility. A number of genes have been identified to potentially affect diabetic nephropathy, however their influence was only demonstrated in defined ethnic subpopulations but not in the majority of patients. This
is probably because of a non-simplistic Mendelian inheritance mode with several genes likely involved \(^{361}\).

All cell types of the kidney are affected by diabetes-induced injury, including podocyte, mesangial cell, endothelial cell, tubular epithelial cell, interstitial fibroblast, and vascular endothelial cell \(^{59,128}\). The earliest clinical manifestations of glomerular capillary damage is increased urinary albumin excretion rate and hyperfiltration \(^{59,128}\). These functional abnormalities are accompanied with morphological changes in the glomeruli including mesangial cell proliferation and hypertrophy, accumulation of extracellular matrix (ECM) proteins in the mesangium, thickening of the basement membrane, and glomerulosclerosis \(^{15}\). Similar changes are also observed in the tubulointerstitial compartment including tubular hypertrophy, thickening of the tubular basement membrane (TBM), and interstitial fibrosis \(^{129,130}\). As nephropathy progresses, the glomerular filtration rate (GFR) gradually declines along with a progressive rise in albuminuria. This continues to a point where the kidney is no longer able to filter wastes and toxic substances out of the body and ESRD ensues. The progression of diabetic nephropathy takes 10 to 30 years \(^{15}\).

The very early manifestations in the disease process are the haemodynamic changes including hyperfiltration and hyperperfusion, they are viewed as pivotal in the initiation of diabetic nephropathy \(^{362}\). Decrease in both afferent and efferent arteriolar resistance increases glomerular capillary pressure because the former is more dilated than the latter \(^{362}\). The elevation in glomerular capillary pressure leads to increased glomerular plasma flow and transcapillary hydraulic pressure gradient. In normal physiological settings, an increase in perfusion pressure would trigger preglomerular vasoconstriction and restore glomerular filtration rate to normal rate \(^{363}\). However in diabetic nephropathy, a defect in autoregulation prevents the restoration of normal glomerular filtration rate. This is affected by a variety of factors including prostanoids, nitric oxide, atrial natriuretic factors, growth hormone, glucagon, insulin, and angiotensin II \(^{362}\). Elevated intraglomerular pressure induces mesangial matrix expansion, thickening of the glomerular basement membrane, and glomerulosclerosis \(^{364,365}\). It is well accepted that both the haemodynamic and structural changes in the glomerulus are important for the manifestation of diabetic nephropathy \(^{362}\). High glucose induces the production of angiotensin II, angiotensin II in turn exerts haemodynamic, trophic, inflammatory and profibrogenic effects on kidney cells \(^{364}\).
Vascular endothelial growth factors (VEGFs) and transforming growth factor beta (TGF-β) have all been shown to influence glomerular haemodynamics under diabetic environment. Altered glomerular haemodynamics induces shear stress and mechanical strain, which influence the autocrine and/or paracrine release of cytokines and growth factors. In vitro studies exposing mesangial cells, glomerular endothelial cells, or podocytes to shear stress induces specific cellular responses including activation of certain signal transduction systems, growth responses, enhanced synthesis of hormones and cytokines, increased angiotensin II and TGF-β concentrations, and increased production of extracellular matrix proteins. These findings suggest that local haemodynamic effects contribute, at least partially, to the structural changes of diabetic kidney by the local activation of cytokines and growth factors.

Abnormality in sodium reabsorption has been implicated in glomerular hyperfiltration in diabetic nephropathy. This suggests that an increase in reabsorption of sodium chloride in proximal tubules or loops of Henle leads to an increase in the glomerular filtration rate by an intact macula-densa mechanism. Diabetes-induced hypertrophy of tubules that mediate sodium chloride reabsorption could be a determinant of this process, linking again structural changes with haemodynamic adaptation in diabetic nephropathy.

Diabetes induces injury in almost all renal compartments, including mesangial expansion, glomerulosclerosis (diffuse, nodular), fibrin cap lesion, capsular drop lesion, basement membrane thickening (glomerular and tubular), endothelial foam cells, podocyte abnormalities and apoptosis, tubular atrophy, interstitial inflammation, interstitial fibrosis, and arteriosclerosis. The earliest morphological change of diabetic nephropathy is the mesangial matrix expansion, as a result of extracellular matrix protein deposition and mesangial cell hypertrophy. A highly significant inverse correlation between glomerular filtration rate and mesangial expansion has been shown by structural-functional relationship studies. Mesangial cells become arrested in the G1-phase of the cell cycle after a short period of proliferation under high glucose environment. This G1-phase arrest is mediated by an inhibitor of cyclin-dependent kinases, p27Kip1. High glucose increases p27Kip1 expression through the activation of mitogen-activated protein kinases. Deletion of the p27Kip1 gene attenuates high glucose-induced mesangial cell hypertrophy. Angiotensin II further enhances p27Kip1 production and blockade of angiotensin II attenuates high glucose-induced mesangial cell hypertrophy.
Thickening of the glomerular basement membrane (GBM) can be found 1 year after onset of type 1 diabetes \(^{376}\). Thickening of the GBM is progressive over many years and is thought to be contributed to by both increased synthesis and impaired removal of the extracellular matrix proteins. Several biochemical alterations of the GBM occur in diabetic nephropathy \(^{376}\). There is an increase in collagen type IV deposition, and decrease in the expression of heparan sulphate and the extent of sulphation. Mesangial matrix mainly contains type IV collagen α1 and α2, in contrast the GBM contains α3, α4, and α5 chains \(^{377,378}\). In diabetic nephropathy, α1(IV) and α2(IV) chains are upregulated in mesangial cells, whereas in GBM, α3(IV) and α4(IV) expression is increased \(^{378}\). Collagen type I and III deposition in the mesangial area occurs late in glomerulosclerosis \(^{377}\).

Podocytes are glomerular epithelial cells which directly cover the GBM. There is recent evidence that changes in structure and function of podocytes occur at the onset of albuminuria and occur early in diabetic nephropathy \(^{379-381}\). Podocytes interacts with the GBM through α3β1 and α2β1 integrins. Hyperglycaemia is associated with changes in integrin expression profile, influencing the adherence of podocytes to the GBM \(^{377}\). Longitudinal studies in human patients with diabetic nephropathy showed a reduction in podocyte number which closely correlated with proteinuria \(^{379}\). Steffes and coworkers counted the glomerular cell number in patients with type 1 diabetes and compared those numbers with age-matched normal individuals \(^{380}\). They observed a reduction in the podocyte number in diabetic patients of all ages, even in patients who had diabetes for a short duration \(^{380}\). In addition, renal biopsies from type 2 diabetic Pima Indians showed a concomitant reduction in the number of podocytes per glomerulus and a broadening in podocyte foot processes \(^{381}\). Vestra and colleagues found a significant reduction in podocytes per glomerulus in patients with type 2 diabetes that were normoalbuminuric \(^{382}\). Podocyte structure abnormality is also found in rodent models of diabetic nephropathy. In a rat model of type 2 diabetes, damage to podocytes, including foot process effacement and cytoplasmic accumulation of lipid droplets were found early during progression of diabetic nephropathy. These rats ultimately developed segmental glomerulosclerosis \(^{383}\). Early progressive podocyte damage antedates the development of glomerulosclerosis and tubulointerstitial damage in this model \(^{384}\).

Currently there is no cure for either diabetes mellitus or diabetic nephropathy. Although poor glycemic control is an important risk factor for diabetic nephropathy, difference in glycemia levels does not fully explain why only a subset of diabetic patients progress to ESRD \(^{14}\).
Therapeutic interventions that improve glycemic control and lessen hypertension may slow but cannot prevent the progression of diabetic nephropathy\textsuperscript{16}. The molecular mechanisms responsible for initiation and progression of diabetic nephropathy have not been fully elucidated. A number of signaling pathways and intracellular events have been implicated to link high-glucose to injury, including increased flux of polyols and hexosamines, generation of reactive oxygen species (ROS) and advanced glycation end-products (AGEs), activation of the transforming growth factor-β, Janus kinase-signal transducer and activator of transcription (JAK-STAT), and protein kinase C (PKC) pathways, deposition of ECM proteins, inhibition of ECM-degrading enzymes, and metalloproteinases\textsuperscript{131-141}. The following sections will summarize some of the pathogenetic mechanisms responsible for diabetic kidney injury.

1.1.2 Glucose uptake and metabolism

In kidney cells, glucose is transported into the cell by two major ways: active transporters, such as Na\textsuperscript{+}-glucose-linked transporters, which transport glucose via an electrochemical concentration gradient, and facilitative transporters such as glucose transporter (GLUT)-1 and -4\textsuperscript{142}. Heilig and coworkers overexpressed GLUT-1 in rat mesangial cells and observed increased ECM production even under normal glucose level. Antisense treatment targeting GLUT-1 prevented glucose-induced fibronectin expression in these cells\textsuperscript{143}.

After glucose enters the cell, it is processed by the glycolysis pathway and forms fructose 6-phosphate (F6-P) after a series of reactions\textsuperscript{145}. In high glucose conditions, F6-P may form glycerol phosphate, which is a precursor of diacylglycerol (DAG). DAG is well-known for its role as a second messenger in recruitment and activation of PKC\textsuperscript{146}. F6-P may also enter the hexosamine pathway and generate UDP-N-acetylglucosamine, a precursor of ECM proteins such as the proteoglycans\textsuperscript{147}. Both PKC and hexosamine pathways have been shown to drive the activation of TGF-β1 and plasminogen activator inhibitor 1 (PAI-1) which contribute to fibrosis in the kidney\textsuperscript{146, 147}.

Another signaling event downstream of excess glucose stimulation and linked to renal fibrosis is the activation of the polyol pathway, which produces polyalcohol sorbitol, which is oxidized to fructose by sorbitol dehydrogenase\textsuperscript{144}. This process leads to depletion of NADPH and reduced
glutathione (GSH), an antioxidant compound, therefore resulting in altered cellular oxidative stress.

1.1.3 Advanced glycation end products

Advanced glycation end products (AGEs) form when sugars bind to other molecules such as amino groups of proteins, and then polymerize into macromolecules. Diabetes increases generation of both intracellular and extracellular AGEs. Glyceraldehyde 3-phosphate (G3-P) from the glycolysis pathway can become a precursor of intracellular AGEs under high glucose condition. These AGEs may activate PKC, Mitogen-activated protein kinase (MAPK), nuclear factor-κB (NF-κB) pathways, and these pathways in turn increase the expression of TGF-β1, cytokines and other growth factors to generate ECM proteins, via the increased production of reactive oxygen species (ROS), and decreased availability of nitric oxide (NO).

Glucose may cross-link with ECM structural proteins to form extracellular AGEs, usually collagen type IV, laminin, fibronectin, and proteoglycans. Extracellular AGEs increase half-lives of these ECM proteins by reducing their susceptibility to enzymatic hydrolysis by matrix metalloproteinases. There is also evidence suggesting that glycation of sulfated proteoglycans may change the charge-selective filtration properties of the basement membrane and contribute to albuminuria. Extracellular AGEs may also bind to their receptor, RAGE, and influence cell-matrix interactions, adhesiveness, neurite growth, and hyperpermeability of capillaries.

1.1.4 Protein kinase C pathway

PKC isoforms, PKC-α, -β, -δ, -ε, and -ζ are expressed in the kidney and activated by high glucose. Generation of DAG is primarily responsible for activation of PKC pathway under diabetic condition, polyol pathway and AGE:RAGE interaction also contribute to PKC activation.
PKC activation may lead to decreased expression of endothelial nitric oxide synthase (eNOS) and NO, and increased expression of endothelin 1 and vascular endothelial growth factor (VEGF) \(^\text{160}\). The subsequent pathophysiological effects are altered blood flow, change in capillary permeability and endothelial dysfunction \(^\text{160}\). PKC may also induce oxidative stress by activation of NADPH oxidase \(^\text{161}\), increase ECM protein deposition by upregulation of TGF-\(\beta\)1 and PAI-1 \(^\text{78, 93}\), and contribute to severe inflammatory response and thrombotic angiopathy via activation of NF-\(\kappa\)B pathway \(^\text{78, 93}\). The administration of a PKC inhibitor ruboxistaurin attenuated diabetic kidney injury in a mouse model of type 2 diabetes \(^\text{162}\). All of these suggest that PKC plays an important role in the pathogenesis of diabetes-induced renal damage.

1.1.5 Transforming growth factor-\(\beta\)1

TGF-\(\beta\)1 signaling under high glucose condition is activated by a variety of mediators: connective tissue growth factor (CTGF), AGEs, ROS, DAG, PKC, hexosamines, angiotensin II, endothelin, thromboxane, and stretching and relaxation of mesangial cells \(^\text{60, 163-165}\). Their predominant effect is to increase synthesis and to inhibit degradation of ECM proteins. TGF-\(\beta\)1 upregulation is observed in patients with diabetic nephropathy \(^\text{166}\). In addition, anti-TGF-\(\beta\)1 neutralizing antibody treatment prevents renal hypertrophy, mesangial matrix expansion, fibrosis and albuminuria in mouse models of type 1 and type 2 diabetes.

TGF-\(\beta\)1 exists in the ECM as a latent, dormant form of propeptide binding to TGF-\(\beta\)1-binding proteins. Plasmin, MMP-2, MMP-9 and thrombospondin 1 cleave the TGF-\(\beta\)1 propeptide to release the active form of free TGF-\(\beta\)1 \(^\text{167-170}\). The active TGF-\(\beta\)1 interacts with the type II serine/threonine kinase receptor, which transphosphorylates and activates the type I receptor. The activated TGF-\(\beta\)1 receptor binds to Smad2 and Smad3, as a result a heterodimeric protein is formed. This heterodimeric protein then translocates into the nucleus with the common partner co-Smad4 and drives the transcription of the target genes such as collagen \(\text{Ia1}\), fibronectin, PAI-1, \(\text{Jun B}\) and \(\text{c-Jun}\) \(^\text{167-170}\). TGF-\(\beta\)1 also upregulates the expression of procollagen and fibronectin in mesangial cells via MAPKs and in turn increases ECM protein deposition \(^\text{171-173}\).
1.1.6 Tubulointerstitial injury

Most studies investigating the pathogenesis of diabetic nephropathy have focused on the glomerulus. Only a few reports have paid attention to the pathobiology of the tubulointerstitium. Although the tubulointerstitium is believed to be adversely affected at a later stage during the progression of diabetic nephropathy than the glomerulus, the degree of the damage to this compartment correlates much better with renal dysfunction such as decrease in the glomerular filtration rate than does damage to the glomerular compartment. That the tubulointerstitial compartment represents 90% of the mass of the kidney further demonstrates the importance of this compartment to the pathogenesis of diabetic nephropathy. For example, in the initial stages of diabetic nephropathy with prevalent hyperfiltration and glomerular enlargement, hypertrophy of the tubules contributes mainly to the increased kidney weight. Similarly, in the later stages of diabetic nephropathy, damage to the tubulointerstitium may substantially impair renal function by a variety of pathogenetic mechanisms.

Tubulointerstitial pathobiology and glomerular pathobiology share some mechanisms, but certain processes are different in the two and can selectively affect them. The pathogenetic mechanisms that are common to both glomerular and tubulointerstitial compartments include increased shunting of glucose metabolites into the polyol pathway; generation of AGEs and ROS; and activation of various pathways pertaining to or influencing RAS, PKC-β, and TGF-β signaling. The adverse effects of these mechanisms are supported by both in vivo and in vitro studies. For instance, direct exposure of tubular HK-2 cells to a high glucose environment increases TGF-β activity and collagen production. In situ hybridization and immunohistochemical studies show that, with the inhibition of ACE or PKC-β, there is a significant decrease in TGF-β activity and in the expression of collagen and osteopontin, which is another ECM protein located mainly in the tubulointerstitium.

Adverse changes in the glomerulus such as proteinuria observed in later stages of diabetic nephropathy may also lead to tubulointerstitial injury. Proteinuria has been shown to induce tubulointerstitial damage via several different mechanisms. Large amounts of profibrogenic cytokines presented in glomerular filtrate may damage tubular epithelium and interstitial cells. Tubular epithelium reabsorbs proteins from glomerular filtrate, excess reabsorption may cause lysosomal rupture, increased energy demand, and ultimately apoptosis.
Increased concentrations of many activated components of the complement system and its endogenous regulatory proteins in tubular lamina, such as C3, C3a, C5a, C5b–9, and Crry may further augment tubular epithelial cell injury. Blockade of complement or MCP-1 activity attenuated tubulointerstitial injury in a rat protein overload model. In addition, proteinuria may be associated with epithelial mesenchymal transformation (EMT), a process that involved in transdifferentiation of tubular cells into myofibroblasts. Interestingly, EMT is mediated by integrin-linked kinase and is stimulated by connective tissue growth factor (CTGF). CTGF is also linked to fibrosis and sclerosis in the glomerular compartment, and its activity is regulated by TGF-β.

Hypoxia-induced injury is another recently identified mechanism by which tubulointerstitial damage can occur in diabetic nephropathy, irrespective of the presence or absence of proteinuria. Hypoxia is usually attributed to chronic ischemia, which may occur by intrarenal vasoconstriction secondary to local activation of RAS or loss of NO. Structural impairment of blood flow may also contribute to hypoxia. Interstitial fibrosis affects the peritubular capillaries in scarred tissue and thus restricts delivery of oxygen to the tubules therefore inducing hypoxia. Oxidant stress and deficiency of NO also contribute to hypoxia by inducing capillary endothelial dysfunction in the tubulointerstitial compartment.

Hypoxia causes tubular injury by mediating functional impairment in the mitochondria of the tubular cells; this leads to apoptosis and is frequently observed in animal models of diabetic nephropathy as well as in humans diabetic patients. Hypoxia itself may also induce activation of resident interstitial cells and EMT of the tubular cells that accelerate tubular fibrosis that further compromises peritubular oxygen delivery. In vitro studies of renal interstitial fibroblasts subjected to oxygen deprivation demonstrated an increase in the transcription of collagen genes and TIMP-1 in human renal fibroblasts. The increase in TIMP-1 expression is shown to be related to the increased activity of its promoter, which is regulated by a transcription factor known as hypoxia-inducible factor 1 (HIF-1). Interestingly, HIF-1 can also bind to the promoter region of the profibrogenic cytokine CTGF, which could contribute to fibrosis. HIF-1 is a versatile transcription factor that regulates a number of pathways and the expression of several genes, including vascular endothelial growth factor (VEGF) and erythropoietin (EPO).
In the initial stages of hypoxia, the intact peritubular cells may generate a sufficient amount of EPO to maintain hemoglobin levels and oxygen tension in the interstitium. However, with persistent damage by chronic hypoxia, hyperglycemia, oxidant stress, and endothelial dysfunction, loss of the EPO-producing fibroblasts may occur, along with anemia and progression of interstitial fibrosis, thereby initiating a vicious cycle of hypoxia and tubulointerstitial injury.

1.1.7 Other important pathogenic mechanisms

In addition to the pathways described above, other pathogenic mechanisms related to diabetes-induced kidney injury have also been studied and recognized, including GTP-binding protein pathway, cell-cycle protein pathway, activation of the renin-angiotensin system (described in section 1.2), reactive oxygen species and oxidative stress (described in section 1.3). The work described in this thesis is closely related to the renin-angiotensin system and NADPH oxidase-derived reactive oxygen species. The following sections summarize our current knowledge regarding the biology of the renin-angiotensin system and the NADPH oxidase.

1.2 THE RENIN-ANGIOTENSIN SYSTEM (RAS)

1.2.1 The classical RAS

The RAS was discovered in the 1890s and was identified as an important endocrine system to regulate cardiovascular function. The classical RAS involves the secretion of renin, a glycoprotein, into the circulation. Renin is synthesized by the juxtaglomerular cells of the renal afferent arteriole. In juxtaglomerular cells, prorenin is processed to form renin, and further processed to become active renin. Once released into the blood, renin cleaves liver-derived angiotensinogen to form angiotensin I (Ang I, Fig. 1.1). Ang I is further cleaved by Angiotensin converting enzyme (ACE) to form Angiotensin II (Ang II).
Biologically active octapeptide Ang II was found to interact with two G protein-coupled receptors (GPCR): the Angiotensin type 1 (AT₁) receptor and the Angiotensin type 2 (AT₂) receptor. The AT₁ receptor activates multiple heterotrimeric G-proteins and generates second messengers like reactive oxygen species (ROS), inositol trisphosphate (IP₃) and diacylglycerol (DAG). AT₁ receptor also activates tyrosine kinases, serine/threonine kinases, mitogen-activated protein kinases (MAPKs), 70 kDa ribosomal protein S6 kinase I (p70S6K), protein kinase B (PKB) and protein kinase C (PKC) pathways. The physiological effects associated with activation of these pathways are vasoconstriction, aldosterone secretion, renal tubular Na⁺ reabsorption, thirst, activation of the sympathetic nervous system, cardiac ionotropic and chronotropic actions, inflammation, hypertrophy and fibrosis.

AT₂ receptor is the predominant receptor in the fetus and only shares 34% amino acid homology to AT₁ receptor. Interestingly, AT₂ receptor is expressed in adult vasculature, juxtaglomerular cells, glomeruli, and tubules to counteract effects of the AT₁ receptor. The physiological effects of the activation of AT₂ receptor are vasodilation, natriuresis, anti-inflammation, anti-fibrosis, and inhibition of cell growth. These are mediated by protein tyrosine phosphatase activation, nitric oxide (NO) generation and sphingolipid signaling pathways.

For many years, renin was thought only to be a protease enzyme responsible for generation of Ang I without any other direct biological actions. Recently it has been shown that pro-renin and renin bind to specific pro-renin receptors. Pro-renin / pro-renin receptor interaction leads to activation of many signaling molecules, including promyelocytic zinc fingers (PLZF), protein-phosphatidylinositol-3-kinase (PI3K) and mitogen-activated protein (MAP) kinases (ERK1/2 and p38MAP kinase). These signaling molecules are involved in cell growth and fibrosis in an Ang II-independent manner, in cardiomyocytes, mesangial cells, podocytes, distal tubular cells, vascular endothelial cells and VSMCs. Currently, the specific physiological and pathophysiological implications of the pro-renin receptors are not unclear.
Figure 1.1: The renin-angiotensin system. Angiotensinogen is cleaved by renin to generate angiotensin I. Angiotensin I may be processed by either angiotensin converting enzyme (ACE) to generate angiotensin II, or angiotensin converting enzyme 2 (ACE2) to generate angiotensin-(1-9). Angiotensin II may be further cleaved by ACE2 to give angiotensin-(1-7), and angiotensin-(1-7) may also be generated from cleavage of angiotensin-(1-9) by ACE. Angiotensin II may act through angiotensin type I receptor (AT₁R) to cause vasoconstriction, hypertrophy, inflammation and fibrosis in the kidney. In contrast, angiotensin-(1-7) antagonizes the effect of angiotensin II via the Mas receptor. Interestingly, angiotensin II-angiotensin type 2 receptor (AT₂R) interaction leads to vasodilation, anti-growth, anti-inflammatory and anti-fibrotic effects in the kidney, acting in opposition to the effect of angiotensin II-AT₁R interaction.
1.2.2 Angiotensin converting enzyme 2, Angiotensin-(1-7) and the Mas receptor

Until recently, the RAS was viewed as a linear process with Ang II being the main effector peptide \(^{47}\). The discoveries of angiotensin converting enzyme 2 (ACE2) and the Mas receptor shifted the focus to the biological, physiological and pathophysiological role of the angiotensin metabolite angiotensin-(1-7) (Ang-(1-7)) \(^{40,48-51}\). The heptapeptide Ang-(1-7) can be generated by ACE2 \(^{52,53}\). Expressed mainly in the heart, kidney, and testis, ACE2 shares 42% amino acid homology to ACE and is not inhibited by classical ACE inhibitors \(^{40,48,52,54-56}\). It acts as a monocarboxypeptidase to cleave a single amino acid from the carboxyl terminus of Ang II to form Ang-(1-7), or to cleave Ang I to form Ang-(1-9) \(^{40,48,52}\). Ang-(1-9) may further be cleaved by ACE to generate Ang-(1-7) \(^{52}\).

The biological and physiological effects of Ang-(1-7) are mediated by the G protein-coupled receptor encoded by the Mas protooncogene \(^{57}\). The binding of Ang-(1-7) to Mas receptor leads to inhibition of MAPK, cyclooxygenase-2 (COX-2)-dependent pathways, and activation of NO/cGMP-dependent pathways \(^{40,48,49,58}\). The result is to oppose the actions of Ang II, in which Ang-(1-7) reduces blood pressure, dilates intrarenal blood vessels, increases renal blood flow and glomerular filtration rate, inhibits proximal tubule transport and induces diuresis \(^{40,48}\).

1.2.3 Intrarenal RAS

It is well recognized now that the RAS is a dual vasoactive system acting on both circulating endocrine and local tissue paracrine levels \(^{30-32}\). The existence and functional properties of local RAS has been shown in the kidney, adrenal glands, heart, blood vessels, pancreas, liver, brain and adipose tissues \(^{33-39}\). All major components of the RAS have been demonstrated in the kidney, including angiotensinogen, renin, ACE, AT\(_1\) and AT\(_2\) receptors, ACE2, angiotensin-(1-7) and the Mas receptor \(^{34,40-44}\). In addition, intracrine/intracellular RAS has also been shown to play important physiological and pathophysiological roles in the kidney \(^{45,46}\).
1.2.4 Renin-angiotensin system in diabetic nephropathy

The activation of the RAS and generation of Ang II in the kidney has been shown to be a determinant of diabetic nephropathy. High glucose upregulates the expression of renin and angiotensinogen in mesangial cells, which increases intrarenal concentrations of Ang II and then, via various autocrine and paracrine pathways, lead to the generation of various cytokines and to glomerular extracellular matrix (ECM) accumulation. It has been demonstrated that hyperglycemia-induced generation of Ang II exacerbates hemodynamic injury to the kidney, contributes to hyperplasia and hypertrophy of the renal cells, through the upregulation of cytokines such as transforming growth factor-β (TGF-β), connective tissue growth factor (CTGF), interleukin-6, monocyte chemoattractant protein 1 (MCP-1), and vascular endothelial growth factor-A (VEGF-A), which increase glomerular ECM deposition and decrease glomerular ECM degradation.

There are still many gaps remaining in our knowledge of the RAS in diabetic nephropathy. Our previous publication has shown that the deletion of the ace2 gene had deleterious effects on a mouse model of diabetic nephropathy, indicating a protective role of ACE2. However, the specific mechanisms of counteracting Ang II by Ang-(1-7), the active product of ACE2, in kidney is not well understood. The experiments in this thesis were designed to fill some of the gaps in our understanding of the RAS in diabetic nephropathy: I first confirmed the protective role of ACE2 by treating diabetic mice with human recombinant ACE2, and then studied the signaling pathways downstream of Ang-(1-7)/Mas receptor interaction in glomerular mesangial cells.

1.3 REACTIVE OXYGEN SPECIES AND NADPH OXIDASE

1.3.1 Reactive oxygen species

Reactive oxygen species (ROS) are small molecules with unpaired valence shell electrons, including oxygen radicals: superoxide (O$_2^-$), hydroxyl (·OH), peroxyl (RO$_2^•$), and alkoxyl (RO•) and nonradicals: hypochlorous acid (HOCl), ozone (O$_3$), and hydrogen peroxide (H$_2$O$_2$). They
are highly reactive and are generally good oxidizing agents\textsuperscript{66, 67}. In cells, ROS interact with a large number of biological molecules including proteins, lipids, carbohydrates and nucleic acids. Most interactions lead to damage of the target molecules, and ROS are accepted as major contributors to the aging process\textsuperscript{68}. In order to limit ROS generation, the cell maintains enzymes that are ROS scavengers. For example, superoxide dismutase converts superoxide to hydrogen peroxide, and catalase further processes hydrogen peroxide into oxygen and water.

Until recently, host defense was believed to be the only physiological role of ROS, such as ROS-dependent killing of the microorganisms\textsuperscript{69}, inactivation of microbial virulence factors\textsuperscript{70}, regulation of pH and ion concentration in the phagosome\textsuperscript{71, 72}, and anti-inflammatory activity\textsuperscript{73}. Recently a second important concept of ROS has been discovered and the physiological importance of ROS is increasingly recognized. ROS have been shown to regulate cellular signaling pathways including inhibition of phosphatases, activation of kinases, regulation of intracellular and plasma membrane ion channels, gene expression, activation of apoptosis or proliferation, and cell growth or senescence\textsuperscript{74-82}. ROS are also oxygen sensing molecules in the kidney, carotid body, pulmonary system and cardiac fibroblast\textsuperscript{83-86}. ROS-dependent biosynthesis and protein cross-linking are key events to the iodination of thyroid hormones\textsuperscript{87}. ROS also play active roles in angiogenesis\textsuperscript{88}, regulation of cellular redox potential\textsuperscript{89}, reduction of metal ions\textsuperscript{90}, regulation of matrix metalloproteinases\textsuperscript{91}, and cross-talk with the nitric oxide system\textsuperscript{92}.

1.3.2 Reactive oxygen species in diabetic nephropathy

ROS are produced normally in the kidney in small amounts and are necessary to maintain cellular homeostasis, but hyperglycemia induces rapid generation of ROS and leads to tissue injury\textsuperscript{93}. This is supported by studies in which overexpression of ROS scavenger superoxide dismutase or catalase protected diabetic mice from hyperglycemia-induced kidney injury\textsuperscript{94-97}. Deficiency of the superoxide dismutase accelerated diabetic kidney injury in mice\textsuperscript{98}. There are two major sources of ROS generation in diabetic condition: mitochondria electron transport chain, and NADPH oxidase and many other minor sources\textsuperscript{99-102}.
Oxidative stress occurs when production of oxidants or ROS exceeds local antioxidant capacity. This leads to oxidation of important macromolecules including proteins, lipids, carbohydrates, and DNA.

There are a number of enzymatic and nonenzymatic sources of ROS in the diabetic kidney, including auto-oxidation of glucose, transition metal–catalyzed Fenton reactions, advanced glycation, polyol pathway flux, mitochondrial respiratory chain deficiencies, xanthine oxidase activity, peroxidases, nitric oxide synthase (NOS) and NADPH oxidase. ROS generated from these sources include free radicals such as superoxide (•O$_2^-$), hydroxyl (•OH), and peroxyl (•RO$_2$) and nonradical species such as hydrogen peroxide (H$_2$O$_2$) and hydrochlorous acid (HOCl). It is also important to pay attention to reactive nitrogen species produced from similar pathways, which include the radicals nitric oxide (•NO) and nitrogen dioxide (•NO$_2$-), as well as the nonradical peroxynitrite (ONOO-), nitrous oxide (HNO$_2$), and alkyl peroxynitrates (RONOO). Of these, •O$_2^-$, •NO, H$_2$O$_2$, and ONOO- have been the most widely investigated in the diabetic kidney.

Although animal studies have demonstrated potent inhibition of oxidative stress with certain antioxidants with associated end-organ protection under experimental diabetic conditions, human studies with various antioxidants including α-tocopherol have been generally disappointing.

1.3.3 Antioxidant studies in animals and tissue culture

In response to excess ROS production during respiration and metabolism, animals have evolved a number of antioxidant systems including free radical scavengers and enzymes. The major antioxidant system in cells consists of the glutathione system, superoxide dismutase (SOD), catalase, and glucose 6-phosphate dehydrogenase, which is the principal source of NADPH. The most important of these antioxidant enzymes is superoxide dismutase, which exists in three major cellular forms: copper zinc (CuZnSOD, SOD1), manganese (MnSOD, SOD2), and extracellular (SOD3). These enzymes are responsible for the detoxification of superoxide radicals to hydrogen peroxide and water in different cellular compartments. Glutathione peroxidase (GPx) and catalase are other antioxidant enzymes that catalyze the conversion of
hydrogen peroxide to water. These antioxidants are relatively specific for one of the many reactive oxygen species. For example, superoxide dismutase converts superoxide to hydrogen peroxide and catalase converts hydrogen peroxide to water. Glutathione peroxidase uses reduced glutathione to convert hydrogen peroxide to water with the resultant oxidation of glutathione. Oxidized glutathione is then converted back to reduced glutathione by the enzyme glutathione reductase. The entire antioxidant system relies on a chemical reductant and that reductant is NADPH, which is mainly produced by glucose 6-phosphate dehydrogenase. There have been studies showing increased antioxidant levels, no changes in antioxidant levels, and decreased antioxidant levels in diabetic nephropathy. These differences in results likely reflect heterogeneity of models, including cell type studied, animal model used, and time tissues were examined. Most importantly, a number of these antioxidants have proven to play a minimal if any role in the treatment of diabetic nephropathy in humans. But because there is an overall increase in ROS in diabetic nephropathy, there should be either an inadequate cellular antioxidant response to ROS, or a primary high glucose-mediated antioxidant decrease contributing to the overall increase in ROS.

Some studies reported that the expression and activity of each of these antioxidants were decreased in diabetic microvascular disease. It has been shown that the overexpression of CuZnSOD protects against end organ damage in models of type 2 diabetic nephropathy. Other studies in diabetic mice with genetic deletions of various antioxidant enzymes have also provided insight into the specific relative contributions of MnSOD to the development of diabetic complications. MnSOD mimetics such as MnTBAP have also been shown to be protective in preventing ROS-induced injury in vitro, although the utility of such agents in vivo may be limited. In addition, specific polymorphisms of the MnSOD gene are associated with the development of diabetic nephropathy, further strengthening a potential role for the antioxidant MnSOD.

Interestingly, GPx-1–deficient mice did not show any increased risk for microvascular disease, in particular diabetic nephropathy. This is probably because of redundancy with respect to other renal GPx isoforms, in particular the GPx-3 isoform.

Overexpression of catalase has been shown to be protective in experimental models of type 2 diabetic nephropathy. However, studies in human patients did not find a relationship between
catalase gene polymorphisms that interfere with its cellular expression and the incidence of nephropathy in type 2 diabetic patients. A number of studies demonstrated high glucose–mediated decreases in antioxidants. In a transgenic mouse model of diabetic nephropathy, Fujita et al showed decreases in cytosolic and extracellular superoxide dismutase activity that was not observed in the mice that were resistant to the development of diabetic nephropathy. Another group of researchers showed that glucose 6-phosphate dehydrogenase, the main source of NADPH, decreased in the kidney from diabetic rats. In addition, the antioxidant system has also been observed to be impaired by pathways that lead to excess consumption of NADPH. For example, aldose reductase is activated in diabetes and uses NADPH as a cofactor to produce sorbitol and NADP. NADPH is the principal reductant upon which the entire antioxidant system relies. So increased aldose reductase is deleterious as it leads to a lowering of NADPH and the resultant impairment of antioxidant function.

Thus, in an effort to develop new, effective therapies in the treatment of diabetic nephropathy, therapeutic strategies should be directed at decreasing/inhibiting sources of ROS in diabetic nephropathy, such as mitochondrial electron transport chain and NADPH oxidase, and/or enhancing antioxidants, such as superoxide dismutase and glucose 6-phosphate dehydrogenase.

Many studies in cell culture and animal models of diabetes demonstrated increased ROS. Antioxidants have been shown to be very effective in treating diabetic nephropathy in animals. For example, rats with streptozotocin-induced diabetes had increased glomerular ROS, which was prevented by treatment with the antioxidant vitamin E or probucol. Another antioxidant α-Lipoic acid prevented glomerular podocyte loss in streptozotocin-induced diabetes in rats and mice. α-Lipoic acid also protected the kidneys from diabetic nephropathy as shown by lower urine albumin levels, decreased ROS, and by correcting other factors associated with diabetic kidney disease in a model of streptozotocin-induced diabetic mice with apolipoprotein E deficiency. In a rabbit model of alloxan-induced diabetes, administration of α-lipoic acid reduced urine albumin levels and attenuated the glomerulopathy.

There have also been many studies directed at specific sources of ROS such as mitochondrial electron transport chain-mediated superoxide production and NADPH oxidase. High glucose stimulates mitochondrial superoxide production via the electron transport chain. One of the
consequences of increased superoxide has been demonstrated to be inhibition of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which leads to blockade of glycolysis at an intermediate step and, as a consequence, accumulation of glycolysis metabolites upstream of this enzyme. It has been proposed that the increase in these glycolysis metabolites are then shunted into pathways that cause many of the known mechanisms thought to be responsible for diabetic injury (eg, increased protein kinase C, increased advanced glycation end products, and others) \(^4\). The substrate for GAPDH, glyceraldehyde 3-phosphate, is the substrate for the thiamine-dependent enzyme transketolase, which is part of the pentose phosphate pathway. In vitro studies using benfotiamine, a form of thiamine, to stimulate transketolase, showed attenuation of the increase in ROS and other pathophysiologic mechanisms. In a streptozotocin-induced diabetic rat model, benfotiamine-treated rats had lower urine albumin, increased transketolase activity, and improved other mechanistic factors associated with diabetic nephropathy such as lowering of advanced glycation end products in glomeruli from benfotiamine-treated diabetic rats \(^5\). These results and others suggest that benfotiamine might be a promising treatment for diabetic nephropathy in humans.

Similar potentially clinically useful agents that inhibit NADPH oxidase are not yet available for humans. Apocynin has been used successfully in diabetic animal models as an inhibitor of NADPH oxidase. There have been a number of promising studies. A recent one is illustrative in which diabetic rats were treated with apocynin, ramipril, or both \(^6\). Apocynin treatment decreased urine albumin levels, decreased expression levels of fibronectin and type IV collagen, reduced renal superoxide production, and attenuated diabetes-induced morphologic changes. Interestingly, the combination of apocynin and ramipril was more effective in ameliorating kidney injury compared with either agent given alone.

### 1.3.4 Antioxidant in clinical trials

To date, the clinical trials of antioxidants in humans have shown very little benefit. In 1999, a small study by Bursell and coworkers reported possibly beneficial effects of vitamin E \(^7\). High-dose vitamin E (1800 IU/day) was given to 9 nondiabetic control and 36 type 1 diabetic patients for a duration of 8 months. The study showed that their intervention normalized creatinine clearance. In the subset of patients who had high creatinine clearance thus hyperfiltration, they
observed a decrease in creatinine clearance. However, none of these patients had any evidence of kidney disease though and it has never been showed that normalization of hyperfiltration has any effect on preventing the initiation and progression of diabetic nephropathy. Then 4 years after, in 2003, the Heart Outcomes and Prevention Evaluation (HOPE) trial participants were administered placebo, ramipril, or vitamin E (400 IU/day). These patients had type 2 diabetes, their ages were 55 years or older, and they were followed up for 4.5 years. Study subjects were also assessed for the initiation and progression of kidney disease in the Microalbuminuria Cardiovascular Renal Outcomes arm of the study (MICRO-HOPE). At the end of MICRO-HOPE they had followed a total of 2740 people. The daily dose of vitamin E had no effect on the initiation and progression of diabetic nephropathy, the same percentage of patients developed microalbuminuria, overt nephropathy, and progressed to end-stage kidney disease.

Probufol is another potential antioxidant agent that has also been studied. It has both antioxidant and lipid-lowering effects. In a small study by Endo and colleagues, 102 type 2 diabetic patients were followed for 3 years. Patients who took probufol showed a trend to slowing the rate of decline in creatinine clearance, lower urine protein levels, and possibly a slowing to end-stage kidney disease. However this was a small, open-labeled study and whether the reported effects of probufol could be ascribed to antioxidant effects or lipid-lowering effects are unknown. An extensive search of the literature shows that despite the many cell culture and animal studies on diabetic nephropathy, no studies on the use of α-lipoic acid have been done. Another antioxidant n-acetylcysteine has been used in very limited trials and it is not practical to take on a daily basis.

In 2010, a clinical trial by Alkhalaf et al showed that benfotiamine is beneficial in improving diabetic nephropathy outcomes. Benfotiamine indirectly attenuates high glucose-induced increase in mitochondrial superoxide production in vitro, therefore is effective in improving oxidative stress. In this 12-week, randomized, placebo-controlled study, 39 type 2 diabetic patients were given high-dose benfotiamine (900 mg/day) and 41 patients were given placebo. ACE inhibitors and angiotensin receptor blockers were continued in this study. Despite significant improvement in thiamine status in the patients there were no differences in urinary albumin excretion rates.

The lack of success of antioxidant treatment in human clinical trials likely reflects one or more of the following factors: 1) The agents studied are mostly nonspecific (except in the benfotiamine
study) in that specific pathophysiologic mechanisms are not targeted. 2) It is not clear if any of these agents are effectively delivered and taken into kidney cells in humans. 3) It is not known whether the appropriate dose, and the length of time needed to see an effect was used. Even though these trials have been disappointing to date, the accumulated in vitro evidence supports a central role for ROS in the initiation and progression of diabetic complications. Thus, a robust research effort into fully understanding the basic mechanisms of ROS regulation under high glucose conditions in concert with a major effort to produce medications that specifically target these mechanisms should ultimately lead to effective treatments. The recent study by Pergola and coworkers shed light on clinical trials of antioxidants. They reported that bardoxolone methyl, an oral antioxidant inflammation modulator, improved GFR in patients with advanced chronic kidney disease and type 2 diabetes after 52 weeks of treatment.

1.3.5 Mitochondria electron transport chain

The mitochondria electron transport chain generates ROS as a by-product of oxidative phosphorylation. As electrons are passing through the system, a proton gradient is generated and maintained across the mitochondrial inner membrane in order to drive the formation of ATP as an energy source. However, in high glucose environment, the increased number of electrons passing through the system overwhelmingly exceeds the capacity of the electron transport chain. As a result, electron transport is halted. In order to resume electron transport and free-up the system, electrons are transferred to O$_2$ one at a time and superoxide is generated in this process. Superoxide induces DNA damage in the nucleus, results in activation of the enzyme poly ADP ribose polymerase (PARP). Activated PARP inhibits glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which is a key enzyme in the glycolysis pathway. Without GAPDH, accumulation of glycolysis metabolites force them to enter other signaling pathways that have all been shown to link to injury: polyol pathway and hexosamine flux, advanced glycation end-product (AGE) formation, and DAG-induced PKC activation.
1.3.6 NADPH oxidase

NADPH oxidase was first discovered in phagocytic cells. Phagocytic NADPH oxidase is a multiunit complex consisting of two membrane proteins: NOX2/gp91^phox, p22^phox; and four cytosolic proteins: p40^phox, p47^phox, p67^phox, rac GTPase (Fig. 1.2). NOX2 (also called gp91^phox) is the main subunit with oxidase activity. The activation of NOX2 requires the recruitment of the cytosolic subunits to the membrane subunits. This process can be triggered by phosphorylation of p47^phox. Once activated, NOX2 becomes capable of transferring electrons from NADPH to molecular oxygen to produce superoxide. In phagocytic cells, such as the macrophage and neutrophil, superoxide serves as a host defence mechanism against foreign microbes. However, functional NADPH oxidase is also found in other cells, and there are a number of different NOX enzymes, including NOX1 and NOX4.

NOX2 is expressed in the kidney, along with two other isoforms: NOX1, NOX4. Diabetic nephropathy is associated with upregulation of expression of both NOX2 and NOX4. In vitro studies of rat mesangial cells have shown that high glucose-induced superoxide generation is completely blunted when antisense RNA against p47^phox is transfected into the cells, implicating a role for NOX2 in oxidative stress in glomeruli under diabetic conditions. Consistent with in vitro experimental results, pharmacological blockade of the cytosolic subunits of NADPH with apocynin reduces the severity of diabetic kidney injury.

There is also evidence that NOX4 is a key determinant of diabetic nephropathy. NOX4 NADPH oxidase is very unique because it is constitutively active and does not require the recruitment of cytosolic subunits for activation. Gorin et al exposed rat mesangial cells to high glucose in order to induce superoxide production. They observed that transfecting cells with antisense RNA against NOX4 was also sufficient to prevent high glucose induced superoxide production. They also delivered antisense NOX4 RNA in vivo using osmotic mini-pump, and showed less oxidative stress and reduced glomerular injury in streptozotocin-induced diabetic rats.

Taken together, it is still not clear which of the NADPH oxidase isoforms is the major determinant of initiation and progression of diabetic nephropathy. The research implicating p47^phox-dependent NOX2 comes mainly from in vitro studies and pharmacological studies using apocynin, but the mechanism of action of apocynin is uncertain. In a recent paper by Heumuller...
et al, apocynin was found to be a non-specific antioxidant in non-phagocytic cells\textsuperscript{117}. Fortunately, genetically modified mice lacking the cytosolic subunit, $p47^{\text{phox}}$, are available. Accordingly I utilized a genetic approach in this thesis to determine if there is a role for $p47^{\text{phox}}$ in the initiation and progression of diabetic nephropathy in a mouse model of diabetic nephropathy.
Figure 1.2: NADPH oxidase. (A) Resting state of phagocyte NADPH oxidase. Membrane-bound subunits: NOX2, p22\textsuperscript{phox}; cytosolic subunits: p67\textsuperscript{phox}, p47\textsuperscript{phox}, p40\textsuperscript{phox} and rac. (B) Activated NADPH oxidase. Phosphorylation of the p47\textsuperscript{phox} leads to the recruitment of cytosolic subunits to membrane-bound subunits and forms the multiunit complex.
1.3.7  NADPH oxidase in the pancreas

Oxidative stress is detected in pancreatic islet β-cells under diabetic conditions \(^{118,119}\). Also, there is evidence suggesting that islet cells have a very low antioxidant capacity \(^{120}\). In addition, antioxidant treatment prevents glucose toxicity in \textit{vitro} and in \textit{vivo} \(^{121}\). Interestingly, both mitochondria and NADPH oxidase are found to be sources for ROS generation by islets \(^{122,123}\). NOX2 and NOX4 expressions are detected in β-cells \(^{111,124}\).

\(\text{p47}^{\text{phox}}\) and NOX-generated ROS may play a role in Endoplasmic reticulum (ER) stress-induced beta-cell apoptosis \(^{125,126}\). Accumulation of ROS has also been shown to be both an initiation factor and a consequence of ER stress, and it is an important cellular response linking protein misfolding in the ER to beta-cell apoptosis \(^{127}\). Deletion of \(\text{p47}^{\text{phox}}\) may protect the beta-cell from ER stress-induced injury and preserve beta-cell function over time. Therefore, the effect of deletion of the \(\text{p47}^{\text{phox}}\) gene on β-cell is also studied in this thesis.

1.3.8  Angiotensin II-induced NADPH oxidase activation

Currently, the mechanism of NADPH oxidase activation by angiotensin II is not fully understood. It has been shown that angiotensin II-induced ROS production in vascular smooth muscle cells (VSMCs) from large arteries is mediated by Nox1 activation, delivery of antisense \textit{Nox1} reduced ROS concentrations \(^{466}\). While Nox2 seems to be the angiotensin II-responsive Nox in resistance vessels, as demonstrated in a similar manner in human VSMCs transfected with \(\text{gp91}^{\text{phox}}\) antisense oligonucleotides \(^{467}\). This is shown in the heart, using small interference RNA (siRNA) against \textit{Nox2} \(^{468}\) and in the kidney of \(\text{gp91}^{\text{phox}}\) knockout mice \(^{469}\). Two different phases of ROS production have been suggested: a rapid and transient phase and a delayed and sustained phase. The first peak is a result to an acute activation of NADPH oxidases by angiotensin II, while the second is dependent on the initial burst and is a consequence of upregulation of different NADPH oxidase subunits by angiotensin II. For example, in VSMC, the sustained phase of ROS production is preceded by upregulation of \textit{Nox1} mRNA \(^{466,470}\), \(\text{p22}^{\text{phox}}\) mRNA \(^{471}\) and protein \(^{467}\) and \(\text{p47}^{\text{phox}}\) protein \(^{472,467}\). It has also been reported that angiotensin II can upregulate \textit{Nox4} mRNA in the vasculature \(^{470}\), although this is not observed in another study \(^{473}\).
In VSMCs, activation of the NADPH oxidase and rapid ROS release requires phosphorylation of p47_{phox} by protein kinase C (PKC) and subsequent translocation to the membrane\textsuperscript{467, 474, 475}, where it recruits the activating subunits NoxA1 and Rac\textsuperscript{476, 477}. While each of these subunits has been implicated in the response to angiotensin II, actual formation of this complex in VSMCs remains to be shown biochemically\textsuperscript{493}. Angiotensin II can induce PKC-dependent NADPH oxidase activity via three different phospholipases (PL): PLC\textsuperscript{478}, PLD\textsuperscript{479}, and PLA2\textsuperscript{480}, with PLD likely predominating. In addition, Src is thought to be another important mediator of p47_{phox} phosphorylation\textsuperscript{481, 482}, although it is not clear whether it modulates p47_{phox} directly or by its actions on the cytoskeleton through cortactin, a Src substrate that binds p47_{phox}\textsuperscript{483, 484, 485} and facilitates the proper assembly of subunits and function of NADPH oxidases by angiotensin II\textsuperscript{485}.

Rac1 is also required for angiotensin II-induced activation of Nox1 and Nox2\textsuperscript{486-488}. Stimulation of Rac1 by angiotensin II appears to be mediated by activation of the guanine nucleotide exchange factor (GEF), SOS-1\textsuperscript{489}, although a relationship between SOS-1 and Nox activation remains to be determined. Activation of the Rac-GEF is dependent upon Src-mediated transactivation of the epidermal growth factor receptor (EGFR), which then serves as a binding site for phosphatidylinositol 3-kinase (PI3K), an immediate activator of the Rac-GEF\textsuperscript{475}. This initial activation step seems to occur in caveolae\textsuperscript{490, 491}, the site of Nox1 localization\textsuperscript{492}. The initial activation of NADPH oxidase leads to production of ROS, which activates Src, creating an amplification loop of oxidase activity\textsuperscript{475}. However, persistent generation of ROS requires upregulation of the Nox subunits, p47_{phox} and p22_{phox}\textsuperscript{466, 467, 471}.

1.3.9 Other sources of ROS

Glycolysis

Inside the cell, glucose is first converted to glucose-6-phosphate, then processed to pyruvate through a sequence of reactions in glycolysis. Cellular glycolysis can promote the production of excess ROS. It is intuitive that in diabetes complications, in order to minimize cellular damage, ROS generation and maintain intracellular glucose homeostasis, it is important to restrict cellular glucose uptake in susceptible cell populations. On the other hand, there is evidence suggesting
that restriction of cellular glucose uptake causes production of small quantities of cellular ROS, which ultimately improve cell survival\textsuperscript{443}. This finding is demonstrated by data in C. elegans, which showed that antioxidant treatments impaired cellular survival by restoring glucose uptake\textsuperscript{443}. In order to explain this finding, it is thought that exposure to minor stress and associated slightly elevated ROS concentrations prime cells against pathological damage during extreme changes in cellular glycolysis. To support this explanation, either caloric restriction\textsuperscript{444} or intermittent feeding patterns are shown to be renoprotective in rodent models of diabetic nephropathy\textsuperscript{445}. Caloric restriction mimetics are currently being tested in ageing because a major issue in human health appears to be long-term compliance to such a dietary-oriented regimen and ageing itself is associated with declining renal function\textsuperscript{446}. However, the ultimate effects of these mimetics currently remain unknown. Nevertheless, disruption of glycolysis by both enhancement or suppression can ultimately facilitate the excessive generation of ROS by a number of pathways.

Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PDH) is the rate-limiting enzyme in the pentose phosphate pathway. The pentose phosphate pathway is involved in ribose synthesis, and is the main source of NADPH, glutathione reductase, and aldose reductase. Altered activity of G6PDH has been shown to induce cellular oxidative stress\textsuperscript{447}. Deficiencies in the activity of G6PDH are common human enzymopathies which lead to increased ROS generation and decreases in antioxidants such as glutathione\textsuperscript{448}. G6PDH activity is increased in kidneys from rodents with experimental diabetic nephropathy. Further investigation is needed for the potential of this pathway as a source of ROS in diabetic nephropathy.

Flux through the sorbitol pathway

Increased flux through the sorbitol/polyol pathway was documented more than 40 years ago in high glucose environment. When intracellular glucose concentration is high, the cytosolic enzyme aldose reductase converts glucose to sorbitol using NADPH derived from the pentose phosphate pathway as a cofactor. Deprivation of NADPH by this reaction prevents replenishment of reduced glutathione in high glucose environment, and reduced glutathione is required to maintain glutathione peroxidase activity. The net effect of this is decreased cellular antioxidant activity. On the other hand, sorbitol is oxidized to fructose by sorbitol
dehydrogenase, with NAD+ reduced to NADH, providing increased substrate to complex I of the mitochondrial respiratory chain. Since the mitochondrial respiratory chain is a major source of ROS generation in diabetes, provision of additional electrons for transfer to oxygen-forming superoxide would further increase mitochondrial ROS production. In addition, since sorbitol cannot cross cell membranes, its intracellular accumulation results in osmotic stress. Osmotic stress is linked to increase in cellular cytosolic generation of H$_2$O$_2$. It has been shown that administration of osmotic diuretics protects proximal tubular cells from ROS-mediated apoptosis.

Although aldose reductase blockade which inhibits sorbitol accumulation has been shown to delay, prevent, and reverse experimental diabetic neuropathy at early stages, decades of clinical trials have in general been disappointing. The clinical utility of aldose reductase inhibitors in diabetic nephropathy remains to be studied.

Xanthine oxidase

Xanthine oxidase is the enzyme that catalyzes the oxidation of hypoxanthine to uric acid using molecular oxygen as the electron acceptor. During this process a number of ROS are produced, including •O$_2$-, •OH, and H$_2$O$_2$. Under normal physiological conditions, xanthine oxidase activity is very low and usually unmeasurable in most cell types, although sensitive electron spin technologies have confirmed xanthine oxidase as an important source of vascular superoxide generation in experimental models of type 1 diabetes. However, there is no direct evidence of abnormalities in this pathway within renal tissues in experimental or human diabetes, and thus the contribution of this enzyme to the pathogenesis of diabetic nephropathy remains unknown.

Uncoupling of NOS.

Three major isoforms of NOS are found, including inducible (iNOS), neuronal (nNOS), and endothelial (eNOS). Each of these isoforms requires five cofactors/prosthetics to produce •NO, including flavinmononucleotide (FMN), bihydrobiopterin (BH4), calmodulin, and flavin adenine dinucleotide (FAD). In diabetes, low substrate (L-arginine) concentration or the absence of cofactors leads to uncoupling of NOS, and is thought to produce superoxide in preference to •NO. Indeed, one study in experimental diabetic nephropathy has suggested that uncoupling of NOS and NADPH oxidase provides two major sources of glomerular superoxide. In that
study, restoration of physiological levels of BH4 attenuated ROS production and improved renal function.

The status of •NO and its role in diabetic nephropathy is controversial. Current findings suggest that early diabetic nephropathy is associated with increased intrarenal •NO production mediated primarily by constitutively released •NO (eNOS and nNOS). Indeed, enhanced •NO production may contribute to the hyperfiltration and other hemodynamic changes that occur in early diabetic nephropathy. This is supported by studies in early diabetic nephropathy where NOS inhibitor L-NAME reversed hemodynamic changes and kidney injury.

On the other hand, most studies in advanced diabetic renal disease showed that severe proteinuria, declining renal function, and hypertension are associated with progressive •NO deficiency. Advanced renal changes attributed to •NO deficiency are thought to be mediated through multiple mechanisms, including glucose and AGE quenching and inhibition and/or posttranslational modification of NOS, which changes the activity of both endothelial and inducible isoforms. Indeed, a study reported no effect or aggravation of renal damage by chronic NO inhibition in models of type 1 and type 2 diabetic nephropathy.

Therefore, the clinical applicability of approaches that inhibit NOS activity still remains to be determined.

1.4 MOUSE MODELS OF DIABETIC NEPHROPATHY

1.4.1 Introduction to mouse models of diabetic nephropathy

The use of animal models provides important insights into the pathogenesis and natural history of diabetic nephropathy. An ideal model should exhibit all features of human diabetic nephropathy, be susceptible to genetic modification and analysis, easy to obtain, maintain, relatively low cost, and not time consuming. For these reasons, the most widely used models to study diabetic nephropathy are the murine systems.
A number of rodent models of diabetes have been reported in recent reviews \(^{176-178}\). Most of these models develop spontaneous and acquired hyperglycemia in conjunction with functional and structural renal abnormalities. The functional abnormalities include albuminuria and hyperfiltration; the structural abnormalities include glomerular and renal hypertrophy, mesangial matrix expansion and glomerular basement membrane thickening. All of these are features of human diabetic nephropathy in early stages. However very few of these models develop glomerulosclerosis, which is only observed in advanced human diabetic nephropathy at late stages.

1.4.2 The Animal Models of Diabetic Complications Consortium criteria for mouse models of diabetic nephropathy

In order to better identify and characterize mouse models of diabetic nephropathy, the Animal Models of Diabetic Complications Consortium (AMDCC) was created. Supported by the United States National Institutes of Health, the AMDCC published a consensus with explicit standards for phenotyping murine models of diabetic nephropathy. The AMDCC aims to standardize phenotyping methodology which allows better comparisons of results obtained by different groups using different animal models. The AMDCC initially proposed the following three criteria for an ideal mouse model in 2005: progressive renal insufficiency in the setting of hyperglycemia; albuminuria; and characteristic pathologic changes including basement membrane thickening by electron microscopy, mesangial matrix expansion with nodular mesangial sclerosis, interstitial fibrosis, and arteriolar hyalinosis \(^{176}\).

In 2009, these criteria have been updated to include:

1. greater than 50% decline in glomerular filtration rate over the lifetime of the animal.

2. greater than 10-fold increase in albuminuria compared with controls for that strain at the same age and gender.

3. pathologic changes in kidneys, including advanced mesangial matrix expansion ± nodular sclerosis and mesangiolysis, any degree of arteriolar hyalinosis, glomerular basement membrane thickening by >50% over baseline, and tubulointerstitial fibrosis \(^{177}\).
Additional histologic phenotyping for diabetic nephropathy models with advanced disease should include:

1. quantification of mesangial matrix expression, ideally with morphometric analyses, and with mesangiolysis and microaneurysms detected with appropriate tissue stains.

2. exclusion of immune complex disease with immunohistochemistry on frozen sections for IgG, IgM, and IgA.

3. demonstration of glomerular basement membrane thickening by electron microscopy.

4. demonstration of podocyte loss by a reasonable morphometric system \(^{177}\).

None of the current mouse models meet all criteria provided above, and failure to meet any one specific standard should not prevent the use of the model in studies. These criteria were established as goals for an ultimate model of diabetic nephropathy, i.e. a model that meets most of these criteria should become the best model to study diabetic nephropathy, but these are not absolute standards.

1.4.3 Widely used mouse models of diabetic nephropathy

\textit{db/db} mouse

The most widely used mouse model of diabetic nephropathy is the \textit{db/db} mouse. Deletion of the gene for leptin receptor renders this mutant susceptible to obesity, insulin resistance and type 2 diabetes \(^{179}\). \textit{db/db} mice develop albuminuria, podocyte loss, and mesangial matrix expansion. They are a good model of the early changes of human diabetic nephropathy \(^{176,179,180}\). The limitation of this model is that \textit{db/db} mutation on the C57BL/6 KS background (contains part of the DBA/2 strain genetic endowment) can develop features of diabetic nephropathy but not on the C57BL/6 background.

Akita mouse

The Akita mouse has a single nucleotide substitution in the \textit{Ins2} gene \((\textit{Ins2}^{WT/C96Y})\), so the translated proinsulin peptide has a cysteine residue replaced by a tyrosine. This mutation disrupts
the disulfide bond formation between the A chain and B chain of the proinsulin molecule and results in misfolding of the peptide. As a consequence of protein misfolding, pancreatic β-cells, which express Ins2 gene, exhibit endoplasmic reticulum (ER) stress and apoptosis. Injury to pancreatic β-cell renders the mouse insulin deficient and hyperglycemic \(^{176, 181, 182}\). Akita mice on the C57BL/6 strain develop albuminuria, early hyperfiltration, glomerular and kidney hypertrophy, mesangial matrix expansion and basement membrane thickening. Podocyte loss is also observed in the Akita mouse \(^{180-181}\). In addition to the \(db/db\) mouse, the Akita mouse is not only a good model of the early changes of human diabetic nephropathy, but also easier to breed. Only one mutant allele is required for the Akita mouse to become diabetic, and this is an advantage to the breeding strategy especially if a second mutation of a different gene is introduced to this model.

OVE26 mouse

The OVE26 mouse has pancreas β-cell injury and type 1-like diabetes because of transgenic overexpression of calmodulin in pancreatic β-cells \(^{183}\). This model develops marked albuminuria, increased mesangial matrix, glomerulosclerosis, and podocyte apoptosis on the FVB background. However, the severe diabetic nephropathy diminishes when the transgene is introduced into other strains \(^{183}\). This is a limitation because it is difficult to introduce other genetic mutations from other strains into this model.

Endothelial nitric oxide synthase deficient mice

Nitric oxide (NO) is a major regulator of the vasculature, and depletion of NO availability is an important mechanism underlying initiation and progression of diabetic nephropathy \(^{184, 185}\). Endothelial nitric oxide synthase deletion (\(eNOS^{-/}\)) on \(db/db\) mice develop, in addition to all features of the \(db/db\) mice, hypertension, marked mesangial expansion and mesangiolysis \(^{184, 185}\). However it is extremely difficult to breed these mice because of the combined mutations.

In order to overcome breeding difficulties, streptozotocin (STZ)-induced \(eNOS^{-/-}\) C57BL/6 diabetic mice are generated. These mice develop features of advanced diabetic nephropathy including hypertension, albuminuria, mesangial matrix expansion, mesangiolysis and nodular glomerulosclerosis \(^{186}\), making it a good model to study mechanisms underlying advanced diabetic nephropathy. However there is evidence suggesting that the development of
hypertension is a confounding variable in this model because the phenotypic features of diabetic glomerulopathy may result from hypertension but not hyperglycemia, and could be prevented by administration of hydralazine to lower blood pressure [187].

Black and Tan Brachyuric BTBR ob/ob mouse

The BTBR ob/ob mouse is a model of type 2 diabetes with insulin resistance because of deletion of the gene for leptin (ob/ob) [188]. This model develops proteinuria with early podocyte loss, mesangial expansion, mesangiolysis, basement membrane thickening and interstitial fibrosis [189]. However, it is difficult to breed these mice because they are infertile, and this BTBR strain is not commonly studied so the introduction of other genetic mutations into these mice requires time-consuming backcrossing strategies.

The experiments in this thesis utilized the Akita mouse as a model to study the roles of renin-angiotensin system and p47phox-dependent NADPH oxidase in diabetic nephropathy. As described previously, the Akita mouse is a good model because it reproduces many features of human diabetic nephropathy in a relatively short period of time, and it is easy to breed and to introduce other genetic mutation into itself. The limitation of this model is that it is on the C57BL/6 background, which is very resistant to diabetic kidney injury and does not develop advanced diabetic nephropathy.

1.5 EXPERIMENTAL OUTLINE AND HYPOTHESIS

The experiments in this thesis are designed to better understand the mechanisms involving the renin-angiotensin system and NADPH oxidase that are responsible for initiation and progression of diabetic nephropathy (Fig. 1.3).

Chapter 2 describes the effect of ACE2 on diabetic kidney injury in the Akita mouse and mesangial cells (Fig. 1.4). My hypothesis is that hrACE2 treatment is protective against diabetes-induced kidney injury in the Akita mouse. ACE2 attenuates diabetes-induced kidney injury in the Akita mouse, increases Angiotensin-(1-7) levels in the kidney, and suppresses NADPH oxidase activation in both the Akita mouse and mesangial cells.
Chapter 3 investigates the interaction between Angiotensin II and Angiotensin-(1-7) in mesangial cells (Figure 1.5). My hypothesis is that angiotensin-(1-7) inhibits angiotensin II-induced ERK1/2 phosphorylation and NADPH oxidase activation via the mas-cAMP-PKA pathway. Angiotensin-(1-7) induces ERK1/2 phosphorylation, prevents Angiotensin II-induced ERK1/2 phosphorylation and NADPH oxidase activation in mas-cAMP-PKA-dependent manner.

Chapter 4 studies the effect of deletion of the regulatory NADPH oxidase subunit p47phox in diabetic kidney injury (Figure 1.6). My hypothesis is that deletion of the p47phox reduces diabetic kidney injury and high glucose-induced oxidative stress in the Akita mouse. Deletion of the p47phox not only reduces diabetic kidney injury but also improves glycemia in the Akita mouse. Deletion of the p47phox also attenuates oxidative stress and expression levels of profibrotic markers in both isolated glomeruli from diabetic mice and high glucose-treated mesangial cells.
Figure 1.3: **High glucose-induced kidney injury.** High glucose leads to both activations of the renin-angiotensin system and NADPH oxidase, and they are all linked to kidney injury.
Figure 1.4: The effect of ACE2 on high glucose-induced kidney injury. ACE2 prevents kidney injury in the Akita mouse, and mesangial cells. Administration of human recombinant ACE2 is also associated with increased Angiotensin-(1-7) levels and attenuated high glucose-induced NADPH oxidase activation.
Figure 1.5: The effect of Angiotensin-(1-7) on Angiotensin II-induced ERK1/2 phosphorylation and NADPH oxidase activation. Angiotensin-(1-7) increased ERK1/2 phosphorylation, decreased Angiotensin II-induced ERK1/2 phosphorylation and NADPH oxidase activation in a mas-cAMP-PKA dependent manner in mesangial cells.
Figure 1.6: The effect of deletion of $p47^{phox}$ in diabetic high glucose-induced kidney injury. Deletion of $p47^{phox}$ not only reduced diabetic kidney injury but also improved glycemia in the Akita mouse. Deletion of the $p47^{phox}$ also attenuated oxidative stress and expression levels of pro-fibrotic markers in both isolated glomeruli from diabetic mice and high glucose-treated mesangial cells.
Chapter 2

Human Recombinant ACE2 Reduces the Progression of Diabetic Nephropathy

All the data presented in this chapter are published and reproduced from the following journal:

2.1 RESEARCH SUMMARY

2.1.1 Objective

Diabetic nephropathy is one of the most common causes of end-stage renal failure. Inhibition of ACE2 function accelerates diabetic kidney injury, and renal ACE2 is downregulated in diabetic nephropathy. We examined the ability of human recombinant ACE2 (hrACE2) to slow the progression of diabetic kidney injury.

2.1.2 Research Design and Methods

Male 12-week-old diabetic Akita mice (Ins2\textsuperscript{WT/C96Y}) and control C57BL/6J mice (Ins2\textsuperscript{WT/WT}) were injected daily with placebo or with rhACE2 (2 mg/kg, i.p.) for 4 weeks. Albumin excretion, gene expression, histomorphometry, NADPH oxidase activity, and peptide levels were examined. The effect of hrACE2 on high glucose and angiotensin II (ANG II)–induced changes was also examined in cultured mesangial cells.

2.1.3 Results

Treatment with hrACE2 increased plasma ACE2 activity, normalized blood pressure, and reduced the urinary albumin excretion in Akita \textit{Ins2\textsuperscript{WT/C96Y}} mice in association with a decreased glomerular mesangial matrix expansion and normalization of increased α-smooth muscle actin and collagen III expression. Human recombinant ACE2 increased ANG-(1–7) levels, lowered ANG II levels, and reduced NADPH oxidase activity. mRNA levels for p47\textsuperscript{phox} and NOX2 were also normalized by treatment with hrACE2. In vitro, hrACE2 attenuated both high glucose and ANG II–induced oxidative stress and NADPH oxidase activity.
2.1.4 Conclusions

Treatment with hrACE2 attenuates diabetic kidney injury in the Akita mouse in association with a reduction in blood pressure and a decrease in NADPH oxidase activity. In vitro studies show that the protective effect of hrACE2 is due to reduction in ANG II and an increase in ANG-(1–7) signaling.

2.2 INTRODUCTION

Chronic kidney disease is recognized as an increasing global public health problem due in part to the increasing prevalence of diabetes. Activation of the renin-angiotensin system (RAS) and the generation of angiotensin II (ANG II) play an important pathogenic role in diabetic nephropathy, and blockade of the RAS attenuates the development of diabetic kidney injury. The discovery of a homologue of the classical ACE, ACE2, has introduced a new enzyme in ANG peptide metabolism. Like ACE, ACE2 is membrane bound, but it is a monocarboxypeptidase that generates ANG (1–7) from the octapeptide ANG II. As such, ACE2 serves as an endogenous negative regulator of the renin-angiotensin system.

In animal models of diabetes, early increases in ACE2 mRNA levels, protein expression, and ACE2 activity occur, whereas ACE2 mRNA and protein levels have been found to decrease in older streptozotocin-induced diabetic rats. Loss of ACE2 is associated with age-dependent glomerulosclerosis and albuminuria and exacerbation of diabetic kidney injury in Akita mice and is preventable by angiotensin type 1 (AT1) receptor blockade. In patients with type 2 diabetes, glomerular and tubular ACE2 expressions are reduced in the setting of increased ACE expression. Taken together, these studies suggest that ACE2 may play an early protective role against the development of diabetic nephropathy. We hypothesized that treatment with human recombinant ACE2 (hrACE2) will target the diabetic glomerulus and slow progression of diabetic nephropathy in the Akita mouse (Ins2WT/C96Y), a model of type 1 diabetes.
2.3 RESEARCH DESIGN AND METHODS

2.3.1 Experimental animals and protocol.

C57BL/6J and diabetic heterozygous Akita (Ins2^{WT/C96Y}) mice were purchased from The Jackson Laboratory and bred in our animal facility. Throughout the period of study, animals were provided with free access to water and standard 18% protein rodent chow (Harlan Teklad, Madison, WI). Ins2^{WT/C96Y} (Akita) and Ins2^{WT/WT} mice were treated from 3 months of age with daily injections of placebo or human recombinant ACE2 at a daily dose of 2 mg/kg for 4 weeks. Twenty-four–hour urine volumes were collected at the end of 4 months and animals were killed. All experiments were conducted in accordance with the Canadian Council of Animal Care and Institutional Guidelines.

2.3.2 Generation and characterization of human recombinant ACE2.

The extracellular domain of human ACE2 (amino acid residues 1–740, molecular wt = 101 kDa) (9) was expressed recombinantly in CHO cells under serum-free conditions in a chemically defined medium. The expression product was purified to homogeneity by applying a capture step on a DEAE Sepharose anion exchanger resin (Pharmacia Biotech AB, Uppsala, Sweden). The eluted fractions containing the expression product were submitted to a polishing step on a Superdex 200 gel filtration column (Pharmacia Biotech AB). The expression product was compared with the commercially available ACE2 standard 933-ZN (R&D Systems, Minneapolis, MN). Chemical and immunological properties of both products were almost identical, although rhACE2 showed a 93% enzymatic activity with Mca-APK-(Dnp)-OH substrate in comparison with rhACE2 standard 933-ZN (R&D Systems). The enzymatic turnover of hrACE2 with ANG II substrate was 5.2 ± 0.1 μmol · mg^{-1} · min^{-1}, and the elimination half-life of hrACE2 was 10.4 h in rhesus monkeys. The purity of the expression product was 99.99% measured by high-performance liquid chromatography.
2.3.3 Plasma ACE2 activity and detection of anti-ACE2 antibodies.

Plasma collected from mice injected with hrACE2 (2 mg/kg, i.p.) for 2 weeks were stored at −80 °C. The enzymatic activity of rhACE2 in plasma samples was measured by its ability to cleave the fluorescent peptide substrate Mca-Ala-Pro-Lys(Dnp)-OH. Cleavage was measured in 1:5 diluted samples (final assay dilution) using excitation and emission wavelengths of 320 and 430 nm, respectively, in presence of 100 μmol/l substrate in 50 mmol/l MES, 300 mmol/l NaCl, 10 μmol/l ZnCl2, and 0.01% Brij-30 at pH 6.5. Evaluation was performed by comparing the maximal slope of the fluorescence/time curve to respective maximal slopes of a serial rhACE2 dilution in normal mouse plasma. The response to the specific peptide ACE2 inhibitor DX600 (Phoenix Pharmaceuticals, Burlingame, CA) on the ACE2 activity in murine plasma was also examined.

Serum samples of mice were analyzed using an ACE2 antigen–specific enzyme-linked immunosorbent assay (ELISA) recognizing total anti–ACE2-specific IgG. Recombinant human soluble ACE2 was presented as antigen, coated at 10 μg/ml onto Maxisorp adsorption plates (Nunc, Vedbaek, Denmark) diluted in coating buffer. Remaining active groups were blocked by incubation with 3% skim milk (Dibco) in PBS. Induced antibodies were detected by their constant domains using a rabbit anti-mouse IgG or a rabbit anti-mouse IgM peroxidase-labeled antibody (Zymed). Staining was performed by o-phenylenediamine dihydrochloride (OPD; Sigma-Aldrich) in staining buffer (PAA Laboratories) using H2O2 as substrate according to the manufacturer's instructions. Absorbance at 492 nm was measured using 620 nm as reference wavelength. Quantification was performed by comparison with a commercially available monoclonal mouse anti-human ACE2 antibody (R&D Systems).

2.3.4 Blood glucose, urinary albumin excretion, and tail-cuff blood pressure measurements.

Blood glucose levels were obtained weekly between 8:30 and 10:30 am using an Ascensia Breeze glucometer (Bayer, Toronto, ON, Canada), and hyperglycemia was stable and sustained in Ins2WT/C96Y mice, as previously reported. Twenty-four–hour urine collections were obtained from mice prior to sacrifice by housing them in individual mouse metabolic cages (Nalgene, model 650-0311; Nalge Nunc International, Rochester, NY) with free access to water and rodent
mash. Urinary albumin concentration was measured using an indirect competitive ELISA according to the manufacturer's instructions (Albuwell M; Exocell, Philadelphia, PA).

For the measurement of tail-cuff systolic blood pressure (TC-SBP), conscious mice were placed in the restrainers and their body temperature was maintained at \(~34 \, ^\circ\text{C}\) by the warming chamber. The IITC tail-cuff sensor containing both the inflation cuff and the photoelectric sensor was placed on the tail and attached to the restrainer. The cuff was inflated to a pressure of 200 mmHg and then deflated slowly. Upon reappearance of pulse signals, TC-SBP data from the IITC amplifier were recorded, analyzed, and reported by the IITC software (IITC Life Science Blood Pressure System, Woodland Hills, CA). The mice were trained on three occasions before actual recordings were made, and the corresponding TC-SBPs were averaged from three readings and used for the averaged comparisons.

### 2.3.5 Histopathology and electron microscopy.

Kidneys were harvested for pathological examination and one section was fixed in 10% neutral-buffered formalin (Sigma-Aldrich, St. Louis, MO) for 24 h and then transferred to 90% ethanol for light microscopy and immunohistochemistry, and the remaining sections were used for electron microscopy or snap-frozen for RNA extraction. The formalin-fixed tissue was embedded in paraffin and 3-micron sections were stained with periodic acid Schiff stain.

The slides were then scanned digitally by the Advanced Optical Microscope Facility (Princess Margaret Hospital, Toronto, Canada) and mean cross sectional areas were calculated using Aperio ImageScope software (Aperio Technologies Inc., Vista, CA). Glomerular volume (VG) was calculated from the mean cross sectional area (\(\bar{A}G\)) of 33 glomerular profiles on each animal using the following equation: 

\[
VG = \frac{\beta}{k} \cdot (\bar{A}G)^{3/2},
\]

where \(\beta = 1.38\) is the shape coefficient for spheres (the idealized shape of glomeruli) and \(k = 1.1\) is the size distribution coefficient. For electron microscopy, tissue was fixed in buffered 1% glutaraldehyde-4% formaldehyde, post-fixed in 1% osmium tetroxide, embedded in eponaraldite and then processed for GBM thickness measurements. The mesangial matrix expansion (MME) score was calculated based on a random and blinded assessment by our expert renal pathologist (AMH) of 50 glomeruli per group from PAS-stained sections. Each glomerulus was scored from 0 to 3 with 0 = no mesangial expansion,
1 = mesangial expansion greater than the width of 1 mesangial cell nucleus, 2 = mesangial expansion greater than 2 mesangial cell nuclei width and 3 = mesangial expansion greater than 3 mesangial cell nuclei width. Neutrophils were stained using rat anti-neutrophil antibody (AbD Serotec, Raleigh, NC), and macrophages were stained using F4/80 staining.

2.3.6 Biochemistry, peptide analysis, and Western blot analysis.

Mice were injected intraperitoneally 10–15 min prior to sacrifice with 0.1 ml of heparin sodium (500 IU/ml; LEO Pharma, Thornhill, ON, Canada) to prevent blood clotting. Whole blood was collected from the carotid artery and jugular vein in syringes containing a mixture of rat renin inhibitor and protease inhibitors. Samples were centrifuged at 3,000 rpm at 4°C for 20 min and the plasma was stored at −80°C until analysis. Plasma potassium, glucose and creatinine, and urine creatinine were measured by VITA-TECH (Markham, ON, Canada), as previously described. For the measurement of peptides, isolated kidneys were quickly perfused with ice-cold saline and the renal cortices dissected and snap frozen in liquid nitrogen. Renal cortical and plasma ANG II and ANG-(1–7) concentrations were measured by radioimmunoassay in the Hypertension and Vascular Disease Centre Core Laboratory at Wake Forest University School of Medicine, Winston-Salem, North Carolina, as previously described.

2.3.7 Real-time Taqman PCR.

mRNA expression levels of various genes were determined by TaqMan Real-time PCR using 18S rRNA as the internal standard. Briefly, kidney samples from mice were snap frozen in liquid nitrogen, the cortex was later dissected in an RNA-stabilizing solution (RNAlater; Ambion, Austin, TX), and RNA was extracted using TRIZOL Reagent (Invitrogen, Carlsbad, CA). Total RNA (1 μg) was reverse transcribed, and RNA expression levels were quantified by Taqman RT-PCR using a sequence detection system (Prism 7700; Applied Biosystems, Foster City, CA).
2.3.8 NADPH oxidase activity and dihydroethidium fluorescence.

Harlan Sprague-Dawley rat mesangial cells were cultured in Dulbecco's modified Eagle's medium supplemented with 20% FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in 95% air and 5% CO2. Experiments were carried out in cells between passages 12 and 20. Sprague-Dawley rat mesangial cells were plated on 60-mm dishes with growth medium. The cells were cultured for 72 h to 90% confluence. The cells were then treated with placebo or hrACE2 (25 ng/ml) and then exposed to 100 nM of Ang II for 18 hours with and without 1 hr pretreatment with the specific ACE2 inhibitor, DX600 (1 μM). In a separate set of experiments, cells were pretreated with 10 μM Losartan for 30 minutes, or 250 ng/ml hrACE2 for 1 hour, or both and then exposed to high glucose (25 mM) for 24 hrs. In the third series of experiments, cells were pretreated with 100 nM Ang-(1-7) for 1 hour, 250 ng/ml hrACE2 alone for 1 hour, or 10μM D-Ala7-Ang-(1-7) (A-779) for 2 hours before hrACE2 was added and then exposed to high glucose (25 mM) for 24 hrs. Cells were washed twice with ice-cold PBS, collected, sonicated and centrifuged at 3000 rpm for 20 minutes at 4 °C. NADPH oxidase activity in cultured cells and renal cortical tissue was measured using lucigenin (5 μM) and NADPH (1 mM) at 37°C in an FB12 luminometer (Berthold Detection Systems, Pforzheim, Germany). For DHE fluorescence, Sprague-Dawley rat mesangial cells (MC) were cultured on autoclaved glass coverslips and grown to 80% confluence. Cells were serum-starved and then treated as described above. DHE (4 μM) was applied to the coverslips and incubated in light-protected and humidified chamber at 37 °C for 1 hour. In situ fluorescence was assessed using a Zeiss confocal laser-scanning microscope (LSM 510, Dusseldorf, Germany).

2.3.9 Statistical analysis.

Results are expressed as means ± SEM, unless otherwise specified. Student t test was used for comparison between two groups. Comparisons among multiple groups were performed by one-way ANOVA followed by multiple comparison testing (Student-Newman-Keuls test) using SPSS software (version 10.1; Chicago, IL).
2.4 RESULTS

2.4.1 Human recombinant ACE2 increases serum ACE2 activity and reduces urinary albumin excretion.

Male $\text{Ins2}^{WT/WT}$ (control C57BL/6J mice) and $\text{Ins2}^{WT/C96Y}$ (mutant diabetic Akita mice) were studied at 3 months of age$^{207,215}$. Whereas plasma ACE2 activity in Akita mice injected with placebo was undetectable, daily injection of 2 mg/kg of hrACE2 for 2 weeks resulted in measurable serum ACE2 activity of $3,138 \pm 721$ fluorescence unit/min (n = 6) in Akita mice that was equivalent to $7.14 \pm 2.1 \mu g/ml$ of hrACE2 (n = 6). The specific ACE2 inhibitor, DX600 (1 μmol/l), suppressed $95 \pm 4\%$ of the murine plasma ACE2 activity (n = 3). We hypothesized that the large size of hrACE2 and the increased serum ACE2 activity would target the diabetic glomeruli. Treatment with hrACE2 for 4 weeks reduced the urinary albumin excretion rate by 60% in the diabetic Akita mice ($\text{Ins2}^{WT/C96Y}$) compared with the placebo-treated diabetic Akita mice (Fig. 2.1, A and B). There were no significant differences in the plasma glucose concentrations of the $\text{Ins2}^{WT/C96Y}$ + placebo and $\text{Ins2}^{WT/C96Y}$ + hrACE2 mice (Fig. 2.1C). Despite severe hyperglycemia in the $\text{Ins2}^{WT/C96Y}$ mice, body weights were similar in all four groups of mice (Table 2.1). Assessment of TC-SBP in conscious mice revealed mild hypertension in the Akita mice (Fig. 2.1D) that declined over a 4-week period in response to daily administration of hrACE2 (Fig. 2.1E). Human recombinant ACE2 did not affect the serum creatinine concentrations or potassium levels (Table 2.1).
Figure 2.1: Human recombinant ACE2 reduces the increased urinary albumin excretion rates in diabetic Akita mice independent of hyperglycemia and with a mild blood pressure–lowering effect. A and B: Urinary albumin excretion rate (A) and urinary albumin/creatinine ratio (B) based on 24-h urine samples showing marked reduction in albuminuria after 4 weeks of daily treatment with hrACE2. n = 8 and 10 for urine albumin measurements in Ins2<sup>WT/WT</sup> and Ins2<sup>WT/C96Y</sup> groups, respectively. *P < 0.05 compared with all other groups and #P < 0.05 compared with placebo + Ins2<sup>WT/C96Y</sup> group using ANOVA and multiple comparison testing. C–E: Plasma glucose and tail-cuff systolic blood pressure showing no effect of hrACE2 on the marked hyperglycemia (C) and mild elevation in systolic blood pressure in diabetic Akita mice (D) that was normalized over a 4-week period in response to daily hrACE2 administration (2 mg · kg<sup>-1</sup> · day<sup>-1</sup>) (E). n = 10 for plasma glucose and n = 12 for systolic blood pressure measurements. *P < 0.05 compared with corresponding Ins2<sup>WT/WT</sup> group (C and D) or with Ins2<sup>WT/C96Y</sup> + placebo group (E) using Student t test.
Table 2.1: Morphometry and plasma biochemistry in 4-month-old mice.

<table>
<thead>
<tr>
<th></th>
<th>Ins$_2^{WT/WT}$+ placebo</th>
<th>Ins$_2^{WT/WT}$+ hrACE2</th>
<th>Ins$_2^{WT/C96Y}$+ placebo</th>
<th>Ins$_2^{WT/C96Y}$+ hrACE2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$</td>
<td>8</td>
<td>10</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>BW (g)</td>
<td>25.0 ± 1.2</td>
<td>25.3 ± 1.4</td>
<td>23.1 ± 0.8</td>
<td>23.4 ± 1.3</td>
</tr>
<tr>
<td>KW (g)</td>
<td>0.145 ± 0.06</td>
<td>0.151 ± 0.08</td>
<td>0.262 ± 0.09*</td>
<td>0.254 ± 0.07*</td>
</tr>
<tr>
<td>KW/BW (mg/g)</td>
<td>0.72 ± 0.15</td>
<td>0.63 ± 0.18</td>
<td>1.12 ± 0.23*</td>
<td>1.10 ± 0.29*</td>
</tr>
<tr>
<td>KW/TL (mg/mm)</td>
<td>7.25 ± 0.83</td>
<td>7.14 ± 0.91</td>
<td>11.23 ± 1.65*</td>
<td>10.64 ± 1.47*</td>
</tr>
<tr>
<td>Plasma K$^+$ (mM)</td>
<td>4.12 ± 0.32</td>
<td>4.5 ± 0.36</td>
<td>4.58 ± 0.41</td>
<td>4.2 ± 0.46</td>
</tr>
<tr>
<td>Creatinine (μM)</td>
<td>42.7 ± 5.7</td>
<td>36.7 ± 8.4</td>
<td>45.3 ± 5.1</td>
<td>33.7 ± 8.9</td>
</tr>
</tbody>
</table>

Data are means ± SEM.

*P < 0.05 compared with corresponding nondiabetic control group. BW, body weight; KW, kidney weight; TL, tibial length.
2.4.2  Recombinant human ACE2 reduces mesangial matrix expansion.

Given the marked protective effect of hrACE2 on the urinary albumin excretion in the diabetic mice, we sought to relate this functional change to kidney histomorphology. As expected, kidney hypertrophy (Table 2.1) was associated with an increase in glomerular volume in the $\text{Ins}^2_{\text{WT/C96Y}}$ + placebo mice compared with the control $\text{Ins}^2_{\text{WT/WT}}$ mice, and glomerular volume was reduced by hrACE2 treatment (Fig. 2.2A and C). In accordance with the light microscopic changes, increased glomerular basement membrane (GBM) thickness in the Akita $\text{Ins}^2_{\text{WT/C96Y}}$ mice was also significantly reduced in response to hrACE2 treatment (Fig. 2.2B and D). Diabetic nephropathy is characterized by an accumulation of extracellular matrix proteins in the glomerular mesangium. A semiquantitative and blinded assessment of the mesangial matrix expansion showed a significant increase in the diabetic Akita mice that was reduced by treatment with hrACE2 (Fig. 2.2E).

Immunohistochemical staining for $\alpha$-smooth muscle actin ($\alpha$-SMA) (Fig. 2.3A and C) and collagen III (Fig. 2.3B and D) was significantly increased in the glomeruli of diabetic mice, and expression was normalized by hrACE2 treatment. Inflammation and the accumulation of kidney macrophages can play an important role in diabetic kidney injury \cite{216} and we also observed that anti-ACE2 IgG antibodies developed in 50% of the mice injected with hrACE2, with a mean IgG titer of $11 \pm 7.2$ ng/ml. We therefore performed immunohistochemical studies of macrophage and neutrophil infiltration in the kidneys (Fig. 2.3E and F). There was no evidence of glomerular or tubulointerstitial infiltration by macrophages or neutrophils in the untreated and treated diabetic Akita mice. In addition, the expression profiles of the proinflammatory cytokines, tumor necrosis factor-$\alpha$, interleukin-1$\beta$, and interleukin-6, and the chemokine, monocyte chemoattractant protein-1, were similar in all four groups of mice (Table 2.2).
Figure 2.2: Glomerular mesangial expansion and thickening of basement membrane were reduced by treatment with human recombinant ACE2. A–C: Representative light micrographs of periodic acid Schiff–stained kidney sections from each group of mice (magnification ×630) (A) with quantification of the glomerular volume (C) showing glomerular expansion in the diabetic Akita mice and a marked reduction in response to hrACE2. B and D: Transmission electron microscopy of the glomeruli (B) with quantification of the glomerular basement thickness (D) showing increased glomerular basement membrane thickness in the Akita Ins2 WT/C96Y mice, which was normalized by treatment with hrACE2. White arrows indicate the glomerular basement membrane. E: Mesangial matrix expansion score showing increased mesangial expansion in diabetic Akita kidneys, which was prevented by hrACE2. n = 5 for all groups. *P < 0.05 compared with all other groups and #P < 0.05 compared with placebo + Ins2 WT/WT group using ANOVA with multiple comparison testing.
Figure 2.3: Increased glomerular expression of α-SMA and collagen III in the diabetic Akita kidneys without evidence of inflammatory changes in response to human recombinant ACE2. A–D: Increased glomerular immunostaining for α-SMA (A) and collagen III (B) in diabetic Akita mice that was quantified based on computer image analysis scores of glomerular immunostaining of α-SMA (C) and collagen III (D). Positive controls are shown as staining in renal blood vessels for α-SMA and from a kidney after 14 days of ureteral obstruction for collagen III. n = 5 for all groups. *P < 0.01 compared with all other groups using ANOVA and multiple comparison testing. E and F: Immunohistochemical-specific staining of neutrophil
and macrophage revealed no evidence of inflammation in the diabetic Akita mice without a
differential impact with treatment with hrACE2. Positive controls were taken from mouse spleen
and lung tissue. Scale bar, 100 μm.
**Table 2.2:** Expression Profile of the Mas Receptor, ProInflammatory Cytokines and the Chemokine, MCP-1.

<table>
<thead>
<tr>
<th></th>
<th>WT+Placebo</th>
<th>WT+hrACE2</th>
<th>Akita+Placebo</th>
<th>Akita+hrACE2</th>
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<td>N</td>
<td>6</td>
<td>6</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>MasR/18S</td>
<td>0.51±0.09</td>
<td>0.49±0.07</td>
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<td>0.61±0.073</td>
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<td>1.19±0.17</td>
</tr>
<tr>
<td>Interleukin-6/18S</td>
<td>0.072±0.009</td>
<td>0.076±0.008</td>
<td>0.081±0.011</td>
<td>0.069±0.008</td>
</tr>
<tr>
<td>MCP-1/18S</td>
<td>3.02±0.15</td>
<td>3.87±0.23</td>
<td>3.11±0.17</td>
<td>3.42±0.21</td>
</tr>
</tbody>
</table>

MasR=Mas Receptor (Angiotensin 1-7 Receptor); TNFα=tumor necrosis factor alpha; MCP-1 = monocyte chemoattractant protein-1 (also known as CCL2); p=NS using one-way ANOVA with multiple comparison testing.
2.4.3 Recombinant human ACE2 reduces ANG II levels in the plasma and renal cortex.

Plasma ACE2 activity was increased in the treated mice, so we measured plasma and renal cortical levels of ANG II, a substrate for ACE2 and ANG-(1–7), a product of ACE2, in response to the exogenous hrACE2. In the Akita mice, plasma and renal cortical ANG II levels were significantly reduced by 4 weeks of hrACE2 treatment (Fig. 2.4A). Consistent with the biochemical action of ACE2, renal ANG-(1–7) levels were increased after 4 weeks of treatment with hrACE2. The average plasma ANG-(1–7) levels was higher in the treated mice but the difference did not reach statistical significance (P = 0.092) (Fig. 2.4B). Renal cortical expression of ace (Fig. 2.4C) was reduced in the diabetic mice, whereas expression of ace2 (Fig. 2.4D) was increased in the diabetic Akita mice. The administration of hrACE2 did not influence ace and ace2 expression levels in the kidney, suggesting that the treatment-induced changes in peptide levels were due to exogenous ACE2 activity rather than changes in endogenous kidney expression of the key angiotensin processing enzymes. Similarly, the expression of other components of the RAS known to play a key role in diabetic nephropathy such as the AT1 receptor (Fig. 2.4E), AT2 receptor (Fig. 2.4F), and bradykinin2 receptor (Fig. 2.4G) was not influenced by hrACE2 treatment. Consistent with the measures of mesangial matrix expansion, the mRNA expression of the ANG II–sensitive genes fibronectin (Fig. 2.4H) and pro–collagen III α-1 (Fig. 2.4I) was increased in Akita diabetic mice and normalized by treatment with hrACE2.
Figure 2.4: Human recombinant ACE2 alters angiotensin peptide metabolism without a differential impact on expression of the genes of the renin-angiotensin system while normalizing matrix gene expression in diabetic Akita mice. A and B: Reduction in plasma and renal cortical ANG II levels (A) and increases in plasma and renal cortical ANG-(1–7) levels (B) in diabetic Akita mice after treatment with hrACE2. n = 10 for placebo group and n = 12 for hrACE2-treated group. *P < 0.05 compared with corresponding placebo group using Student t test. C–G: Decreased renal cortical ace (C) and increased ace2 (D) expression, unaltered ANG II type 1 receptor, AT1R (E), and type 2 receptor, AT2R (F), expression, and increased bradykinin type 2 receptor, B2R (G), expression in Akita mice were not affected by treatment with hrACE2. n = 8 for placebo groups; n = 10 for hrACE2 groups. †P < 0.05 compared with corresponding Ins2WT/WT group using Student t test. H and I: Increased renal cortical expression of extracellular matrix genes, fibronectin (H) and pro–collagen III α-1 (I), in diabetic Akita mice was suppressed in response to hrACE2. n = 8 for placebo groups; n = 10 for hrACE2 groups. #P < 0.05 compared with placebo + Ins2WT/C96Y group using ANOVA and multiple comparison testing.
2.4.4 Increased NADPH oxidase activity and PKC expression were suppressed by hrACE2.

Increased renal NADPH oxidase activity and activation of the PKC system play key roles in the pathophysiology of diabetic nephropathy. Given the protective effect of hrACE2 on diabetic kidney injury, we examined the effect of hrACE2 treatment on renal cortical NADPH oxidase activity. In the diabetic Akita mice, renal cortical NADPH activity based on the lucigenin chemiluminescence assay was significantly increased compared with nondiabetic mice (Fig. 2.5A) in association with increased renal cortical mRNA expression of the NADPH oxidase subunits, \( \text{NOX2} (\text{gp}91^{\text{phox}}) \) (Fig. 2.5B) and \( p47^{\text{phox}} \) (Fig. 2.5C). The cortical expression of the other NADPH subunits including \( \text{NOX1} \), \( \text{NOX4} \), \( p22^{\text{phox}} \), \( p40^{\text{phox}} \), and \( p67^{\text{phox}} \) was not significantly altered in our diabetic model (Table 2.3). Treatment with hrACE2 normalized NADPH oxidase activity and mRNA expression of \( \text{NOX2} \) and \( p47^{\text{phox}} \) subunits in the diabetic mice (Fig. 2.5A–C).
Figure 2.5: Reduction of NADPH oxidase activity in response to human recombinant ACE2 in diabetic Akita mice. A–C: Elevated renal cortical NADPH activity (A) and increased expression of \( \text{NOX2 (gp91}^{\text{phox}} \) (B) and \( p47^{\text{phox}} \) mRNA (C) in Akita mice were completely suppressed and normalized by treatment with hrACE2. \( n = 6 \) and 8 in \( \text{Ins2}^{\text{WT/WT}} \) and \( \text{Ins2}^{\text{WT/C96Y}} \) groups, respectively. *P < 0.05 compared with all other groups using ANOVA with multiple comparison testing.
**Table 2.3**: Expression Profile of the NADPH oxidase subunits.

<table>
<thead>
<tr>
<th></th>
<th>WT+Placebo</th>
<th>WT+hrACE2</th>
<th>Akita+Placebo</th>
<th>Akita+hrACE2</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>6</td>
<td>6</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>NOX1/18S</td>
<td>0.23±0.031</td>
<td>0.25±0.022</td>
<td>0.28±0.034</td>
<td>0.31±0.036</td>
</tr>
<tr>
<td>NOX4/18S</td>
<td>219±11.5</td>
<td>187±14</td>
<td>231±22</td>
<td>239±23.7</td>
</tr>
<tr>
<td>p22phox/18S</td>
<td>45.8±4.1</td>
<td>40.2±5.9</td>
<td>49.6±6.3</td>
<td>42.4±4.8</td>
</tr>
<tr>
<td>p40phox/18S</td>
<td>0.71±0.04</td>
<td>0.67±0.05</td>
<td>0.79±0.07</td>
<td>0.81±0.08</td>
</tr>
<tr>
<td>p67phox/18S</td>
<td>0.49±0.05</td>
<td>0.54±0.08</td>
<td>0.63±0.09</td>
<td>0.52±0.07</td>
</tr>
</tbody>
</table>

p=NS using one-way ANOVA with multiple comparison testing.
2.4.5 Human recombinant ACE2 reduces high glucose and ANG II–induced NADPH oxidase activity in mesangial cells: evidence for the potential role of ANG-(1–7).

To address mechanisms responsible for the protective effect of hrACE2 in the diabetic mice, we used cultured primary rat mesangial cells to study the effects of hrACE2 on high glucose and ANG II–induced dihydroethidium fluorescence (DHF) fluorescence and NADPH oxidase activity. High glucose–induced DHF fluorescence was attenuated by both hrACE2 and ANG-(1–7) (Fig. 2.6A–D). NADPH oxidase was also activated by high glucose (Fig. 2.6E), and this effect was attenuated by pretreatment with rhACE2 (Fig. 2.6F). As an osmotic control, d-mannitol did not activate NADPH oxidase (Fig. 2.6E). Consistent with the ability of hrACE2 to metabolize ANG II, hrACE2 also suppressed ANG II–induced DHF fluorescence (Fig. 2.6G–I) and reduced NADPH oxidase activity in a dose-dependent manner (Fig. 2.6J) in mesangial cells. Pretreatment with the specific ACE2 inhibitor, DX600 (1 μmol/l), prevented the ability of hrACE2 to suppress ANG II–mediated activation of NADPH oxidase (Fig. 2.6J). High glucose concentrations can activate the intrarenal RAS and increase the generation of ANG II in mesangial cells, so we studied the effects of hrACE2 on high glucose–induced NADPH oxidase activity in mesangial cells pretreated with either the ANG II type I receptor antagonist, losartan, or the Mas receptor peptide antagonist, d-Ala7-ANG-(1–7). Treatment with the ANG II type I receptor antagonist attenuated the high glucose–induced increase in NADPH oxidase activity with hrACE2, leading to incremental suppression (Fig. 2.6K). The attenuation of high glucose–induced NADPH oxidase activity by hrACE2 was partially prevented by the Mas receptor antagonist, d-Ala7-ANG-(1–7), suggesting that part of the effect was mediated by ANG-(1–7) (Fig. 2.6L). Taken together, these results support the hypothesis that the protective effect of hrACE2 is mediated, at least in part, by a reduction in ANG II and an increase in ANG-(1–7) and that together these changes reduce oxidative stress in the diabetic kidney.
Figure 2.6: Recombinant human ACE2 prevents high glucose (HG) and ANG II–induced oxidative stress and NADPH oxidase activation in cultured rat mesangial cells. A–D: DHF staining in cultured mesangial cells treated for 24 h with normal glucose (NG; 5.6 mmol/l) (A) or high glucose (25 mmol/l) (B) and pretreated with 250 ng/ml of ACE2 for 1 h (C) or 100 nmol/l of ANG-(1–7) for 15 min (D) and then exposed to 25 mmol/l of high d-glucose for 24 h. E: NADPH oxidase activity in response to high d-glucose (25 mmol/l) or d-mannitol for 24 h. F: NADPH oxidase activity in response to 24 h of high d-glucose (25 mmol/l) and the effects of pretreatment with 100 nmol/l ANG-(1–7). G–J: Dihydroethidium fluorescence in cultured mesangial cells treated with placebo (G) or stimulated by 100 nmol/l of ANG II for 18 h (H) and with pretreatment with hrACE2 (25 ng/ml) (I). Suppression of ANG II–induced NADPH oxidase activity in cultured mesangial cells by pretreatment with 25 and 250 ng/ml of hrACE2 was preventable by 1 μmol/l of the specific ACE2 inhibitor, DX600 (J). K: The effects of pretreatment with AT1 receptor blocker, losartan (10 μmol/l), and hrACE2 (250 ng/ml) on high-glucose–induced NADPH oxidase activity. L: The effects of hrACE2 (250 ng/ml) with and without the ANG-(1–7) blocker, d-Ala-ANG-(1–7) (10 μmol/l), on high glucose–induced NADPH oxidase activity. n = 5 for all groups. *P < 0.05 compared with all other groups, #P < 0.05 compared with the 5.6 mmol/l d-glucose group, **P < 0.05 compared with the 25 mmol/l d-
glucose– and losartan-treated group, and ***P < 0.05 compared with the 25 mmol/l d-glucose– and hrACE2-treated group using ANOVA with multiple comparison testing.
2.5 DISCUSSION

Diabetic nephropathy continues to be the most common cause of end-stage renal disease in North America. Activation of the RAS and ANG II play an important role in the development of experimental and clinical diabetic nephropathy, and blockade of the RAS in both experimental and clinical diabetes attenuates the development of diabetic kidney injury\(^{195-197, 207}\). However, ACE inhibitors and angiotensin receptor blockers provide only partial long-term benefits in patients with type 1\(^{224}\) and type 2\(^{195, 196, 225}\) diabetes. The recent discovery of an ACE homologue, ACE2, has revised our understanding of the renin-angiotensin system\(^{200, 201, 226}\). In a long-standing diabetic rat model, renal ACE2 expression is reduced\(^{205}\), whereas there is an early increase in ACE2 expression and activity in the kidneys of the diabetic db/db\(^{204}\) and Akita\(^{207}\) mice. Deletion of the ace2 gene and pharmacological inhibition of ACE2 is associated with accelerated glomerular injury in Akita diabetic mice\(^{207}\) and in streptozocin-induced diabetes\(^{227, 228}\), providing definitive evidence that ACE2 is renoprotective and that reduced ACE2 activity contributes to the progression of kidney disease\(^{208, 209}\). Kidney disease in patients with type 2 diabetes is associated with a reduction in ACE2 mRNA and protein expression\(^{209}\). Accordingly, we evaluated the ability of hrACE2 to reduce the functional and structural changes of diabetic nephropathy in male Akita (\(Ins2^{\text{WT/C96Y}}\)) mice, a model of type 1 diabetes that is associated with the development of changes in the kidney that are similar to human diabetic nephropathy\(^{207, 215, 229}\).

We observed early and sustained increases in the blood glucose concentrations in our Akita mice, as reported previously\(^{207, 215, 229}\). Our major finding is that treatment with exogenous hrACE2 slows the progression of diabetic nephropathy. The Akita diabetic mice develop an increase in the urinary albumin excretion rate in association with renal and glomerular hypertrophy, mesangial matrix expansion, and an increase in GBM thickness compared with littermate nondiabetic mice. The increase in albumin excretion, an early functional abnormality in the natural history of nephropathy in patients with diabetes\(^{230, 231}\), was markedly reduced by hrACE2 treatment. ACE2 activity increased in the plasma of treated mice; plasma and renal ANG II levels declined whereas ANG-(1–7) levels rose in the treated Akita mice. These observations are consistent with the hypothesis that ACE2 plays an important role in the processing of angiotensin peptides in the plasma and kidney\(^{202, 204, 205}\) and that ANG II–
dependent injury (via the AT1 receptors) in the diabetic kidney is accelerated by reduced ACE2 activity\textsuperscript{207}. Whether the changes in renal angiotensin peptide levels reflect the changes in plasma angiotensin levels and/or an active intrarenal process remains to be clarified. Consistent with previous studies\textsuperscript{207,229}, we observed mild hypertension in the diabetic Akita mice and hrACE2 treatment lowered blood pressure in association with the decrease in plasma ANG II levels, an effect that may contribute to renal protection.

Glomerular hypertrophy and mesangial matrix expansion, early features of human diabetic nephropathy, were reduced by hrACE2 treatment, confirming that modulation of angiotensin peptide metabolism and its downstream effects can attenuate diabetic kidney injury in the diabetic mouse. The RAS is activated in the diabetic milieu and increasing ACE2 activity may provide an alternate and important strategy to limit the role of the RAS in progressive diabetic nephropathy. Increased renal NADPH oxidase activity and activation of the PKC system are two canonical pathways known to play a fundamental role in the pathophysiology of diabetic nephropathy\textsuperscript{217-219}. Expression analysis of the renal cortical NADPH subunits revealed that both NOX2 (gp91\textsuperscript{phox}) and p47\textsuperscript{phox} subunits were increased in diabetic Akita mice in agreement with previous findings in a type 1 diabetic model\textsuperscript{218}. Along with these changes in NADPH oxidase subunit expression, NADPH oxidase activity increased in the kidney cortex of our diabetic Akita mice. Importantly, hrACE2 treatment normalized NADPH oxidase subunit expression and activity in diabetic Akita mice.

We used an \textit{in vitro} system of cultured primary rat mesangial cells to provide further insights into the mechanisms responsible for renoprotective effect of hrACE2 treatment in our diabetic Akita mice. Both high glucose concentrations and ANG II increased NADPH oxidase activity in vitro, and hrACE2 treatment attenuated high glucose– and ANG II–induced DHE staining and NADPH oxidase activation in the mesangial cells. Blockade of ANG-(1–7) signaling with a Mas receptor peptide antagonist limited the protective effect of hrACE2 in vitro. Taken together with our finding that kidney cortical levels of ANG-(1–7) were increased in the treated diabetic mice, these in vitro findings support the hypothesis that the protective effect of hrACE2 on diabetic injury was mediated, at least in part, by an increase in ANG-(1–7) levels and attenuation of oxidative stress. Indeed, treatment with ANG-(1–7) reduces renal NADPH oxidase activity and urinary albumin excretion in diabetic hypertensive rats\textsuperscript{232}, whereas the loss of ANG-(1–7) receptor (Mas receptor) leads to glomerular hyperfiltration and albuminuria\textsuperscript{233}, changes that are
characteristic of early diabetic nephropathy. Finally, our data also suggest that hrACE2-induced reduction in NADPH oxidase activity in vivo is due in part to the decrease in plasma and kidney ANG II levels.

In summary, we have shown that hrACE2 treatment improves kidney function and structure in a murine model of diabetic nephropathy. The ability of hrACE2 to suppress high glucose– and ANG II–induced activation of NADPH oxidase and to limit diabetic nephropathy is consistent with the notion that it functions as a negative regulator of the RAS. These beneficial effects of hrACE2 were not due to changes in plasma glucose levels, although there was a normalizing effect on blood pressure that may contribute to the renoprotection. Enhancing ACE2 activity may represent a novel therapeutic strategy to minimize the rate of progression of diabetic kidney disease. Additional work will be required to determine whether hrACE2 provides an incremental benefit over AT1 receptor blockade and/or ACE inhibition.
Chapter 3

Angiotensin-(1-7)-Induced Activation of ERK1/2 is cAMP/Protein Kinase A-dependent in Glomerular Mesangial Cells

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### 3.1 ABSTRACT

The renin-angiotensin system (RAS) plays an important role in renal physiology and kidney injury. Although the cellular effects of the RAS activation are generally attributed to angiotensin II (ANG II), the recent identification of angiotensin-converting enzyme 2 has shifted the focus to other peptides including Ang-(1–7). The G protein-coupled receptor for Ang-(1–7), mas, is expressed by mesangial cells (MC) but the signal transduction pathways activated by Ang-(1–7) in MC have not been fully elucidated. Accordingly, we studied the effect of Ang-(1–7) on extracellular signal-related kinase (ERK)1/2 activation in rat MC. Ang-(1–7)-induced ERK1/2 phosphorylation in MC is time- and concentration-dependent. Pretreatment of MC with the mas receptor antagonist A-779 but not the AT1 antagonist losartan or the AT2 antagonist PD123319 abrogated ERK1/2 activation. Neither pretreatment with the NADPH oxidase inhibitors diphenyleneiodonium and apocynin nor pretreatment with the epidermal growth factor (EGF) receptor antagonists AG1478 and PD158780 attenuated Ang-(1–7)-induced activation of ERK1/2, although each of these compounds abolished ANG II-induced activation of ERK1/2. Ang-(1–7) increased intracellular cAMP levels and activated protein kinase A (PKA) and inhibition of either adenylyl cyclase or PKA activity attenuated Ang-(1–7)-induced ERK1/2 activation. Pre-treatment with Ang-(1–7) reduced Ang II-induced NADPH oxidase activity and ERK1/2 activation in a cAMP-PKA-dependent manner. In conclusion, Ang-(1–7)-induced activation of ERK1/2 is cAMP/PKA-dependent in MC, but independent of NADPH oxidase and the EGF receptor.

### 3.2 INTRODUCTION

The renin-angiotensin system (RAS) plays a central role in the development and progression of chronic kidney disease including diabetic nephropathy. Although the main effector peptide of the RAS is angiotensin II (ANG II), angiotensin-converting enzyme 2 (ACE2) has been shown to be protective under many pathological settings. ACE2 converts ANG I to Ang-(1–9) which can be converted to Ang-(1–7) by ACE. In addition, ACE2 can also convert ANG II to Ang-(1–7). Ang-(1–7) activates the mas receptor and is associated with vasodilating, natriuretic, diuretic, and antiproliferative effects. It has been suggested that Ang-(1–7) is an important...
counterbalancing mechanism within the RAS. Studies support a protective role for Ang-(1–7) because Ang-(1–7) prevented ANG II-induced MAPK activation in proximal tubular cells and vascular smooth muscle cells. In this regard, Ang-(1–7) infusion has been reported to attenuate experimental glomerulonephritis. However, other studies suggest that some of the cellular effects of Ang-(1–7) may be deleterious; for example, stimulation of growth factor expression and cell proliferation by Ang-(1–7) in MC have been reported. In addition, deletion of the gene for the mas receptor in mice is associated with decreased inflammation in kidneys subjected to unilateral ureteral obstruction.

The mas receptor is expressed by glomerular mesangial cells (MC) but the intracellular signal transduction pathways activated by Ang-(1–7) have not been fully elucidated. Accordingly, the goal of the current study was to elucidate the intracellular signaling pathways linking Ang-(1–7) to the activation of the canonical mitogen-activated protein kinase extracellular signal-related kinase (ERK)1/2 in MC. We observed that the activation of ERK1/2 by Ang-(1–7) was dependent on generation of cAMP and the subsequent activation of protein kinase A (PKA).

### 3.3 MATERIALS AND METHODS

#### 3.3.1 Cell culture.

Harlan Sprague-Dawley rat MC (SD-MC) were cultured in DMEM supplemented with 20% fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 U/ml) at 37°C in 95% air-5% CO2. Before treatment, the cells were starved in serum-free medium overnight. Experiments were carried out using cells between passages 11 to 20.

#### 3.3.2 Chemicals.

Angiotensin-(1–7), ANG II, DB-cAMP, H-89 and pCPT-cAMP, DPI, NAC, catalase, and BAPTA-AM were obtained from Sigma (St. Louis, MO). SQ22536, KT5720, AG1478, PD158780, and apocynin were obtained from Calbiochem (Merck KGaA, Darmstadt, Germany).
A-779 was purchased from Bachem California (Torrance, CA). A23187 was purchased from Alexis (Enzo Life Sciences, Ann Arbor, MI). Ang-(1–7) (10^{-11} \text{ M}) was used for experiments unless mentioned otherwise.

3.3.3 Western blot analysis.

After treatment, cells were washed twice with ice-cold PBS and transferred to microcentrifuge tubes and centrifuged at 18,000 g for 30 s. Cell pellets were resuspended and lysed on ice with lysis buffer (Cell Signaling, Beverly, MA) containing 20 mM Tris· HCl (pH 7.5), 150 nM NaCl, 1% Triton X-100, 1 mM Na₂ EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 μg/ml leupeptin, and 1 mM phenylmethyl sulfonzy fluoride. After 20 min of incubation in the lysis buffer, a 10-min centrifugation at 18,000 g at 4°C was performed. The total cell lysate was collected, and the protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). Samples with equal concentrations of cellular protein (25 μg) were mixed with 6× SDS sample loading buffer containing 125 mM Tris· HCl (pH 6.8), 2% SDS, 20% glycerol, 0.2% bromophenol blue, and 5% β-mercaptoethanol and were boiled for 5 min, and separated by a 10% SDS-PAGE gel and then transferred onto Immobilon polyvinylidene fluoride membrane (Millipore, Bedford, MA). After being blocked with 5% skim milk, primary antibodies were applied (1:1,000 phospho-ERK1/2, 1:1,000 total-ERK1/2, purchased from Cell Signaling) and allowed to incubate overnight at 4°C. After extensive washing in TBS-Tween 20, the membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG for 1 h at room temperature. After being washed, the membranes were incubated with enhanced chemiluminescence system detection kit (Millipore, Billerica, MA). Total ERK1/2 was used as loading control, and densitometry was measured using Scion Image software (Scion, Frederick, MD).

3.3.4 cAMP assay and PKA assay.

MCs were cultured in 60-mm culture dishes to confluence and subsequently serum deprived for 18 h. The cells were then treated with 10^{-11} \text{ M} Ang-(1–7) for 15 min and lysed. The amount of cAMP extracted from each dish was quantified by cAMP enzyme immunoassay kit from Sigma.
according to the manufacturer's instructions and normalized against total protein as determined by Bradford assay. For PKA assay, MCs were cultured in 60-mm culture dishes to confluence and subsequently serum deprived for 18 h. The cells were pretreated with the potent PKA-selective inhibitor H89 (1 μM) for 30 min before Ang-(1–7) stimulation. The cells were lysed and adjusted to the same concentration of protein in each sample. An IMAP-PKA Assay Kit (Molecular Devices, Sunnyvale, CA) was used to measure PKA enzyme activity according to the manufacturer's instruction.

3.3.5 Fluorescence imaging.

SD-MCs were transferred to six-well plates containing autoclaved glass slips and grown to 80% confluence. Cells were serum-starved overnight. Cells were pretreated by the inhibitors and/or 10⁻¹¹ M Ang-(1–7) for 15 min at 37°C. After treatment, cells were washed three times with ice-cold PBS. Cells were fixed with 4% paraformaldehyde for 30 min at room temperature and quenched for 10 min with 100 mM glycine in PBS. Cells were then washed three times with PBS, permeabilized with 0.1% Triton X-100 for 30 min, and then washed again. Blocking with 5% goat serum was performed at room temperature over 1 h, and cells were then incubated overnight at 4°C with anti-phospho-ERK1/2 (1:50). Cells were washed five times with PBS and then the secondary Alexa-488 goat anti-rabbit antibody (1:100; Molecular Probes, Eugene, OR) was applied for 1 h. After being washed with PBS, the coverslip was placed directly on a glass slide using DAKO fluorescence mounting medium. Cells were imaged with a Zeiss confocal laser-scanning microscope (LSM 510, Dusseldorf, Germany).

3.3.6 Lucigenin chemiluminescence for NADPH oxidase activity.

SD-MCs were plated on 60-mm dishes with growth medium. Cells were cultured for 72 h to 90% confluence, then exposed to experimental reagents for the indicated times and concentrations, washed with ice-cold PBS twice, collected, and sonicated. After 20 min of centrifugation at 1,000 rcf and 4°C, 100 μl of supernatant were assayed by adding lucigenin (5 μM, Sigma), NADPH (1 mM, Merck KGaA), and detected using an FB12 luminometer (Huntsville, AL). The protein concentration was determined with the Bradford assay (Bradford Laboratories).
3.3.7 Statistical analysis.

Results are expressed as means ± SE, unless otherwise specified. Student's t-test was used for comparison between two groups. Comparisons between multiple groups were performed by one-way ANOVA followed by Bonferroni correction using GraphPad software.

3.4 RESULTS

3.4.1 Ang-(1–7) induces phosphorylation of ERK1/2.

We first examined the time- and concentration-dependent effect of Ang-(1–7) on the phosphorylation of ERK1/2 in our primary rat MC (Fig. 3.1, A and B). As expected, there was a time-dependent activation of ERK1/2 and we observed a peak effect at 5 min. Although we observed a stronger intensity for phospho-ERK2 (p42 MAPK) following treatment with Ang-(1–7), the responses of phospho-ERK1 and phospho-ERK2 paralleled one another. ERK1/2 was activated at a dose of $10^{-12}$ M Ang-(1–7) and there was only a modest dose-dependent effect on the phosphorylation of ERK1/2 at higher concentrations of the peptide. Ang-(1–7) ($10^{-11}$ M) was used in subsequent experiments.
Figure 3.1: Time and concentration-dependent ERK1/2 phosphorylation by Ang-(1-7). A: time course of $10^{-11}$ M Ang-(1–7)-induced extracellular signal-related kinase (ERK)1/2 phosphorylation. Cells were exposed to $10^{-11}$ M Ang-(1–7) for the indicated times. B: concentration-dependent Ang-(1–7)-induced ERK1/2 phosphorylation. Total cell lysates were immunoblotted with antibodies specific for phospho-ERK1/2 or total ERK1/2, and densitometry was performed. *P < 0.001, #P < 0.01 compared with baseline. Each experiment was performed in triplicate.
3.4.2 Effect of A-779 on Ang-(1–7)-induced ERK1/2 phosphorylation.

To relate activation of ERK1/2 by Ang-(1–7) to the mas receptor, we studied the effect of the mas receptor antagonist A-779. A-779 inhibited Ang-(1–7)-induced ERK1/2 phosphorylation in a dose-dependent manner (Fig. 3.2A). Pretreatment with the AT1 blocker losartan did not prevent Ang-(1–7)-induced ERK1/2 phosphorylation (Fig. 3.2B), although the dose of losartan effectively blocked ANG II-induced ERK1/2 phosphorylation (Fig. 3.2C). Pretreatment with the AT2 blocker PD123319 did not attenuate Ang-(1–7)-induced ERK1/2 phosphorylation (Fig. 3.2B) or ANG II-induced ERK1/2 phosphorylation in the primary SD-MC (Fig. 3.2C).
Figure 3.2: Effect of A-779, losartan, and PD123319 on Ang-(1–7)- or ANG II-induced ERK1/2 phosphorylation. A: Sprague-Dawley rat glomerular mesangial cells (SD-MC) were pretreated with 1 μM or 10 μM A-779 for 5 min and then treated with $10^{-11}$ M Ang-(1–7) for 5 min. B: SD-MC were pretreated with 1 μM losartan or 1 μM PD123319 for 1 h and then treated with $10^{-11}$ M Ang-(1–7) for 5 min. There was no statistical difference between cells treated with Ang-(1–7) alone and cells treated with Ang-(1–7) but pretreated with either losartan or PD123319. C: SD-MC were pretreated with 1 μM losartan or 1 μM PD123319 for 1 h and then treated with $10^{-7}$ M ANG II for 5 min. *P < 0.001 compared with untreated control cells. #P < 0.01 compared with cells treated with Ang-(1–7) alone. Experiments were performed in triplicate.
3.4.3 Effect of DPI and apocynin on Ang-(1–7)-induced ERK1/2 phosphorylation.

To better understand the transduction pathways linking Ang-(1–7)/mas receptor interaction(s) to the phosphorylation of ERK1/2, we first studied the effect of NADPH oxidase inhibition. Cells were pretreated with the NADPH oxidase inhibitors DPI (10 μM) or apocynin (100 μM) for 60 min before the administration of Ang-(1–7). Neither NADPH oxidase inhibitors reduced Ang-(1–7)-induced ERK1/2 phosphorylation (Fig. 3.3A), although pretreatment with the inhibitors significantly attenuated ANG II-induced phosphorylation of ERK1/2 (Fig. 3.3B).
Figure 3.3: Effect of DPI and apocynin on the activation of ERK1/2 in SD-MC on Ang-(1–7) (A) or ANG II (B)-induced ERK1/2 phosphorylation. A: cells were pretreated with 10 μM DPI or 100 μM apocynin for 1 h before exposure to $10^{-11}$ M Ang-(1–7) for 5 min. B: cells were pretreated with 10 μM DPI or 100 μM apocynin for 1 h before exposure to $10^{-7}$ M ANG II for 5 min. *P < 0.05 vs. untreated control cells. #P < 0.05 compared with cells treated with ANG II alone. †P < 0.001 compared with cells treated with ANG II alone. Each experiment was performed in triplicate.
3.4.4 Ang-(1–7)-induced ERK1/2 phosphorylation is independent of the epidermal growth factor receptor.

Transactivation of the epidermal growth factor receptor (EGFR) has been linked to ERK1/2 signaling by ANG II\textsuperscript{259}. To assess the role of EGFR in Ang-(1–7)-induced ERK1/2 phosphorylation, we pretreated SD-MC with the EGFR kinase inhibitors AG1478 and PD158780. Neither EGFR kinase inhibitors attenuated Ang-(1–7)-induced ERK1/2 phosphorylation (Fig. 3.4A), although the equivalent doses prevented both EGF-induced ERK1/2 phosphorylation (Fig. 3.4B) and ANG II-induced ERK1/2 phosphorylation (Fig. 3.4C).
Figure 3.4: Effect of epidermal growth factor (EGF) receptor antagonists on activation of ERK1/2 in SD-MC. A: cells were preincubated with 0.2 μM AG-1478 for 20 min or 10 μM PD158780 for 2 h and then treated with $10^{-11}$ M Ang-(1–7). B: cells were preincubated with 0.2 μM AG-1478 for 20 min or 10 μM PD158780 for 2 h and then treated with 100 ng/ml EGF for 5 min. C: cells were preincubated with 0.2 μM AG-1478 for 20 min or 10 μM PD158780 for 2 h and then treated with $10^{-7}$ M ANG II for 5 min. Values are means ± SE. *P < 0.001 compared with untreated SD-MC. #P < 0.001 compared with SD-MC treated with ligand alone. †P < 0.05 compared with cells treated with ANG II alone. Each experiment was performed in triplicate.
3.4.5 Ang-(1–7) increases cAMP levels in SD-MC and Ang-(1–7)-induced ERK1/2 phosphorylation is cAMP-dependent.

In some cell types, mainly of neuronal origin, activation of ERK1/2 downstream of Gs-α-coupled receptors is cAMP-dependent. Ang-(1–7) increased cAMP levels in the SD-MC, an effect that was abrogated by pretreatment with the mas receptor antagonist A-779 (Fig. 3.5A). The selective adenyl cyclase inhibitor SQ22536 also prevented Ang-(1–7)-induced increase in cAMP levels (Fig. 3.5A). Taken together, these findings suggest that the mas receptor is coupled to Gs and that the binding of Ang-(1–7) activates adenyl cyclase in SD-MC. We then studied the effect of SQ22536 on Ang-(1–7)-induced activation of ERK1/2, and we found that pretreatment with the adenyl cyclase inhibitor prevented phosphorylation of ERK1/2 (Fig. 3.5B).
Figure 3.5: Effect of mas receptor antagonist A-779 and adenylyl cyclase inhibitor SQ22536 on Ang-(1-7)-induced cAMP generation and ERK1/2 phosphorylation. A: effect of Ang-(1-7) on intracellular cAMP concentration. SD-MC were treated with 10^{-11} M Ang-(1-7) for 15 min, alone or after pretreatment with 10 \mu M A-779 (5 min) or 1 mM SQ22536 (30 min). Intracellular cAMP concentrations were determined by enzyme immunoassay. B: effect of SQ22536 on Ang-(1-7)-stimulated phosphorylation of ERK1/2. Cells were preincubated with 0.1 or 1 mM SQ22536 for 30 min and then treated with 10^{-11} M Ang-(1-7) for 5 min. *P < 0.05 compared with control cells. #P < 0.05 compared with cells treated with Ang-(1-7) alone. Experiments were performed in triplicate.
3.4.6  Ang-(1–7) increases PKA activity in SD-MC and Ang-(1–7)-induced ERK1/2 phosphorylation is PKA-dependent.

It has been proposed that there is a signal transduction pathway that includes cAMP, PKA, and MEK leading to the activation of ERK1/2. We first found that Ang-(1–7) increased PKA activity, an effect that was inhibited by the reversible PKA inhibitor H89 (Fig. 3.6A). We then studied the effect of the reversible PKA inhibitors H89 and KT5720 on Ang-(1–7)-induced activation of ERK1/2 and found that pretreatment with the PKA inhibitors prevented phosphorylation of ERK1/2 (Fig. 3.6, B and C). To further relate cAMP and PKA to ERK1/2 phosphorylation in our primary SD-MC, we studied the effect of the cell-permeable cAMP analog pCPT-cAMP on ERK1/2 phosphorylation. Treatment with the pCPT-cAMP led to a time-dependent activation of ERK1/2 in the SD-MC (Fig. 3.7A). This analog leads to the activation of both PKA- and cAMP-activated guanine-nucleotide exchange factors (Epac) so we also examined the effect of inhibiting PKA on pCPT-cAMP-induced phosphorylation of ERK1/2. Inhibition of PKA by H89 attenuated pCPT-cAMP-induced phosphorylation of ERK1/2, as did pretreatment with the MEK inhibitor PD98059 (Fig. 3.7B). Taken together, these findings suggest that the signal transduction pathway linking Ang-(1–7) to the activation of ERK1/2 is dependent on both cAMP and PKA and does not proceed through Epac.
Figure 3.6: Effect of PKA inhibitor H89 and KT5720 on Ang-(1-7)-induced PKA activation and ERK1/2 phosphorylation. A: effect of Ang-(1–7) on protein kinase A (PKA) activity. SD-MC were treated with Ang-(1–7) for 15 min, alone or after pretreatment with 10 μM H89 (30 min). PKA activity was measured with an IMAP-PKA assay kit. B: SD-MC were pretreated with 1 or 10 μM H89 for 30 min before treatment with 10^{-11} M Ang-(1–7) for 5 min. C: SD-MC were pretreated with 0.1 or 1 μM KT5720 for 30 min before treatment with 10^{-11} M Ang-(1–7) for 5 min. *P < 0.05 compared with untreated control cells. #P < 0.05 compared with SD-MC treated with Ang-(1–7) alone. Experiments were performed in triplicate.
Figure 3.7: Effect of the cell-permeable cAMP analog pCPT-cAMP on ERK1/2 phosphorylation in SD-MC. A. Cells were exposed to 200 μM pCPT-cAMP for the indicated times. Total cell lysates were immunoblotted with antibodies specific for phospho-ERK1/2 or total ERK1/2, and densitometry was performed. *P < 0.05 compared with baseline. B: cells were pretreated with 1 μM H89 (30 min) or 10 μM PD98059 (60 min) and then exposed to 200 μM pCPT-cAMP for 5 min. *P < 0.001 compared with untreated SD-MC. #P < 0.001 compared with SD-MC treated with pCPT-cAMP alone. Experiments were performed in triplicate.
3.4.7 Effect of Ang-(1–7) on the phosphorylation and nuclear translocation of ERK1/2.

We compared the effect of PKA inhibition and the effect of EGFR inhibition on Ang-(1–7)-induced nuclear translocation of phospho-ERK1/2 by confocal immunofluorescence imaging. In accord with our Western blot analyses, inhibition of PKA prevented the phosphorylation and nuclear translocation of ERK1/2 while pretreatment with the EGFR kinase inhibitor had no effect (Fig. 3.8).
Figure 3.8: Ang-(1–7)-induced nuclear localization of phospho-ERK1/2 in SD-MC. A: untreated cells. B: cells exposed to $10^{-11}$ M Ang-(1–7) for 15 min. C: cells were pretreated with 1 μM H89 for 30 min before exposed to Ang-(1–7) for 15 min. D: cells were pretreated for 20 min with 0.2 μM AG-1478 before exposure to Ang-(1–7) for 15 min.
3.4.8  The inhibitory effect of Ang-(1-7) on Ang II-induced phosphorylation of ERK1/2.

It has been reported that Ang-(1-7) inhibits Ang II-induced activation of MAP kinases in proximal tubule cells but the mechanism(s) responsible for this effect have not been fully elucidated. In this regard, in some cell types Gs-alpha coupled receptors can inhibit ERK1/2 activation in a cAMP-PKA-dependent manner by inhibiting Raf-1, upstream of ERK1/2. We first observed that pretreatment with Ang-(1-7) for 30 minutes attenuated the subsequent activation of ERK1/2 in MC (Fig. 3.9A). This effect of Ang-(1-7) was prevented by the PKA inhibitor, H89 (Fig. 3.9B). Dibutryl-cAMP (Db-cAMP), a stable cAMP analogue, reproduced the inhibitory effect of Ang-(1-7) on activation of ERK in a PKA-dependent manner (Fig. 3.9C).
Figure 3.9: Effect of Ang-(1-7) on Ang II-induced activation of ERK1/2 in MC. A. MC cells were pretreated $10^{-11}$ M Ang-(1-7) for 30 min, then treated with $10^{-7}$ M Ang II for 5 min. *P<0.01 compared with untreated MC. # P<0.05 compared with Ang II-treated MC. B. MC cells were treated with $10^{-7}$ M Ang II for 5 min before and after pretreatment with $10^{-11}$ M Ang-(1-7) (30 min) and/or 10 µM H89 (30 min). *P<0.05 compared with untreated MC; **P<0.001 compared with MC treated with Ang II alone; #P<0.001 compared with MC treated with Ang II and Ang-(1-7). C. SD-MC cells were treated with $10^{-7}$ M Ang II for 5 min before and after pretreatment with 2mM DB-cAMP for 20min or 10 µM H89 alone for 10 min. Values are mean ± SE. * P<0.01 compared with untreated MC. # P<0.05 compared with MC treated with Ang II alone. Experiments were performed in triplicate.
3.4.9 The inhibitory effect of Ang-(1-7) on Ang II-induced activation of NADPH oxidase.

Ang II-induced activation of NADPH oxidase is upstream of ERK activation. We therefore studied the effect of Ang-(1-7) on Ang II-induced NADPH oxidase activation, measured by lucigenin enhanced chemiluminescence, as described in the Methods. Pre-treatment of MC with Ang-(1-7) blocked Ang-II induced activation of NADPH oxidase (Fig. 3.10), and this effect was also cAMP and PKA dependent because treatment with either the adenylyl cyclase inhibitor, SQ22536 or the PKA inhibitor, H89, prevented the inhibitory actions of Ang-(1-7) (Fig. 3.10).
Figure 3.10: cAMP inhibitor SQ22536 and PKA inhibitor H89 on the effect of Ang-(1-7) on Ang II-induced NADPH oxidase activity. SD-MC cells were treated with Ang II (10^{-7} M) alone or Ang II (10^{-7} M) 30 minutes after pretreatment with Ang-(1-7) (10^{-9} M). SD-MC were also treated with SQ22536 (0.1 mM) or H89 (1 µM, 18 hours) prior to the addition of Ang-(1-7) and Ang II. NADPH oxidase activity was measured by lucigenin enhanced chemiluminescence assay after 18 hours. Values are mean ± SE. *p<0.001 compare to untreated MC; **p<0.001 compare to MC treated with Ang II along; #p<0.005 compare to MC treated with Ang-(1-7) and Ang II. The data are representative of three separate experiments.
3.5 DISCUSSION

The RAS plays a key role in both renal physiology and the pathogenesis of chronic kidney disease. All components of the RAS are expressed in the kidney and ANG II is generated at higher levels in the kidney than in the systemic circulation. The recent discovery of ACE2 has refocused attention on angiotensin peptide processing and the generation of other biologically relevant angiotensin peptides. ACE2 is highly expressed in the kidney and generates Ang-(1–7). In vivo studies suggest that Ang-(1–7) may exert a protective effect in the kidney. For example, Giani et al. 251 showed that Ang-(1–7) reduced proteinuria and renal fibrosis in hypertensive rats. Similarly, Zhang et al. 256 found that Ang-(1–7) infusion attenuated glomerulosclerosis in rats, and more recently attenuated kidney injury in mice with type 2 diabetes mellitus 266. However, the signal transduction pathways downstream of Ang-(1–7) in kidney cells have not been fully elucidated.

Our first major observation was that Ang-(1–7) activated ERK1/2 in SD-MC in a mas receptor/cAMP/PKA-dependent manner. Although MCs express the G protein-coupled receptor mas, it has been reported that Ang-(1–7) can interact with the AT1 receptor in rat heart and renal cortex 267, 268, and more recently that Ang-(1–7) exhibits modest affinity for the AT2 receptor 269. However, we found that activation of ERK1/2 was downstream of the mas receptor because the mas receptor antagonist A-779 prevented ERK1/2 phosphorylation while neither the AT1 receptor blocker losartan nor the AT2 receptor antagonist PD123319 had an effect on ERK1/2 phosphorylation, in accord with previous studies 257. Given the above reports on Ang-(1–7)/AT1 receptor interactions we also determined that the dose of losartan, we used inhibited ANG II-induced activation of ERK1/2.

We consistently observed a stronger intensity for phospho-ERK2 (p42 MAPK) immunostaining than for phospho-ERK1 (p44 MAPK) immunostaining following treatment with Ang-(1–7) and ANG II in the Western blot analyses. Although there is evidence suggesting different biologic roles for ERK1 and ERK2 270-272, it is widely accepted that ERK1 and ERK2 are regulated in a similar manner. In addition, despite the difference in the intensity of the signals for phospho-ERK1 and phospho-ERK2, the responses to our experimental manoeuvres paralleled one another. We therefore did not distinguish between ERK1 and ERK2 in the presentation of our data.
A number of signal transduction pathways link G protein-coupled receptors to the activation of ERK1/2. One central pathway utilized by the AT1 receptor is NADPH oxidase activation and several studies have shown that inhibition of NADPH oxidase attenuates AT1R-dependent activation ERK1/2. In addition, transactivation of the EGFR by ANG II/AT1 is also an important determinant of ANG II-dependent activation of ERK1/2. We therefore studied the effect of two inhibitors of NADPH oxidase and two inhibitors of EGFR kinase activity and found that while these reagents effectively inhibited ANG II-induced activation of ERK1/2, there was no effect on Ang-(1–7)-induced ERK1/2 activation. These findings suggest that neither NADPH oxidase nor transactivation of the EGFR plays a role in the signal transduction pathway linking Ang-(1–7)/mas receptor to ERK1/2, unlike ANG II/AT1 receptor cell signaling, and that there is a fundamental difference in the mechanisms linking these two peptide ligands and their cognate receptors to the activation of ERK1/2.

Initially, we did not expect that cAMP/PKA signaling would play a role in the activation of ERK1/2 because Gs-α-coupled receptors inhibit ERK1/2 activation in many cell types, due at least in part to a cAMP/PKA-dependent inhibition of the serine threonine kinase Raf-1 that is upstream of MEK and ERK1/2. Moreover, there have been conflicting reports on the effect of Ang-(1–7) on cAMP levels in kidney cells in vitro. However, it has also been reported that ERK1/2 is activated in neuronal cells by Gs-α-coupled receptors. Although the signal transduction pathway linking Gs-coupled receptors to ERK1/2 is not well-defined, it may proceed through cAMP, PKA, and MEK because other studies have implicated a role for the GTP-binding Ras and Raf-1. Our second major observation was that inhibition of adenylyl cyclase attenuated Ang-(1–7)-induced activation of ERK1/2. We therefore measured cAMP levels in our SD-MC and we found a modest increase that was prevented by pretreatment of the cells with the mas receptor antagonist A-779 and the adenylyl cyclase inhibitor SQ22536. These novel findings suggest that the mas receptor is coupled to Gs-α and that ligand binding activates Gs-α and its effector molecule adenylyl cyclase.

The Ang-(1–7)-induced increase in cAMP levels was also associated with an increase in PKA activity in the SD-MC. However, cAMP can lead to an increase in ERK1/2 independent of PKA, an effect that may be mediated by cAMP-activated guanine-nucleotide exchange factors (cAMP-GEFs, also known as Epacs). This pathway has been described in kidney cells. We next treated our SD-MC with two different PKA inhibitors before exposure to Ang-(1–7) to determine
whether ERK1/2 activation was dependent on PKA. Both compounds inhibited the Ang-(1–7)-induced ERK1/2 phosphorylation. Taken together with our finding that a stable cAMP analog activated ERK1/2 in the SD-MC in a PKA- and MEK-dependent manner, these findings support the conclusion that Ang-(1–7) signals to ERK1/2 via cAMP/PKA and not via cAMP-GEFs. Finally, we compared the effect of PKA inhibition and EGFR inhibition on the activation and nuclear translocation of ERK1/2 by confocal microscopy and fluorescence imaging. In accord with the Western blot analyses, PKA inhibition but not EGFR kinase inhibition prevented nuclear translocation of ERK1/2.

Su and colleagues have shown that Ang-(1-7) inhibits Ang II-induced activation of MAPKs in renal proximal tubule cells, and a similar effect of Ang-(1-7) has also been reported in human endothelial cells. The mechanisms responsible for this inhibition of Ang II signaling by Ang-(1-7) has not been fully elucidated. We extended these observations by studying MC, and we found that pre-treatment with Ang-(1-7) also attenuated the activation of ERK1/2 by Ang II in these cells. We also observed that this interaction was dependent on the activation of PKA by Ang-(1-7). In addition, treatment of the MC with a stable cAMP analogue was also sufficient to inhibit Ang II-induced phosphorylation of ERK1/2 by Ang II, supporting the conclusion that Ang-(1-7) induced cAMP/PKA signaling, while responsible for activating ERK1/2 is also responsible for the subsequent inhibition of Ang II activation of ERK1/2.

The interaction between RAS and NADPH oxidase has been shown to play an important role in many pathological settings. We have reported that Ang II-induced activation of NADPH oxidase is inhibited by Ang-(1-7) in vitro and more recently Ang-(1-7) infusion has been reported to reduce NADPH oxidase activity in the kidneys of mice with experimental type 2 diabetes mellitus. In this regard, our final observation is that this effect is dependent on cAMP/PKA signaling. Taken together with our analyses of ERK1/2 activation, this finding suggests that the mechanism responsible, at least in part, for the attenuation of Ang II signaling by Ang-(1-7) may be inhibition of NADPH oxidase because compounds that inhibit NADPH oxidase also prevent the activation of ERK1/2 by Ang II. In addition, it remains possible that PKA-mediated phosphorylation and inhibition of Raf-1 may contribute to decreased ERK1/2 phosphorylation.
We believe that these findings have some relevance to experimental models of kidney injury, especially with respect to a protective role of Ang-(1–7). Infusion of Ang-(1–7) attenuates experimental glomerulonephritis and diabetic nephropathy and the inhibition of NADPH oxidase and ERK1/2 may contribute to these beneficial effects. On the other hand, it is difficult to reconcile these findings with reports that Ang-(1–7) promoted TGF-β1 and extracellular matrix protein production in MC in vitro and contributed to epithelial-to-mesenchymal transformation. It may be that there are model- and cell-specific effects that account for reports that suggest that Ang-(1–7) is deleterious. In support of this notion is the recent report that infusion of Ang-(1–7) does not improve or worsen glomerular injury and function in mice subjected to partial renal ablation. Further studies are necessary to better define the role of Ang-(1–7) in kidney injury.

In summary, these studies show that Ang-(1–7) activates ERK1/2 in SD-MC in a mas receptor/cAMP/PKA/MEK-dependent manner. These findings extend our understanding of the signal transduction pathways activated by Ang-(1–7) in MC, especially with respect to ERK1/2, and these interaction(s) may account for the observed protective effect of Ang-(1–7) in some experimental models of kidney injury.
Chapter 4

Deletion of $p47^{phox}$ Attenuates the Progression of Diabetic Nephropathy and Reduces the Severity of Diabetes in the Akita Mouse

Excerpts from the chapter were published and reproduced from the following journal:

4.1 ABSTRACT

4.1.1 Aims/hypothesis

Reactive oxygen species (ROS) contribute to diabetes-induced glomerular injury and endoplasmic reticulum (ER) stress-induced beta-cell dysfunction but the source of ROS has not been fully elucidated. Our aim was to determine if p47phox-dependent activation of NADPH oxidase is responsible for hyperglycemia-induced glomerular injury in the Akita mouse, a model of type 1 diabetes mellitus due to ER stress-induced beta-cell dysfunction.

4.1.2 Methods

We examined the effect of deletion of the gene for the NADPH oxidase subunit p47phox on diabetic nephropathy in the Akita mouse (Ins2WT/C96Y) by studying four groups of mice: 1) Non-diabetic mice (Ins2WT/WT/p47phox+/-); 2) Non-diabetic p47phox-null mice (Ins2WT/WT/p47phox-/-); 3) Diabetic mice (Ins2WT/C96Y/p47phox+/-); and 4) Diabetic, p47phox-null mice (Ins2WT/C96Y/p47phox-/-). We measured the urinary albumin excretion rate, oxidative stress, mesangial matrix expansion, plasma and pancreatic insulin concentrations in 16 week-old mice; glucose tolerance test and insulin sensitivity, islet and glomerular NADPH oxidase activity and subunit expression, and pro-fibrotic gene expression in 8 week-old mice. We measured NADPH oxidase activity, subunit expression, and pro-fibrotic gene expression in high glucose-treated murine mesangial (MC) cells. The relationship between p47phox and collagen Iα1 mRNA expression was determined in kidney biopsies from normal and diabetic subjects.

4.1.3 Results

Deletion of p47phox reduced kidney hypertrophy, albuminuria, oxidative stress, and mesangial matrix expansion but also reduced hyperglycemia by increasing pancreatic and circulating insulin concentrations. p47phox-/- mice exhibited improved glucose tolerance but modestly decreased insulin sensitivity. Deletion of p47phox attenuated high glucose-induced activation of
NADPH oxidase and pro-fibrotic gene expression in glomeruli and MC. There was a positive correlation between \( p47^{phox} \) and collagen Iα1 mRNA levels in renal biopsy samples from control subjects and subjects with diabetic nephropathy.

4.1.4 Conclusions/interpretation

Deletion of \( p47^{phox} \) attenuates diabetes-induced glomerular injury and beta-cell dysfunction in the Akita mouse.

4.2 INTRODUCTION

High glucose-induced generation of reactive oxygen species (ROS) is an important contributor to the pathogenesis of diabetic nephropathy \(^{278-285}\). Although superoxide can be generated from mitochondrial electron transport, uncoupled eNOS activity, or activation of the family of NADPH oxidase (NOX) enzymes \(^{286-289}\), the contribution of each of these pathways to kidney injury has not been fully elucidated.

Several isoforms of NADPH oxidase (NOX) have been identified in the kidney including NOX1, NOX2, and NOX4 \(^{286}\). Activation of membrane-bound NOX isoforms, NOX1 and NOX2, is dependent on recruitment and phosphorylation of several cytosolic subunits, including \( p47^{phox} \) \(^{290-292}\), and \( p47^{phox} \) has been implicated in the generation of superoxide in rat mesangial cells under high glucose conditions \(^{293}\). Apocynin, a non-specific antioxidant that targets \( p47^{phox} \), reduces albuminuria and mesangial expansion in diabetic mice \(^{294-297}\). \( p47^{phox} \)-independent NOX4 has also been implicated in diabetes-induced oxidative stress in the glomerulus and proximal tubule \(^{298-301}\). Therefore, although NOX-induced ROS may play a role in the progression of diabetic nephropathy, the role of specific NOX isoforms and the cytosolic subunits that regulate their activation remains uncertain.

Accordingly, we examined the effect of deletion of the gene for the NADPH oxidase subunit, \( p47^{phox} \), on diabetic kidney injury in the Akita mouse model of type 1 diabetes mellitus to test the hypothesis that \( p47^{phox} \)-dependent activation of NOX is an important determinant of experimental diabetic nephropathy.
4.3 RESEARCH DESIGN AND METHODS

4.3.1 Animals.

Four groups of male mice were studied: 1) Non-diabetic mice (Ins2\textsuperscript{WT/WT}/p47\textsuperscript{phox+/-} (NDWT)); 2) Non-diabetic \textit{p}47\textit{phox}-null mice (Ins2\textsuperscript{WT/WT}/\textit{p}47\textit{phox}-/- (NDKO)); 3. Diabetic Akita mice (Ins2\textsuperscript{WT/C96Y}/\textit{p}47\textit{phox+/-} (DMWT)); and 4) Diabetic \textit{p}47\textit{phox}-null mice (Ins2\textsuperscript{WT/C96Y}/\textit{p}47\textit{phox}-/- (DMKO)). All mice were on C57BL/6 background and provided with free access to water and standard 18% protein rodent chow. Blood glucose levels and body weights were measured weekly from 4 to 16 weeks of age. Albumin excretion rates were determined from 24-hour urine samples at age 8 and 16 weeks of age using Albuwell M ELISA kits (Exocell Inc. Philadelphia, PA, USA). Systolic blood pressure was measured as previously described\textsuperscript{316,317}. At 8 and 16 weeks kidney were fixed in 10% formalin (wt./vol.) and/or snap frozen. Pancreatic tissue was placed into 10 ml of Acid-Ethanol (1.5% hydrochloric acid in 70% ethanol) for 18 hours at -20°C. The tissue was homogenized, incubated overnight at -20°C, and then centrifuged at 200 g for 15 minutes at 4°C. The aqueous layer was transferred into a 15 ml tube. 100 µl of Acid-Ethanol extract was neutralized with 100 µl of 1 mol/l Tris at pH 7.5. Insulin content was measured with a rat insulin RIA kit that cross-reacts with mouse insulin (Linco Research Inc., St. Charles, MO, USA). Total protein was measured by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). NADPH oxidase was measured in tissue and cells as previously described\textsuperscript{318}. All procedures were conducted in accordance with the guidelines of the University of Toronto Animal Care Committee.

4.3.2 Primary mouse mesangial cell culture.

Mesangial cells were isolated as previously described\textsuperscript{316}. Cells were maintained in serum free medium for 18 hours then treated with 5.6 mmol/l or 30 mmol/l D-glucose for 16 hours.
4.3.3 Glomeruli isolation.

8 week-old mice were anesthetized by isoflurane and perfused with 10 ml PBS containing 200 µl Dynabeads M-450 (4 x 10^8 beads/ml. Invitrogen Dynal AS, Oslo, Norway) through the left ventricle. Both kidneys were harvested, minced with a razor blade, and incubated with 10 ml HBSS containing 0.01 g of collagenase A (Roche Diagnostics, Indianapolis, IN, USA) and 7.5 µl Deoxyribonuclease I (Invitrogen, Carlsbad, CA, USA) at 37°C for 30 minutes. After incubation, samples were passed through a 100 µm cell strainer (BD Falcon, BD Biosciences, Bedford, MA, USA), washed with 10 ml ice-cold HBSS and then centrifuged at 200 g, 4°C for 5 minutes. Glomeruli were collected with a magnetic particle concentrator and stored at -80°C until use.

4.3.4 Renal histology.

Frozen kidney tissue sections (10µm thick) were incubated with dihydroethidium (DHE) (2 µmol/l; Molecular Probes, Invitrogen Canada Inc. Burlington, ON, Canada) at 37°C for 1 hour and scanned with a Zeiss confocal laser-scanning microscope (LSM 510, Dusseldorf, Germany). The image color intensity of DHE stained kidney sections was scored blindly on a scale of 0 to 4 (0 for dark and 4 for the strongest intensity). Formalin-fixed paraffin-embedded kidneys were sectioned and stained with Periodic Acid Schiff (PAS), Picrosirius Red, and Masson's Trichrome reagents as previously described. Mouse glomeruli (approximately 60 to 100) were scored blindly for severity of diabetic glomerulosclerosis in PAS-stained sections by a nephropathologist. Each glomerulus was given a score of 0 (normal), 1 (mild, mesangial matrix (MM) increase approximately 2 times the width of a mesangial cell nucleus), 2 (moderate, MM increase approximately 3 to 4 times the width of a mesangial cell nucleus), or 3 (severe, MM increase >4 times the width of a mesangial cell nucleus). The mean glomerular MM score was then calculated for each animal. Glomerular volume was calculated using ImageScope software (Aperio, Vista, CA, USA) from scanned PAS slides. Formalin-fixed paraffin-embedded sections were used for immunohistochemical analysis. Anti-collagen I primary antibody was from Cedarlane (1:20, Cedarlane, Burlington, ON, Canada). All slides were scanned digitally at the Advanced Optical Microscope Facility (Princess Margaret Hospital, Toronto, ON, Canada) and Aperio ImageScope software was used to quantify collagen I immunostaining.
4.3.5  WT-1 staining and podocyte quantitation.

De-paraffinized mouse kidney slides were blocked with serum for 20 minutes at room temperature and then incubated with WT-1 antibody (1:200 in 1% BSA, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and then incubated with anti-rabbit IgG antibody (Vectastain ABC Kit, Vector Laboratories Inc., Burlington, ON, Canada). All slides were scanned digitally at the Advanced Optical Microscope Facility and AperioImageScope software (Aperio Technologies Inc., Vista, CA) was used to count WT-1 positive nuclei in the glomerular profiles.

4.3.6  Quantitative real-time PCR.

RNA was extracted from isolated glomeruli and cultured primary mouse mesangial cells using RNeasy Mini kit (Qiagen Inc. Canada, Mississauga, ON, Canada). mRNA expression levels for NOX2, NOX4, p47\textsuperscript{phox}, p22\textsuperscript{phox}, p67\textsuperscript{phox}, p40\textsuperscript{phox}, collagen type I α1, collagen type I α2, fibronectin, PAI-1, TGF-β1 and nephrin were quantified by realtime TaqMan PCR using ABI Prism 7900 sequence detection system as previously described\textsuperscript{316}. Specific mouse primer sets were purchased from ABI (Applied Biosystems, Foster City, CA, USA).

4.3.7  Western blot.

Western Blot analysis of protein lysates from isolated glomeruli or mesangial cells was performed as previously described\textsuperscript{316} using primary antibodies for p47\textsuperscript{phox}, NOX2, and β-actin (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA). β-actin was used as loading control and densitometry was measured using Scion Image software (Scion Corp. Frederick, MD, USA).

4.3.8  Mouse islet isolation.

Under isoflurane anesthesia, the common bile duct was clamped at the point where it entered the duodenum. 2 ml of collagenase A (2 mg/ml, Roche Diagnostics, Indianapolis, IN, USA) solution was injected into the common bile duct. The pancreas was removed and placed in a 50 ml tube,
then incubated in a 37°C water bath for 17 minutes. 20 ml of ice-cold HBSS/HEPES solution was added to stop collagenase digestion, prior to centrifugation at 450 g for 1 minute. The pellet was washed with ice-cold HBSS/HEPES, filtered through gauze and centrifuged at 450 g for 1 minute. The pellet was re-suspended in ice-cold HBSS/HEPES. Islets were isolated under a dissecting microscope, transferred to a 35 mm culture dish with medium (RPMI-1640, 10% FBS, 1 mmol/l sodium pyruvate, 11 mmol/l glucose, 2 mmol/l L-glutamate, and 50 U/ml penicillin/streptomycin) and recovered at 37°C overnight.

4.3.9 Intraperitoneal glucose tolerance test (IPGTT) and insulin sensitivity test.

*p47phox* wild-type and -null mice were fasted from 7 a.m. for 6 hours. IPGTT was done according the Animal Models of Diabetic Complications Consortium protocol. 10 µl/g body weight of D-glucose (100 mg/ml) was injected intraperitoneally into the mice. For insulin sensitivity test, 0.75 U/kg body weight of Humulin® R (Eli Lilly and Company, Indianapolis, IN, USA.) was injected intraperitoneally into the animals. Blood glucose values were measured at 0, 5, 15, 30, 60, and 120 minutes using tail venous blood by Contour® Meter (Bayer Inc., Toronto, ON, Canada).

4.3.10 Statistical analysis.

Results are expressed as mean ± SEM unless otherwise specified. Two-tailed Student’s t test was used for comparison between two groups. Comparisons between multiple groups were performed by one-way ANOVA followed by Bonferroni post-hoc test. GraphPad Prism software was used for statistical tests (GraphPad Software, Inc., La Jolla, CA, USA).
4.4 RESULTS

4.4.1 Kidney studies in 16 week-old mice.

Four groups of mice were studied: non-diabetic $p47^{phox}$ wild-type mice ($Ins2^{WT/WT}/p47^{phox+/+}$), non-diabetic $p47^{phox}$-null mice ($Ins2^{WT/WT}/p47^{phox-/-}$), diabetic $p47^{phox}$ wild-type mice ($Ins2^{WT/C96Y}/p47^{phox+/+}$); and diabetic $p47^{phox}$-null mice ($Ins2^{WT/C96Y}/p47^{phox-/-}$). The onset of hyperglycemia occurred between 4 and 6 weeks of age in both of diabetic groups (Fig. 4.1A). Mean values for blood glucose diverged after 10 weeks of age and at 16 weeks were approximately 10 mmol/l greater in $Ins2^{WT/C96Y}/p47^{phox+/+}$ mice compared to $Ins2^{WT/C96Y}/p47^{phox-/-}$ mice (Table 4.1). All mice gained weight over 16 weeks although values for the $Ins2^{WT/WT}/p47^{phox-/-}$ mice tended to be higher (Fig. 4.1B and Table 1). Both groups of diabetic mice exhibited an increase in the urinary albumin excretion rate which was attenuated but not normalized by deletion of $p47^{phox}$ in the $Ins2^{WT/C96Y}/p47^{phox-/-}$ diabetic mice (Fig. 4.1C).
Figure 4.1: Blood glucose, body weight and urinary albumin excretion rate in four groups of mice follow to 16 weeks of age. A. Blood glucose and B. body weight in four groups of mice followed to 16 weeks of age. C. 24 hour urinary albumin excretion in 16 week-old mice. (ND: non-diabetic, DM: diabetic, WT: p47^phox^+/+, KO: p47^phox^-/-; * p < 0.05 compared with non-diabetic groups, # p < 0.05 compared with the diabetic p47^phox^ wild-type group, † p < 0.05 compared with the diabetic p47^phox^-null group, ‡ some values from diabetic p47^phox^+/+ mice
exceeded the measurable range of the glucometer (33.3 mmol/l) therefore were recorded as 33.3 mmol/l on the graph).

**Table 4.1:** Whole animal data and kidney morphology of 16 week-old mice.

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetic</th>
<th>Diabetic</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Ins2&lt;sup&gt;WT/WT&lt;/sup&gt;</em>&lt;sup&gt;p47phox&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td><em>Ins2&lt;sup&gt;WT/WT&lt;/sup&gt;</em>&lt;sup&gt;p47phox&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td><em>Ins2&lt;sup&gt;WT/C96Y&lt;/sup&gt;</em>&lt;sup&gt;/p47phox&lt;sup&gt;&lt;sup&gt;+/+&lt;/sup&gt;&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>BG (mmol/l)</strong></td>
<td>5.48 ± 0.27</td>
<td>5.93 ± 0.30</td>
<td>35.20 ± 1.58&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>BW (g)</strong></td>
<td>26.98 ± 1.54</td>
<td>34.87 ± 1.49&lt;sup&gt;c&lt;/sup&gt;</td>
<td>26.03 ± 0.63</td>
</tr>
<tr>
<td><strong>KW (g)</strong></td>
<td>0.1625 ± 0.0038</td>
<td>0.1899 ± 0.0091</td>
<td>0.2304 ± 0.0105&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>KW / BW</strong></td>
<td>0.00602 ± 0.00011</td>
<td>0.00545 ± 0.00017</td>
<td>0.00885 ± 0.00049&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Glomerular Volume (µm³)</strong></td>
<td>170100 ± 5296</td>
<td>149129 ± 5681</td>
<td>260944 ± 16863&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Mesangial Matrix Score</strong></td>
<td>1.00 ± 0.39</td>
<td>1.98 ± 0.71</td>
<td>6.70 ± 1.64&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>UAE (µg/24hr)</strong></td>
<td>15.64 ± 1.61</td>
<td>26.69 ± 2.48</td>
<td>111.0 ± 8.98&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM.


<sup>a</sup> p < 0.05 compared with non-diabetic groups.
b p < 0.05 compared with the diabetic \( p47^{phox} \) wild-type group.

c p < 0.05 compared with all other groups.
At 16 weeks of age, both diabetic groups exhibited greater kidney/body (KW/BW) weight ratios compared to their non-diabetic littermates (Table 4.1). Both kidney hypertrophy and the KW/BW ratios were reduced by deletion of $p47^{phox}$ in the diabetic mice ($Ins2^{WT/C96Y/p47^{phox}+/+}$ mice). Deletion of $p47^{phox}$ also attenuated diabetic glomerular hypertrophy (Table 4.1).

Oxidative stress in glomeruli was assessed in the four groups of mice using dihydroethidium (DHE) staining to detect superoxide (Fig. 4.2A-D). DHE staining was increased three-fold in the glomeruli of $Ins2^{WT/C96Y/p47^{phox}+/+}$ mice compared to $Ins2^{WT/WT/p47^{phox}+/+}$ mice, and the increase was attenuated by deletion of $p47^{phox}$ (Fig. 4.2E). Increased oxidative stress was associated with a significant increase in the mesangial matrix score in the glomeruli of $Ins2^{WT/C96Y/p47^{phox}+/+}$ mice compared to $Ins2^{WT/WT/p47^{phox}+/+}$ mice (Fig. 4.3 and Table 4.1). In parallel with the mesangial matrix score, glomerular collagen I immunostaining was increased in the $Ins2^{WT/C96Y/p47^{phox}+/+}$ mice compared to the $Ins2^{WT/WT/p47^{phox}+/+}$ mice and reduced by deletion of $p47^{phox}$ (Fig. 4.4E). There were no differences in the number of glomerular WT1-positive cells in the four groups of mice (Fig. 4.4J), and no significant differences in the glomerular basement membrane thickness across the four groups (Fig. 4.5). Renal cortical nephrin mRNA expression was similar in all four groups (Fig. 4.6).
Figure 4.2: Renal superoxide levels in four groups of 16 week-old mice. Sections of 16 week-old mouse kidneys were stained with dihydroethidium to detect superoxide levels. (A-D) Representative images from each group are shown. (E) The intensity of emission from dihydroethidium stained sections was scored for each of the four groups of mice. (ND: non-diabetic, DM: diabetic, WT: \(p47^{phox+/+}\), KO: \(p47^{phox-/-}\); * \(p < 0.05\) compared with non-diabetic groups, † \(p < 0.05\) compared with the diabetic \(p47^{phox}\) wild-type group).
Figure 4.3: Mesangial expansion in four groups of 16 week-old mice. Sections of 16 week-old mouse kidneys were stained with Periodic Acid Schiff (A-D), Picrosirius Red (E-H), and Masson's Trichrome (I-L) reagents. Representative images showing glomeruli from each group: (A, E, I) non-diabetic, p47phox wild-type; (B, F, J) non-diabetic, p47phox-null; (C, G, K) diabetic, p47phox wild-type; (D, H, L) diabetic, p47phox-null; (M) mesangial matrix score derived from four groups of 16 week-old mice. (ND: non-diabetic, DM: diabetic, WT: p47phox+/+, KO: p47phox−/−; * p < 0.05 compared with non-diabetic groups, † p < 0.05 compared with the diabetic p47phox wild-type group).
Figure 4.4: Collagen I protein expression and podocyte number in four groups of 16 week-old mice. Sections of 16 week-old mouse kidneys were stained with anti-collagen I and anti-WT-1 primary antibodies, representative images showing glomeruli from each group. (A, F) non-diabetic, $p47^{phox}$ wild-type; (B, G) non-diabetic, $p47^{phox}$-null; (C, H) diabetic, $p47^{phox}$ wild-type; (D, I) diabetic, $p47^{phox}$-null; (E) Semi-quantitative analysis of collagen positive area from kidney sections stained with collagen I primary antibody; (J) number of WT-1 positive cells per glomerular profile (open bars = non-diabetic mice; closed bars = diabetic mice; * p < 0.05 compared with non-diabetic groups; # p < 0.05 compared with diabetic $p47^{phox}$ wild-type group).
Figure 4.5: Glomerular basement membrane thickness measured in four groups of 16 week-old mice. (DM: diabetic, WT: $p47^{phox}+/+$, KO: $p47^{phox}$−/−).

Figure 4.6: Nephrin mRNA expression in kidney cortex of four groups of 16 week-old mice (DM: diabetic, WT: $p47^{phox}+/+$, KO: $p47^{phox}$−/−).
4.4.2 The effect of deletion of $p47^{phox}$ on plasma insulin concentration and pancreatic insulin content in 16 week-old diabetic mice.

Blood glucose levels were significantly lower in diabetic mice with a deletion of $p47^{phox}$ compared to the $\text{Ins}^{\text{WT/C96Y}}/p47^{\text{phox}+/+}$ diabetic mice (Fig. 4.7A and Table 4.1), a difference that emerged at 10 weeks of age (Fig. 4.1A). Plasma insulin levels and pancreatic insulin content were both significantly greater in the diabetic mice with a deletion of $p47^{phox}$ suggesting that beta-cell function was better preserved in these mice at 16 weeks of age (Fig. 4.7B and C).
Figure 4.7: Blood glucose, plasma insulin, and pancreatic insulin contents in two groups of 16 week-old diabetic mice. Blood glucose (A), plasma insulin (B), and pancreatic insulin (C) contents in two groups of diabetic mice at 16 weeks of age. * p < 0.05 compared with the $p47^{phox}$ wild-type group.
4.4.3 The effect of deletion of $p47^{phox}$ on beta-cell function and insulin sensitivity in non-diabetic mice.

In order to test the hypothesis that deletion of $p47^{phox}$ might maintain better beta-cell function we studied 8 week-old non diabetic $Ins2^{WT/WT}/p47^{phox+/+}$ and $Ins2^{WT/WT}/p47^{phox-/}$ mice. We found that mice with a deletion of $p47^{phox}$ exhibited significantly better glucose tolerance than their wild-type littermates (Fig. 4.8B), though insulin sensitivity of $p47^{phox}$-null mice was slightly lower compared to wild-type mice (Fig. 4.8C). We then isolated pancreatic islets from the four groups of mice and measured NADPH oxidase activity. $Ins2^{WT/C96Y}$ mutation increased NADPH oxidase activity three to four-fold in the isolated islets of $Ins2^{WT/C96Y}/p47^{phox+/+}$ mice. Deletion of $p47^{phox}$ markedly attenuated this response (Fig. 4.8D).
Figure 4.8: Plasma insulin, intraperitoneal glucose tolerance test, insulin sensitivity test, and islets NADPH oxidase activity of 8 week-old mice. Plasma insulin (A), intraperitoneal glucose tolerance test (B), insulin sensitivity test (C), and islets NADPH oxidase activity (D) of 8 week-old mice (A-C: non-diabetic groups; D: islets were isolated from all four groups; Ins2: insulin 2 gene; WT: $p47^{phox+/+}$, KO: $p47^{phox-/-}$; * $p < 0.05$ compared with the $p47^{phox}$ wild-type group,‡ $p < 0.05$ compared with all other groups).
4.4.4 Kidney studies in 8 week-old mice.

To determine the effect of deletion of $p47^{phox}$ on the kidney response to hyperglycemia we studied another four groups of mice at 8 weeks of age when there were no differences in the blood glucose levels (Fig. 4.9A and Table 4.2). Urinary albumin excretion rates were increased to a similar extent in both of the diabetic groups; mean values for systolic blood pressure were also similar in the four groups (Table 4.2). There were no differences in BW between the groups although the diabetic groups both exhibited an increase in KW and KW/BW ratios (Table 4.2). Mean values for glomerular volume tended to be greater in the diabetic groups and there were early (but not significant) increases in mesangial matrix scores in both of the diabetic groups compared to the non-diabetic groups (Table 4.2). NADPH oxidase activity was increased twofold in isolated glomeruli from the $Ins2^{WT/C96Y/p47^{phox}++}$ mice and attenuated by deletion of $p47^{phox}$ (Fig. 4.10A). DHE staining was also increased in the $Ins2^{WT/C96Y/p47^{phox}++}$ mice compared to the $Ins2^{WT/C96Y/p47^{phox}---}$ mice (Fig. 4.10B-E).
Figure 4.9: Blood glucose and body weight in four groups of 8 week-old mice. Blood glucose (A), and body weight (B) in four groups of mice followed to 8 weeks of age. (ND: non-diabetic, DM: diabetic, WT: $p47^{phox+/+}$, KO: $p47^{phox-/-}$; * $p < 0.05$ compared with non-diabetic groups, # $p < 0.05$ compared with the diabetic $p47^{phox}$ wild-type group, † $p < 0.05$ compared with the diabetic $p47^{phox}$-null group).
Table 4.2: Whole animal data and kidney morphology of 8 week-old mice.

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetic</th>
<th>Diabetic</th>
<th>Diabetic</th>
<th>Diabetic</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$Ins2^{WT/WT/p47^{phox+}}$</td>
<td>$Ins2^{WT/WT/p47^{phox-}}$</td>
<td>$Ins2^{WT/C96Y/p47^{phox+}}$</td>
<td>$Ins2^{WT/C96Y/p47^{phox-}}$</td>
</tr>
<tr>
<td>BG (mmol/l)</td>
<td>6.73 ± 0.34</td>
<td>5.71 ± 0.25</td>
<td>30.73 ± 0.79a</td>
<td>29.05 ± 1.06a</td>
</tr>
<tr>
<td>BW (g)</td>
<td>24.68 ± 0.53</td>
<td>28.03 ± 0.97</td>
<td>22.61 ± 0.90a</td>
<td>22.44 ± 1.22a</td>
</tr>
<tr>
<td>KW (g)</td>
<td>0.1419 ± 0.0038</td>
<td>0.1863 ± 0.0089</td>
<td>0.2301 ± 0.0153a</td>
<td>0.2282 ± 0.0221a</td>
</tr>
<tr>
<td>KW / BW</td>
<td>0.00575 ± 0.00017</td>
<td>0.00665 ± 0.00043</td>
<td>0.01018 ± 0.00065a</td>
<td>0.01017 ± 0.00050a</td>
</tr>
<tr>
<td>Glomerular Volume (µm3)</td>
<td>146556 ± 6341</td>
<td>158184 ± 10022</td>
<td>167433 ± 7932</td>
<td>174291 ± 8958</td>
</tr>
<tr>
<td>Mesangial Matrix Score</td>
<td>1.00 ± 0.05</td>
<td>1.13 ± 0.03</td>
<td>1.24 ± 0.04a</td>
<td>1.29 ± 0.03a</td>
</tr>
<tr>
<td>UAE (µg/24hr)</td>
<td>12.31 ± 1.43</td>
<td>14.32 ± 1.12</td>
<td>61.51 ± 5.64a</td>
<td>64.80 ± 3.60a</td>
</tr>
<tr>
<td>BP (mmHg)</td>
<td>111.4 ± 7.6</td>
<td>116.0 ± 5.1</td>
<td>120.2 ± 6.9</td>
<td>108.0 ± 1.4</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM.


$^a$ p < 0.05 compared with non-diabetic groups.
Figure 4.10: NADPH oxidase activity and superoxide concentration in isolated glomeruli from 8 week-old mice. (A) NADPH oxidase activity of isolated glomeruli measured by a lucigenin chemiluminescence method. (B-E) Sections of 8 week-old mouse kidneys were stained with dihydroethidium to detect superoxide levels. Representative images from each group are shown. (ND: non-diabetic, DM: diabetic, WT: $p47^{phox+/+}$, KO: $p47^{phox-/-}$; ‡ p < 0.05 compared with all other groups).
4.4.5  NADPH oxidase subunit expression in isolated glomeruli.

NADPH oxidase is a multiunit complex so we looked at mRNA expression of the NADPH oxidase subunits in isolated glomeruli from the four groups of mice (Table 4.3). Hyperglycemia-induced activation of NADPH oxidase was associated with significant increases in expression of $p47^{phox}$, NOX2, $p22^{phox}$, $p40^{phox}$, and $p67^{phox}$ in the glomeruli of $Ins2^{WT/C96Y}/p47^{phox}+/+$ mice compared to $Ins2^{WT/WT}/p47^{phox}+/+$ mice. Mean values for NOX4 mRNA levels tended to increase but the difference did not reach statistical significance. Interestingly, deletion of $p47^{phox}$ attenuated the increase in expression of NOX2, $p22^{phox}$, $p40^{phox}$, and $p67^{phox}$ induced by hyperglycemia but did not affect NOX4 mRNA expression. We then related changes in mRNA to protein expression for $p47^{phox}$ and NOX2 in isolated glomeruli from the four groups of mice. Western Blot analysis showed $p47^{phox}$ and NOX2 protein expression paralleled the changes in mRNA levels (Fig. 4.11A-C).
**Table 4.3:** NADPH oxidase subunits, extracellular matrix proteins and pro-fibrotic markers mRNA expression of glomeruli isolated from 8 week-old mice.

<table>
<thead>
<tr>
<th></th>
<th>Non-diabetic</th>
<th>Diabetic</th>
<th>Diabetic</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Ins2</em>&lt;sup&gt;WT/WT&lt;/sup&gt;/p47&lt;sup&gt;pox&lt;/sup&gt;+/+</td>
<td><em>Ins2</em>&lt;sup&gt;WT/WT&lt;/sup&gt;/p47&lt;sup&gt;pox&lt;/sup&gt;-/-</td>
<td><em>Ins2</em>&lt;sup&gt;WT/C96Y&lt;/sup&gt;/p47&lt;sup&gt;pox&lt;/sup&gt;+/+</td>
<td><em>Ins2</em>&lt;sup&gt;WT/C96Y&lt;/sup&gt;/p47&lt;sup&gt;pox&lt;/sup&gt;-/-</td>
</tr>
<tr>
<td><strong>p47&lt;sup&gt;pox&lt;/sup&gt;</strong></td>
<td>1.00 ± 0.09</td>
<td>non-detectable</td>
<td>2.85 ± 0.76&lt;sup&gt;a&lt;/sup&gt;</td>
<td>non-detectable</td>
</tr>
<tr>
<td><strong>NOX2</strong></td>
<td>1.00 ± 0.14</td>
<td>1.01 ± 0.11</td>
<td>2.24 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.37 ± 0.13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>NOX4</strong></td>
<td>1.00 ± 0.03</td>
<td>0.88 ± 0.09</td>
<td>1.30 ± 0.29</td>
<td>1.47 ± 0.28</td>
</tr>
<tr>
<td><strong>p22&lt;sup&gt;pox&lt;/sup&gt;</strong></td>
<td>1.00 ± 0.08</td>
<td>0.82 ± 0.12</td>
<td>1.71 ± 0.42</td>
<td>0.94 ± 0.12</td>
</tr>
<tr>
<td><strong>p40&lt;sup&gt;pox&lt;/sup&gt;</strong></td>
<td>1.00 ± 0.19</td>
<td>1.20 ± 0.18</td>
<td>3.99 ± 0.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.21 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>p67&lt;sup&gt;pox&lt;/sup&gt;</strong></td>
<td>1.00 ± 0.10</td>
<td>1.09 ± 0.10</td>
<td>4.53 ± 0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.89 ± 0.27&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Collagen <em>Ia1</em></td>
<td>1.00 ± 0.11</td>
<td>1.10 ± 0.18</td>
<td>2.71 ± 0.60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.25 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Collagen <em>Ia2</em></td>
<td>1.00 ± 0.13</td>
<td>0.87 ± 0.14</td>
<td>4.70 ± 1.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.30 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>1.00 ± 0.12</td>
<td>1.07 ± 0.23</td>
<td>3.58 ± 0.51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.60 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>1.00 ± 0.08</td>
<td>1.22 ± 0.14</td>
<td>4.76 ± 0.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.67 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PAI-1</td>
<td>1.00 ± 0.15</td>
<td>0.97 ± 0.21</td>
<td>3.27 ± 0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.11 ± 0.16&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM.

<sup>a</sup> p < 0.05 compared with non-diabetic groups.

<sup>b</sup> p < 0.05 compared with the diabetic *p47<sup>pox</sup>* wild-type group.
Figure 4.11: Protein expression of p47^{phox} and NOX2 in glomeruli isolated from four groups of mice. Glomeruli were isolated from 8 week-old mice, protein expression levels of p47^{phox} and NOX2 were determined by Western blot. (A) Representative Western blot of p47^{phox}, NOX2, and β-actin; (B, C) Quantitative densitometry analysis of Western blot for p47^{phox} (B) and NOX2 (C) (ND: non-diabetic, DM: diabetic, WT: p47^{phox}+/+, KO: p47^{phox}−/−; N. D.: non-detectable; * p < 0.05 compared with the non-diabetic p47^{phox} wild-type group, ‡ p < 0.05 compared with all other groups).
4.4.6  Pro-fibrotic gene expression in isolated glomeruli.

In order to relate these early effects of deletion of \( p47^{phox} \) on NADPH oxidase activation to pro-fibrotic gene expression we measured mRNA levels for collagen Iα1, collagen Iα2, fibronectin, transforming growth factor-β1 (TGF-β1), and plasminogen activator inhibitor -1 (PAI-1) in isolated glomeruli. Mean values for these pro-fibrotic genes were increased in the glomeruli of \( \text{Ins2}^{WT/C96Y}/p47^{phox+/+} \) mice compared to both the \( \text{Ins2}^{WT/WT}/p47^{phox+/+} \) mice and the \( \text{Ins2}^{WT/C96Y}/p47^{phox-/-} \) mice (Table 4.3).

4.4.7  Studies on NADPH oxidase activation in primary mouse mesangial cells.

Our \textit{in vivo} data showed that diabetic nephropathy was attenuated by deletion of \( p47^{phox} \). In order to relate this finding to a cellular response to high glucose we studied primary mesangial cells derived from \( \text{Ins2}^{WT/WT}/p47^{phox+/+} \) mice and \( \text{Ins2}^{WT/WT}/p47^{phox-/-} \) mice. Primary mesangial cells were exposed to 5.6 mmol/l or 30 mmol/l D-glucose. 30 mmol/l D-glucose increased NADPH oxidase activity two-three fold in wild-type mesangial cells from \( \text{Ins2}^{WT/WT}/p47^{phox+/+} \) mice (Fig.4.12A); this effect was not due to an osmotic stimulus (Fig.4.12B) and the high glucose-induced increase in NADPH oxidase was attenuated in mesangial cells from the \( \text{Ins2}^{WT/WT}/p47^{phox-/-} \) mice.
Figure 4.12: NADPH oxidase activity in high glucose-treated wild-type and p47phox-null mesangial cells. A. NADPH oxidase activity of p47phox wild-type and p47phox-null mouse mesangial cells treated with 5.6 mmol/l or 30 mmol/l D-glucose. B. NADPH oxidase activity of p47phox wild-type mouse mesangial cells treated with 5.6 mmol/l, or 30 mmol/l D-glucose, or 5.6 mmol/l D-glucose with 24.4 mmol/l D-mannitol. (WT: p47phox+/+, KO: p47phox−/−. * p < 0.05 compared with non-diabetic groups, † p < 0.05 compared with the diabetic p47phox wild-type group, ‡ p < 0.05 compared with all other groups).
4.4.8 NADPH oxidase subunit expression in primary mesangial cells.

High glucose-induced activation of NADPH oxidase was associated with an increase in the mRNA levels for the NADPH oxidase subunits: \(p47^{\text{phox}}\), NOX2, NOX4, \(p22^{\text{phox}}\), \(p67^{\text{phox}}\), and \(p40^{\text{phox}}\) in the primary mesangial cells, consistent with the \textit{in vivo} data. Deletion of \(p47^{\text{phox}}\) attenuated but did not normalize the increases in expression levels of NOX2, \(p22^{\text{phox}}\), \(p67^{\text{phox}}\) and \(p40^{\text{phox}}\), while there was no effect on NOX4 (Table 4.4). Western Blot analysis showed \(p47^{\text{phox}}\) and NOX2 protein expression paralleled the changes in mRNA levels (Fig. 4.13A-C).
Table 4.4: NADPH oxidase subunits, extracellular matrix proteins and pro-fibrotic markers mRNA expression of mouse mesangial cells.

<table>
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<tr>
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<th>5.6 mmol/l glucose</th>
<th>30 mmol/l glucose</th>
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<tr>
<td></td>
<td>p47phox+/+</td>
<td>p47phox/-</td>
</tr>
<tr>
<td>p47phox</td>
<td>1.00 ± 0.05</td>
<td>non-detectable</td>
</tr>
<tr>
<td>NOX2</td>
<td>1.00 ± 0.30</td>
<td>0.84 ± 0.19</td>
</tr>
<tr>
<td>NOX4</td>
<td>1.00 ± 0.07</td>
<td>0.93 ± 0.06</td>
</tr>
<tr>
<td>p22phox</td>
<td>1.00 ± 0.06</td>
<td>0.95 ± 0.04</td>
</tr>
<tr>
<td>p40phox</td>
<td>1.00 ± 0.40</td>
<td>0.70 ± 0.15</td>
</tr>
<tr>
<td>p67phox</td>
<td>1.00 ± 0.07</td>
<td>0.95 ± 0.05</td>
</tr>
<tr>
<td>Collagen Iα1</td>
<td>1.00 ± 0.14</td>
<td>1.06 ± 0.09</td>
</tr>
<tr>
<td>Collagen Iα2</td>
<td>1.00 ± 0.19</td>
<td>1.10 ± 0.11</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>1.00 ± 0.08</td>
<td>0.92 ± 0.06</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>1.00 ± 0.08</td>
<td>0.97 ± 0.06</td>
</tr>
<tr>
<td>PAI-1</td>
<td>1.00 ± 0.07</td>
<td>0.95 ± 0.05</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM.

\textsuperscript{a} p < 0.05 compared with 5.6 mmol/l glucose-treated groups.

\textsuperscript{b} p < 0.05 compared with the 30 mmol/l glucose-treated p47phox wild-type group.
Figure 4.13: Protein expression of p47\textsuperscript{phox} and NOX2 in high glucose-treated wild-type and p47\textsuperscript{phox}-null mesangial cells. p47\textsuperscript{phox} wild-type and p47\textsuperscript{phox}-null mouse mesangial cells were treated with 5.6 mmol/l or 30 mmol/l D-glucose, protein expression levels of p47\textsuperscript{phox} and NOX2 were determined by Western blot. (A) Representative Western blot of p47\textsuperscript{phox}, NOX2, and β-actin; (B, C) Quantitative densitometry analysis of Western blot for p47\textsuperscript{phox} (B) and NOX2 (C) (WT: p47\textsuperscript{phox}\textsuperscript{+/+}, KO: p47\textsuperscript{phox}\textsuperscript{−/−}; N. D.: non-detectable; * p < 0.05 compared with the 5.6 mmol/l D-glucose-treated p47\textsuperscript{phox} wild-type group, ‡ p < 0.05 compared with all other groups).
4.4.9 Pro-fibrotic gene expression in mouse mesangial cells.

In order to relate the effect of deletion of $p47^{phox}$ on high glucose-induced pro-fibrotic gene expression we measured mRNA levels for collagen Iα1, collagen Iα2, fibronectin, TGF-β1, and PAI-1 in primary mesangial cells. Mean values for these pro-fibrotic genes were increased by 30 mmol/l glucose in mesangial cells from both $Ins2^{WT/WT}/p47^{phox+/+}$ mice and $Ins2^{WT/WT}/p47^{phox/-}$ mice but the magnitude of the increase was significantly reduced in cells from the $Ins2^{WT/WT}/p47^{phox/-}$ mice (Table 4.4).

4.4.10 $p47^{phox}$ and Collagen Iα1 mRNA expression levels in human renal biopsy samples.

Deletion of the gene for $p47^{phox}$ had beneficial effects in both diabetic mice and primary mouse mesangial cells exposed to high glucose concentrations, but the role of p47$^{phox}$ in human diabetic kidney injury is not known. Therefore we measured $p47^{phox}$ and collagen I mRNA levels in microdissected kidney biopsy samples from three diabetic patients and three healthy control subjects. The average age for health controls was 62 ± 11 years, and the average age for diabetic patients was 48 ± 8 years. All of the diabetic patients were hypertensive, and their average HbA1C value was 7.4 ± 0.8%. Total mRNA was extracted from the biopsy samples and expression levels of $p47^{phox}$ and collagen Iα1 were measured real time PCR. Mean values for both $p47^{phox}$ and collagen Iα1 mRNA levels were numerically higher in the diabetic subjects compared to the non-diabetic control subjects although the differences did not reach statistical significance. However, there was a positive correlation between the expression levels of two genes were observed (Fig. 4.14).
Figure 4.14: Expression levels of \( p47^{phox} \) and Collagen I\( \alpha 1 \) mRNA in human renal biopsy samples. A. \( p47^{phox} \) mRNA expression; B. Collagen I\( \alpha 1 \) mRNA expression; C. A linear correlation of mRNA expression levels of Collagen I\( \alpha 1 \) and \( p47^{phox} \) in 3 diabetic and 3 control human subjects (open circles = control subjects; closed circles = subjects with type 2 diabetes mellitus).
4.5 DISCUSSION

Oxidative stress is postulated to play a central role in the pathogenesis of diabetic nephropathy. In this report we sought to focus on the role of NADPH oxidase (NOX) and specifically the cytosolic subunit p47phox in the generation of superoxide in a high glucose environment. The rational for focusing on p47phox is based on \textit{in vitro} studies of rat mesangial cells exposed to high glucose, and \textit{in vivo} studies of diabetic mice by Ohshiro and coworkers.

Our first major observation was that deletion of the p47phox gene attenuated diabetic nephropathy in the Akita mouse. Hyperglycemia was associated with increased urinary albumin excretion rates, kidney and glomerular hypertrophy, and mesangial matrix expansion. These diabetes-induced changes were associated with increased renal oxidative stress. Deletion of p47phox lessened oxidative stress and reduced (but did not normalize) the albumin excretion rate and there were significant reductions in kidney and glomerular hypertrophy, and mesangial matrix expansion. The observed protective effect of deletion of the p47phox gene on diabetic kidney injury was at least partially dependent on improved glycemic control.

Our second major observation was that deletion of p47phox also lessened the severity of diabetes even though the onset and early phase of hyperglycemia were similar in both groups of diabetic mice. This effect of p47phox emerged and was significant after 10 weeks of age and the difference persisted through to 16 weeks of age. The Akita mouse (Ins2\textsubscript{WT/C96Y}) harbours a mutation of the insulin 2 gene (Ins2) (Cys96Tyr) that disrupts a disulfide bond between A and B chains of the insulin molecule. This mutation leads to C/EBP homologous protein (CHOP)-dependent ER stress in the beta-cell and the subsequent apoptosis leads to insulin deficiency and hyperglycaemia. p47phox and NOX-generated ROS may play a role in ER stress-induced beta-cell apoptosis. Accumulation of ROS has also been shown to be both an initiation factor and a consequence of ER stress, and it is an important cellular response linking protein misfolding in the ER to beta-cell apoptosis. Our data showing attenuation of the severity of
diabetes in the Akita mouse suggests that deletion of $p47_{phox}$ may have partially protected the beta-cell from ER stress-induced injury and sustained beta-cell function over time.

To further explore the effect of deletion of $p47_{phox}$ on beta-cell function, we measured plasma insulin concentrations and pancreatic insulin content in the 16 week-old diabetic mice. The difference in blood glucose levels was associated with significant increases in both pancreatic insulin content and circulating insulin levels in Akita diabetic mice with a deletion of $p47_{phox}$. In non-diabetic mice, deletion of $p47_{phox}$ was also associated with improved glucose tolerance compared to wild-type littermates, independent of an effect on insulin sensitivity, suggesting that beta-cell function was enhanced in non-diabetic mice by deletion of $p47_{phox}$. Finally we examined NOX activity in isolated islets from the four groups of mice at 8 weeks of age when blood glucose levels were similar in the two diabetic groups. Hyperglycemia was associated with increased NOX activity in isolated islets from $Ins2^{WT/C96Y/p47_{phox}++/}$ mice while this response was attenuated in $Ins2^{WT/C96Y/p47_{phox}--/}$ mice supporting our hypothesis that deletion of $p47_{phox}$ protects pancreatic beta-cells from injury by attenuating oxidative stress.

The 10 mmol/l difference in blood glucose levels could have contributed, at least in part, to the protective effect of $p47_{phox}$ deletion on glomerular injury in $Ins2^{WT/C96Y/p47_{phox}--/}$ mice, so our next series of studies was designed to more directly test the hypothesis that $p47_{phox}$ deletion attenuated the glomerular response to hyperglycemia. We studied mice at 8 weeks of age when the blood glucose levels were similar in the two groups of diabetic mice. Our third major observation was that deletion of $p47_{phox}$ attenuated NOX activity in isolated glomeruli of diabetic mice independent of blood glucose levels.

NOX is a protein complex consisting of two membrane subunits: NOX and $p22_{phox}$. There are several NOX isoforms including NOX1, NOX2, and NOX4. The activation of NOX1 or NOX2 is dependent on recruitment of four cytosolic proteins: $p40_{phox}$, $p47_{phox}$, $p67_{phox}$, and rac GTPase to the cell membrane. The phosphorylation of $p47_{phox}$ is a critical event in this recruitment.
In contrast to NOX1 and NOX2, NOX4 is constitutively active and does not require the cytosolic subunits for activation \(^{309-311}\). All of these subunits are expressed in the kidney \(^ {290,291}\), and our findings suggest that p47\(^{\text{phox}}\)-dependent NOXs are important sources of superoxide in the diabetic glomerulus. We also measured the mRNA levels of NOX subunits in isolated glomeruli from the four groups of mice. It has been reported that diabetes is associated with increased expression of both NOX2 \(^{294,298,312,313}\), and NOX4 \(^ {298-301,314}\) in kidney cortex. We found that increased NOX activity was associated with significant increases in the mRNA expression of the cytosolic subunits p47\(^{\text{phox}}\), p40\(^{\text{phox}}\), and p67\(^{\text{phox}}\), and the membrane subunits p22\(^{\text{phox}}\) and NOX2 in the glomeruli of diabetic mice. NOX2 and p47\(^{\text{phox}}\) protein levels in isolated glomeruli paralleled the changes in mRNA levels. These effects were attenuated by deletion of p47\(^{\text{phox}}\) suggesting that NOX-mediated oxidative stress may exert a positive feedback loop on subunit expression in the diabetic glomerulus. Although we did not address the mechanism responsible to this effect, studies by Bondi et al \(^ {326}\) provide a possible explanation that reactive oxygen species (ROS)-induced TGF-\(\beta\) may function in an autocrine manner to increase NOX subunits expression.

Our fourth major finding was that the early increases in NOX subunit expression and activity were associated with increased expression of the extracellular matrix proteins collagen I and fibronectin, and the pro-fibrotic factors, TGF-\(\beta\) and PAI-1 \(^ {301-308}\) in isolated glomeruli. These early changes preceded significant differences in the mesangial matrix scores. These findings are consistent with in vivo studies of the effect of apocynin on the severity of diabetic kidney injury, although apocynin functions as a general antioxidant as opposed to a specific antagonist of p47\(^{\text{phox}}\) in non-phagocytic cells \(^ {294-297}\). Previous studies of PKC-beta and diabetic nephropathy have also implicated a role for p47\(^{\text{phox}}\) in the development of kidney injury \(^ {292}\).

One of the limitations of our study is that we did not measure blood pressure at 16-weeks of age. Although blood pressure values were similar at 8-weeks of age, it remains possible that these differences could have contributed to the attenuation of diabetic nephropathy. Fujita and coworkers showed that an antioxidant, apocynin, reduced albuminuria and mesangial matrix expansion in the Akita mouse at 14 weeks of age, but did not lower blood pressure. They also
found that a blood pressure reduction of 13 mmHg with a calcium channel blocker did not attenuate albuminuria or mesangial matrix expansion in the Akita mouse, unless the agents block the renin-angiotensin system.\textsuperscript{327}

Glomerular injury in the Akita mouse has been related to effects on both the podocyte and mesangial cell.\textsuperscript{295, 316-319} Although we did not detect a difference in the number of podocytes in our four groups of mice, we sought to link our \textit{in vivo} observations to a direct cellular effect of p47\textsuperscript{phox} deletion in the mesangial cell. High glucose-induced activation of NOX was attenuated but not normalized in primary mesangial cells derived from p47\textsuperscript{phox}-null mice. We observed similar changes in expression of the NOX subunits in the \textit{in vitro} and \textit{in vivo} studies. Changes in ECM protein expression and pro-fibrotic factors like TGF-β1 and PAI-1 were attenuated but not normalized in mesangial cells from p47\textsuperscript{phox}-null mice. These findings confirm previous work in rat mesangial cells\textsuperscript{293, 297}, and together support the hypothesis that deletion of p47\textsuperscript{phox} attenuates the mesangial cell response to high glucose.

Although our studies focused on p47\textsuperscript{phox}-dependent NOX activation, other pathways may also contribute to oxidative stress in diabetic glomeruli \textit{in vivo}. More recently, Chacko and coworkers reported that treatment with a mitochondria-targeted ubiquinone reduced glomerular injury in diabetic Akita mice, implicating a role for mitochondrial-derived superoxide.\textsuperscript{315} In addition, Gorin \textit{et al} delivered NOX4 antisense \textit{in vivo} using osmotic mini-pumps, and showed less oxidative stress and reduced glomerular injury in streptozotocin-induced diabetic rats\textsuperscript{298}. Experimental studies of diabetic mice with deletion of the gene for NOX4 have not been reported and recent studies in the heart suggest that NOX4 may play a protective role.\textsuperscript{325}

In order to relate these findings in the mouse to human diabetic nephropathy, we micro-dissected kidney biopsy samples from humans with type 2 diabetes mellitus to measure p47\textsuperscript{phox} and collagen Iα1 expressions in glomerular and tubulointerstitial compartments. We were unable to obtain enough glomeruli to extract and amplify the RNA so we focused on the tubulointerstitial compartment which yielded sufficient RNA and did not require amplification before real time – PCR analysis. The tubulointerstitium is also a target for progressive injury in humans with
diabetes mellitus. In a limited number of samples, there was a numerical increase in $p47^{phox}$ and $collagen\ I\alpha1$ expression in the diabetic samples compared to the control samples but the difference did not reach statistical significance. However, there was a positive correlation between expression levels of the $p47^{phox}$ and $collagen\ I\alpha1$, in the kidney samples from human subjects, mirroring our in vivo and in vitro studies in mice. These findings suggest that $p47^{phox}$-dependent activation of NOX may also be a mechanism of kidney injury that is relevant to human diabetic nephropathy.

In summary, our studies support the hypothesis that $p47^{phox}$-dependent activation of NOX is an important determinant of both glomerular injury and beta-cell dysfunction in the Akita mouse model of type 1 diabetes mellitus. The renoprotective effect of deletion of $p47^{phox}$ on the kidney is dependent in part on improved glycemic control. The NADPH oxidase cytosolic subunit, $p47^{phox}$, may be a treatment target for humans with diabetic nephropathy.
Chapter 5

Discussion and Future Directions
Diabetic nephropathy is the leading cause of end-stage renal disease in North America. Although the exact mechanisms for the initiation and progression of diabetic nephropathy have not been fully elucidated, some studies have postulated that the activation of renin-angiotensin system, which allows generation of angiotensin II, and its interaction with the angiotensin II type I receptor, triggering vasoconstriction, hypertrophy, inflammation and fibrosis in the kidney. All components of the RAS are expressed in the kidney and angiotensin II is generated at higher levels in the kidney than in the systemic circulation. It has been shown in both in vitro and in vivo settings, that high glucose drives the activation of the renin-angiotensin system, increases angiotensin II levels, and causes glomerular and tubular injury. Inhibition of the renin-angiotensin system attenuated high glucose-induced kidney injury 195-197, 207.

Enhancing angiotensin converting enzyme 2 (ACE2) activity as therapeutic strategy.

The renin-angiotensin system (RAS) is an important mediator of vascular and renal homeostasis. Major functions of the systemic RAS are regulation of blood pressure, natriuresis, and blood volume control. On the local level, the RAS regulates regional blood flow, and controls trophic and fibrotic responses to a variety of stimuli. The complicated RAS consists of a number of different effector molecules and regulatory components (see Chapter 1, Fig. 1.1). The major effector angiotensin II interacts with the AT$_1$ and AT$_2$ receptors, exerting important physiologic and pathologic roles. However, this view of the RAS has been changed after the recent discovery of ACE2 and angiotensin-(1-7). Angiotensin-(1-7) is produced in the kidney at concentrations comparable to angiotensin II, and through interactions with mas receptor, it regulates vasodilation, modulation of sodium and water transport, and stimulation of nitric oxide synthase.

ACE2 is found in most tissues with highest expression observed in the kidney, endothelium and heart. It is a type 1 integral membrane glycoprotein with a single catalytic metallopeptidase domain contained in the extracellular surface. This catalytic domain shares 42% sequence identity and 61% sequence similarity with the N-terminal catalytic domain of somatic ACE. ACE2 is an exopeptidase that catalyzes the conversion of Ang I to the nonapeptide angiotensin-(1-9) and the conversion of angiotensin II to the heptapeptide angiotensin-(1-7). The primary role of ACE2 is converting angiotensin II into angiotensin-(1-7) with an efficacy > 400-fold greater
than that of the hydrolytic action of ACE2 in forming angiotensin-(1-9)\textsuperscript{334}. ACE2 is associated with a reduction in angiotensin II and an increase in angiotensin-(1-7) levels. ACE2 protein levels are significantly decreased in the kidneys of hypertensive patients, and patients with late diabetic nephropathy\textsuperscript{200}.

Angiotensin-(1-7) is a heptapeptide generated from angiotensin-(1-9) or angiotensin II. While angiotensin II has vasoconstrictor, proliferative, and profibrotic effects in the circulation, cardiac, vascular and renal tissues, angiotensin-(1-7) acts opposing the effects of angiotensin II\textsuperscript{258, 333}. Moon JY et al showed that angiotensin-(1-7) attenuated angiotensin II-mediated NADPH oxidase activation and reactive oxygen species production in diabetic mouse glomeruli and mesangial cells\textsuperscript{266}. Angiotensin-(1-7) interacts with the mas receptor, which is a seven transmembrane protein with domains containing sequences characteristic of the G-protein coupled receptor. The Mas receptor is highly expressed in renal proximal tubular cells, afferent arterioles, cardiac myocytes, and neuronal cells. Angiotensin-(1-7)/mas receptor interaction activates downstream signaling pathways and transcriptional factors.

In Chapter 2 of this thesis, I have examined the therapeutic potential of human recombinant angiotensin converting enzyme 2 (ACE2) in the pathogenesis of experimental diabetic nephropathy. ACE2 is an enzyme expressed in the kidney, and of relevance to my projects, it cleaves angiotensin II into angiotensin-(1-7). Angiotensin-(1-7) acts on the mas receptor and this interaction serves an important counter-regulating function in the renin-angiotensin system. Angiotensin-(1-7) has been linked to vasodilation, anti-growth, anti-inflammatory and anti-fibrotic effects in the kidney. The presence of ACE2 therefore, in theory, should decrease angiotensin II and increase angiotensin-(1-7), protecting the kidney from diabetic nephropathy. Our group and others have previously shown that deletion of the ace2 gene, and pharmacological blockade of the ACE2 enzyme accelerated progression of diabetic nephropathy\textsuperscript{207-209, 227}. In addition, patients with type 2 diabetes who developed diabetic nephropathy showed reduced ace2 expression in both mRNA and protein levels\textsuperscript{209}. I therefore sought to examine whether injection of the exogenous hrACE2 would attenuate diabetic kidney injury by limiting renal angiotensin II levels and enhancing renal angiotensin-(1-7) levels.

I demonstrated for the first time that treatment with hrACE2 protected the kidney from diabetes-induced injury in a mouse model of type 1-like diabetes. Diabetes is associated with both
functional and structural changes in the kidney. In my Akita diabetic mice, I observed higher urinary albumin excretion rate, renal/glomerular hypertrophy, mesangial matrix expansion and GBM thickening compared to their non-diabetic littermates. Treatment with hrACE2 significantly reduced these changes. The attenuation of injury is associated with decreased plasma and renal angiotensin II levels and increased angiotensin-(1-7) levels, consistent with the hypothesis that ACE2 plays a protective role in the processing of angiotensin peptides in the kidney. My experiment, however, did not specify the source of angiotensin peptides in the kidney. These changes in the angiotensin peptide levels could reflect the changes in plasma angiotensin levels and/or as a result of an active intrarenal process. Although a recent study published by Matsusaka and colleagues demonstrated that liver angiotensinogen is the main source of renal angiotensin II, it is still unknown if this is also true under pathological settings such as diabetic nephropathy. It will be very interesting to identify the source of renal angiotensin peptides using tissue specific knock-out mice with deletion of the angiotensinogen gene either in the liver or in the kidney, and observe the effect of diabetes on these mice.

A possible confounder to this study is the mild hypertension observed in the diabetic Akita mice. hrACE2 treatment reduced blood pressure in association with the decreased plasma angiotensin II levels. Although hypertension is a risk factor contributing to chronic kidney disease, there is evidence suggesting that mild hypertension observed in the Akita mice does not play an important role in kidney injury. Fujita and colleague reduced blood pressure in the Akita mice with a calcium channel blocker Amlodipine. They found that normalization of blood pressure to control level does not attenuate albuminuria, hyperfiltration, renal hypertrophy, and mesangial expansion. In order to determine whether this is also true in my Akita mice, I would treat them with a blood pressure lowering drug and to compare their renal damage to diabetic mice treated with hrACE2. This would allow me to assess if the renal protective effect of hrACE2 is independent of the hemodynamic effect of reduced systemic angiotensin II levels.

Activation of the NADPH oxidase is also known to play a fundamental role in the pathophysiology of diabetic nephropathy. The interaction between angiotensin II and NADPH oxidase has been studied in many pathological settings. Angiotensin II-stimulated NADPH oxidase-mediated generation of superoxide has been shown to be upregulated in hypertension, atherosclerosis and diabetes. Angiotensin-(1-7), on the other hand, has been shown to inhibit many signaling cascades that can be activated by angiotensin II. Therefore, the decreased
Angiotensin II level and increased angiotensin-(1-7) level associated with ACE2 treatment should reduce NADPH oxidase activation. I found that hrACE2 normalized diabetes-induced NADPH oxidase activation in the Akita mice. This reduction in NADPH oxidase activity is associated with decreased subunit expression. The mRNA and protein expression levels of both NOX2 and p47phox subunits of the NADPH oxidase were increased in diabetic Akita mice treated with placebo but not with hrACE2. It is not entirely clear 1) how angiotensin-(1-7) is able to inhibit effects of angiotensin II; and 2) which specific NADPH oxidase isoform is responsible for diabetes-induced kidney injury. The effect of interaction between angiotensin II and angiotensin-(1-7) on NADPH oxidase is therefore examined in Chapter 3, and the role of p47phox-dependent NADPH oxidase in diabetic nephropathy is examined in Chapter 4.

In order to further study the mechanisms responsible for a renoprotective effect of hrACE2 treatment in my diabetic mice, I used an in vitro system of cultured primary rat mesangial cells. Consistent with my in vivo findings, high glucose and angiotensin II increased NADPH oxidase activity and superoxide concentration in the mesangial cells. hrACE2 treatment attenuated the effects of high glucose and angiotensin II. Mas receptor antagonist A-779 removed the protective effect of hrACE2, suggesting at least partially, an angiotensin-(1-7)/mas receptor-mediated renoprotection by hrACE2. In addition to mesangial cells, Nadarajah and colleagues overexpressed human ACE2 specifically in podocytes and attenuated kidney injury in mice with streptozotocin-induced diabetes.

My finding strongly suggests that hrACE2 has the therapeutic potential to minimize the rate of progression of diabetic kidney disease. However, there are questions that remain to be answered. ACE2 treatment protects the kidney from diabetes-induced injury by reducing angiotensin II levels and by increasing angiotensin-(1-7) levels. It is important to assess how much protection each of these two effects offers, since AT1R blockade and ACE inhibition are current therapies to treat diabetic nephropathy, both of them limit the effects of angiotensin II. If angiotensin-(1-7) provides additional benefit over AT1R blockade and/or ACE inhibition per se, then ACE2 treatment provides a greater protection. My experiment in mouse mesangial cells indicated that this is the case in vitro, however this was not done in vivo. It has been demonstrated previously that ACE inhibitors and receptor blockers can affect the ACE2/angiotensin-(1-7) system. Inhibition of ACE increases the production of angiotensin I, which is converted to angiotensin-(1-9) by ACE2, and further converted to angiotensin-(1-7) by endopeptidase. Increased
production of angiotensin-(1-7) is responsible for the antihypertensive actions of ACE inhibitors. This is supported by the studies of Luque et al, they observed that urine samples from hypertensive patients treated with the ACE inhibitor captopril had higher amount of angiotensin-(1-7)\textsuperscript{332}. Increased production of angiotensin-(1-7) is also observed when AT\textsubscript{1}R blocker is used, since the accumulation of angiotensin II will stimulate angiotensin-(1-7) generation. Hypertensive rats treated with AT\textsubscript{1}R blocker showed higher plasma angiotensin-(1-7) levels\textsuperscript{333}.

It will be very interesting to treat diabetic mice with hrACE2 in combination with the mas receptor antagonist A-779, and compare to the diabetic mice treated with hrACE2 alone, this will allow me to determine how much protection is mediated by angiotensin-(1-7)/mas receptor pathway. On the other hand, I could also treat diabetic mice with AT\textsubscript{1}R blocker and/or ACE inhibitor, compare them to mice treated with hrACE2, to determine if there is incremental benefit of hrACE2 over AT\textsubscript{1}R blockade and/or ACE inhibition. Or, a third way, to treat diabetic mice with AT\textsubscript{1}R blocker in combination with angiotensin-(1-7), compared to diabetic mice treated with AT\textsubscript{1}R blocker alone. Either way may separate the effect of angiotensin-(1-7) and effect of angiotensin II blockade.

In addition to diabetic nephropathy, ACE2 treatment may be protective in other pathophysiological settings. Activation of the renin-angiotensin system and generation of angiotensin II has been shown in both chronic and acute kidney injury models. The beneficial effect of ACE2 has been implied. For example, deletion of \textit{ace2} gene increased kidney damage in mice with unilateral ureteral obstruction, a model of chronic kidney disease\textsuperscript{330}. Our laboratory confirmed this finding and also found that acute renal ischemia reperfusion injury worsened in \textit{ace2}-null mice. Dilauro and colleagues used a pharmacological inhibitor of ACE2, MLN-4760, on mice following (5/6) nephrectomy and reported an increase in albuminuria via an AT\textsubscript{1}R-dependent mechanism. The same group also found that angiotensin-(1-7) treatment did not improve albuminuria in these mice\textsuperscript{335}. These studies suggest a protective role of ACE2 in different models of renal injury. In order to further assess the therapeutic potential of hrACE2, it is tempting to deliver hrACE2 into these different injury models and evaluate its effect on the kidney.
The effect of angiotensin-(1-7) on mesangial cells.

As mentioned above, ACE2 is associated with a decrease in angiotensin II levels and an increase in angiotensin-(1-7) levels. The mechanisms linking angiotensin II and tissue injury have been studied extensively. Angiotensin II influences kidney function by various ways including hyperfiltration, opening of nonselective pores in the ultrafiltration barrier, modifying the composition of the GBM, and reducing nephrin expression on podocytes. Angiotensin II also increases tubular reabsorption of ultrafiltered proteins and this leads to tubular inflammation and fibrosis. Angiotensin II activates NF-κB and induces proinflammatory effects via AT₁ and AT₂ receptor interactions, contributing to proteinuria and interstitial fibrosis. These events occur through the upregulation of cytokines such as TGF-β, CTGF, interleukin-6, monocyte chemoattractant protein 1 (MCP-1), and VEGF-A, which modulate glomerular ECM pathobiology. In addition, angiotensin II-induced phosphorylation of the Extracellular signal-Regulated Kinase (ERK) 1/2 contributes to renal oxidative stress and fibrosis. Transactivation of the EGFR by angiotensin II/AT₁R interaction is an important determinant of angiotensin II-dependent activation of ERK1/2. Angiotensin-(1-7) counterregulates angiotensin II signaling in kidney cells, however the specific pathways mediating this effect are not clear.

In Chapter 3 of my thesis, I sought to understand how angiotensin-(1-7) inhibits the effect of angiotensin II. In vivo studies suggest that Angiotensin-(1–7) may exert a protective effect in the kidney. For example, Giani et al. showed that angiotensin-(1–7) reduced proteinuria and renal fibrosis in hypertensive rats. Similarly, Zhang et al. found that angiotensin-(1–7) infusion attenuated glomerulosclerosis in rats, and more recently attenuated kidney injury in mice with type 2 diabetes mellitus. However, the signal transduction pathways downstream of ang-(1–7) in kidney cells have not been fully elucidated.

My first major observation was that angiotensin-(1–7) activated ERK1/2 in mesangial cells in a mas receptor/cAMP/PKA-dependent manner. Although mesangial cells express the G protein-coupled receptor mas, it has been reported that angiotensin-(1–7) can interact with the AT₁ receptor in rat heart and renal cortex, and more recently that angiotensin-(1–7) exhibits modest affinity for the AT₂ receptor. However, I found that activation of ERK1/2 was downstream of the mas receptor because the mas receptor antagonist A-779 prevented ERK1/2
phosphorylation while neither the AT1 receptor blocker losartan nor the AT2 receptor antagonist PD123319 had an effect on ERK1/2 phosphorylation, in accord with previous studies \(^{257}\). Given the above reports on angiotensin-(1–7)/AT1 receptor interactions I also determined that the dose of losartan I used was sufficient to inhibit angiotensin II-induced activation of ERK1/2.

ERK1/2 plays important roles in gene transcription to regulate expressions of key proteins, and can contribute to loss of epithelial cells and epithelial-mesenchymal transformation (EMT) \(^{340,341}\). Both angiotensin II and angiotensin-(1-7) activate ERK1/2 phosphorylation, however, through completely independent mechanisms. Angiotensin II induces ERK1/2 phosphorylation via AT\(_1\)R/EGFR/NADPH oxidase pathway, whereas angiotensin-(1-7)-induced ERK1/2 phosphorylation, as shown in Chapter 3, is dependent on the mas receptor/cAMP/PKA pathway. They may also have distinct effects on the subsequent cellular response, which is not studied in my thesis. In order to further understand the difference between angiotensin II and angiotensin-(1-7)-induced ERK1/2 phosphorylation, future experiment should focus on signaling events downstream of ERK1/2, including activation of substrates Elk1, phospholipase A2 and p90Rsk1. ERK1/2 activation by angiotensin II and angiotensin-(1-7) may also differ by localization of action. ERK1/2 is directed to specified sites of action by different stimuli with distinct functions on membranes, in the nucleus, and on cytoskeleton \(^{342-344}\). ERK1/2 phosphorylation may have different consequences in different cellular compartments.

ERK1/2 has a great influence on polymerization dynamics of cytoplasmic microtubules therefore about half of ERK1/2 is bound to them \(^{345-347}\). ERK1/2 is also found at cell adherens junctions and focal adhesions, at sites of cell-cell and cell-matrix contact. In addition, ERK1/2 plays an important role in transcription, differentiation and transformation in the nucleus \(^{348}\). Most of the ERK1/2 is localized in the cytoplasm and the nucleus of a resting cell \(^{346,348}\). Mislocalization of EKR1/2 has been implicated to impact disease \(^{349-354}\). Proteins that increase the nuclear localization of ERK1/2 has been shown to induce renal cancers \(^{355}\). Proteins that promote nuclear export and prevent nuclear entry of ERK2 are associated with decreased glucose-stimulated insulin secretion in cultured pancreatic beta cells \(^{353,354}\). Taken together, the localization of EKR1/2 in response to angiotensin II or angiotensin-(1-7) may be different and therefore contributes differently to subsequent cellular effects.
A number of signal transduction pathways link G protein-coupled receptors to the activation of ERK1/2. One central pathway utilized by the AT1 receptor is NADPH oxidase activation and several studies have shown that inhibition of NADPH oxidase attenuates AT1R-dependent activation ERK1/2. In addition, transactivation of the EGFR by angiotensin II/AT1R is also an important determinant of angiotensin II-dependent activation of ERK1/2. I therefore studied the effect of two inhibitors of NADPH oxidase and two inhibitors of EGFR kinase activity and found that while these reagents effectively inhibited angiotensin II-induced activation of ERK1/2, there was no effect on angiotensin-(1–7)-induced ERK1/2 activation. These findings suggest that neither NADPH oxidase nor transactivation of the EGFR plays a role in the signal transduction pathway linking angiotensin-(1–7)/mas receptor to ERK1/2, unlike angiotensin II/AT1 receptor cell signaling, and that there is a fundamental difference in the mechanisms linking these two peptide ligands and their cognate receptors to the activation of ERK1/2.

Initially, I did not expect that cAMP/PKA signaling would play a role in the activation of ERK1/2 because Gs-α-coupled receptors inhibit ERK1/2 activation in many cell types, due at least in part to a cAMP/PKA-dependent inhibition of the serine threonine kinase Raf-1 that is upstream of MEK and ERK1/2. Moreover, there have been conflicting reports on the effect of angiotensin-(1–7) on cAMP levels in kidney cells in vitro. However, it has also been reported that ERK1/2 is activated in neuronal cells by Gs-α-coupled receptors. Although the signal transduction pathway linking Gs-coupled receptors to ERK1/2 is not well-defined, it may proceed through cAMP, PKA, and MEK because other studies have implicated a role for the GTP-binding Ras and Raf-1. My second major observation was that inhibition of adenyl cyclase attenuated angiotensin-(1–7)-induced activation of ERK1/2. We therefore measured cAMP levels in our mesangial cells and found a modest increase that was prevented by pretreatment of the cells with the mas receptor antagonist A-779 and the adenyl cyclase inhibitor SQ22536. These novel findings suggest that the mas receptor is coupled to Gs-α and that ligand binding activates Gs-α and its effector molecule adenyl cyclase.

The Angiotensin-(1–7)-induced increase in cAMP levels was also associated with an increase in PKA activity in mesangial cells. However, cAMP can lead to an increase in ERK1/2 independent of PKA, an effect that may be mediated by cAMP-activated guanine-nucleotide exchange factors (cAMP-GEFs, also known as Epacs). This pathway has been described in kidney cells. I next treated my mesangial cells with two different PKA inhibitors before exposure to
angiotensin-(1–7) to determine whether ERK1/2 activation was dependent on PKA. Both compounds inhibited the angiotensin-(1–7)-induced ERK1/2 phosphorylation. Taken together with my finding that a stable cAMP analog activated ERK1/2 in the mesangial cells in a PKA- and MEK-dependent manner, these findings support the conclusion that angiotensin-(1–7) signals to ERK1/2 via cAMP/PKA and not via cAMP-GEFs. Finally, I compared the effect of PKA inhibition and EGFR inhibition on the activation and nuclear translocation of ERK1/2 by confocal microscopy and fluorescence imaging. In accord with the Western blot analyses, PKA inhibition but not EFGR kinase inhibition prevented nuclear translocation of ERK1/2.

Su and colleagues have shown that angiotensin-(1–7) inhibits angiotensin II-induced activation of MAPKs in renal proximal tubule cells $^{223}$, and a similar effect of angiotensin-(1–7) has also been reported in human endothelial cells $^{253}$. The mechanisms responsible for this inhibition of angiotensin II signaling by angiotensin-(1-7) has not been fully elucidated. I extended these observations by studying mesangial cells, and found that pre-treatment with angiotensin-(1-7) also attenuated the activation of ERK1/2 by angiotensin II in these cells. I also observed that this interaction was dependent on the activation of PKA by angiotensin-(1-7). In addition, treatment of the MC with a stable cAMP analogue was also sufficient to inhibit angiotensin II-induced phosphorylation of ERK1/2 supporting the conclusion that angiotensin-(1-7) induced cAMP/PKA signaling, while responsible for activating ERK1/2 is also responsible for the subsequent inhibition of angiotensin II activation of ERK1/2.

The interaction between RAS and NADPH oxidase has been shown to play an important role in many pathological settings $^{274,275}$. We have previously reported that angiotensin II-induced activation of NADPH oxidase is inhibited by angiotensin-(1-7) in vitro and more recently angiotensin-(1-7) infusion has been reported to reduce NADPH oxidase activity in the kidneys of mice with experimental type 2 diabetes mellitus $^{266}$. In this regard, my final observation is that this effect is dependent on cAMP/PKA signaling. Taken together with our analyses of ERK1/2 activation, this finding suggests that the mechanism responsible, at least in part, for the attenuation of angiotensin II signaling by angiotensin-(1-7) may be inhibition of NADPH oxidase because compounds that inhibit NADPH oxidase also prevent the activation of ERK1/2 by angiotensin II. However, it is not addressed in my thesis how angiotensin-(1-7) signaling inhibits NADPH oxidase activity. Although NADPH oxidase activity can be activated by many upstream signaling pathways, protein kinase C (PKC) has been shown to play a major role in
high glucose and angiotensin II-induced NADPH oxidase activation. Angiotensin-(1-7) may have an influence on PKC by inhibiting its activation, and therefore subsequently reduces NADPH oxidase activity. In addition, it remains possible that PKA-mediated phosphorylation and inhibition of Raf-1 may contribute to decreased ERK1/2 phosphorylation. This will provide further insight into the mechanism that angiotensin-(1-7) inhibits angiotensin II-induced cellular responses.

I believe that these findings have some relevance to experimental models of kidney injury, especially with respect to a protective role of angiotensin-(1–7). Infusion of angiotensin-(1–7) attenuates experimental glomerulonephritis and diabetic nephropathy and the inhibition of NADPH oxidase and ERK1/2 may contribute to these beneficial effects. On the other hand, it is difficult to reconcile these findings with reports that angiotensin-(1–7) promoted TGF-β1 and extracellular matrix protein production in mesangial cells in vitro and contributed to epithelial-to-mesenchymal transformation. It may be that there are model- and cell-specific effects that account for reports that suggest that angiotensin-(1–7) is deleterious. In support of this notion is the recent report that infusion of angiotensin-(1–7) does not improve or worsen glomerular injury and function in mice subjected to partial renal ablation. Further studies are necessary to better define the role of angiotensin-(1–7) in kidney injury. For example, measuring pro-fibrotic marker (i.e., collagen, fibronectin, TGF-β1 and PAI-1), and pro-inflammatory marker (i.e., TNF-α, MCP-1, interleukin-6) expression levels in cells treated with both angiotensin-(1-7) and angiotensin II, and compare them to cells treated with angiotensin-(1-7) or angiotensin II alone.

In summary, these studies show that angiotensin-(1–7) activates ERK1/2 in mesangial cells in a mas receptor/cAMP/PKA/MEK-dependent manner. These findings extend our understanding of the signal transduction pathways activated by angiotensin-(1–7) in mesangial cells, especially with respect to ERK1/2, and these interactions may account for the observed protective effect of angiotensin-(1–7) in some experimental models of kidney injury.
Inhibiting NADPH oxidase activity as a therapeutic strategy.

The main reason that leads to the conclusion that angiotensin-(1-7) is protective in mesangial cells is that it prevented angiotensin II-induced activation of NADPH oxidase. The role of NADPH oxidase and oxidative stress in pathogenesis of diabetic nephropathy has been implicated in many studies\textsuperscript{278-283}. However, the role of specific NOX isoforms and the cytosolic subunits that regulate their activation remains uncertain. The rational for focusing on p47\textsuperscript{phox} is based on in vitro studies of rat mesangial cells exposed to high glucose\textsuperscript{293, 297}, and in vivo studies of diabetic mice by Ohshiro and coworkers\textsuperscript{292}. In Chapter 4 of my thesis, I examined the effect of deletion of the gene for the NADPH oxidase subunit, p47\textsuperscript{phox}, on diabetic kidney injury in the Akita mouse model of type 1 diabetes mellitus to test the hypothesis that p47\textsuperscript{phox}-dependent activation of NOX is an important determinant of experimental diabetic nephropathy.

My first major observation was that deletion of the p47\textsuperscript{phox} gene attenuated diabetic nephropathy in the Akita mouse. Hyperglycemia was associated with increased urinary albumin excretion rates, kidney and glomerular hypertrophy, and mesangial matrix expansion\textsuperscript{316, 317}. These diabetes-induced changes were associated with increased renal oxidative stress. Deletion of p47\textsuperscript{phox} lessened oxidative stress and reduced (but did not normalize) the albumin excretion rate and there were significant reductions in kidney and glomerular hypertrophy, mesangial matrix expansion. The observed protective effect of deletion of the p47\textsuperscript{phox} gene on diabetic kidney injury was at least partially dependent on improved glycemic control.

My second major observation was that deletion of p47\textsuperscript{phox} also lessened the severity of diabetes even though the onset and early phase of hyperglycemia were similar in both groups of diabetic mice. This effect of p47\textsuperscript{phox} emerged and was significant after 10 weeks of age and the difference persisted through to 16 weeks of age. The Akita mouse (Ins2\textsuperscript{WT/C96Y}) harbours a mutation of the insulin 2 gene (Ins2) (Cys96Tyr) that disrupts a disulfide bond between A and B chains of the insulin molecule\textsuperscript{320}. This mutation leads to C/EBP homologous protein (CHOP)-dependent ER stress in the beta-cell and the subsequent apoptosis leads to insulin deficiency and hyperglycaemia\textsuperscript{320-322}. p47\textsuperscript{phox} and NOX-generated ROS may play a role in ER stress-induced beta-cell apoptosis\textsuperscript{322, 323}. Accumulation of ROS has also been shown to be both an initiation factor and a consequence of ER stress, and it is an important cellular response linking protein misfolding in the ER to beta-cell apoptosis\textsuperscript{324}. Our data showing attenuation of the severity of
diabetes in the Akita mouse suggests that deletion of $p47^{phox}$ may have partially protected the beta-cell from ER stress-induced injury and sustained beta-cell function over time.

To further explore the effect of deletion of $p47^{phox}$ on beta-cell function, we measured plasma insulin concentrations and pancreatic insulin content in the 16 week-old diabetic mice. The difference in blood glucose levels was associated with significant increases in both pancreatic insulin content and circulating insulin levels in Akita diabetic mice with a deletion of $p47^{phox}$. In non-diabetic mice, deletion of $p47^{phox}$ was also associated with improved glucose tolerance compared to wild-type littermates, independent of an effect on insulin sensitivity, suggesting that beta-cell function was enhanced in non-diabetic mice by deletion of $p47^{phox}$. Finally we examined NOX activity in isolated islets from the four groups of mice at 8 weeks of age when blood glucose levels were similar in the two diabetic groups. Hyperglycemia was associated with increased NOX activity in isolated islets from $Ins2^{WT/C96Y/p47^{phox}+/+}$ mice while this response was attenuated in $Ins2^{WT/C96Y/p47^{phox}+/-}$ mice supporting our hypothesis that deletion of $p47^{phox}$ protects pancreatic beta-cells from injury by attenuating oxidative stress.

The 10 mmol/l difference in blood glucose levels could have contributed, at least in part, to the protective effect of $p47^{phox}$ deletion on glomerular injury in $Ins2^{WT/C96Y/p47^{phox}+/-}$ mice, so our next series of studies was designed to more directly test the hypothesis that $p47^{phox}$ deletion attenuated the glomerular response to hyperglycaemia. We studied mice at 8 weeks of age when the blood glucose levels were similar in the two groups of diabetic mice. Our third major observation was that deletion of $p47^{phox}$ attenuated NOX activity in isolated glomeruli of diabetic mice independent of blood glucose levels.

In an effort to better separate the kidney specific effect of deletion of the $p47^{phox}$ and the effect of improved glycaemia on ameliorating kidney injury, I implanted insulin pellets to the 16 week-old diabetic mouse subcutaneously and tried to reduce their blood glucose levels to levels comparable to diabetic mouse with a deletion of the $p47^{phox}$ gene (25 mmol/l). This effort failed because the insulin pellets could not deliver the same effect in reduction in blood glucose to all experimental animals. Some mice responded less to insulin pellets, with higher blood glucose levels than anticipated and some mice responded more, with lower blood glucose levels than anticipated. Therefore this approach was abandoned.
Another approach that may solve this issue and thus remove blood glucose difference as a confounding variable is to generate tissue-specific $p47^{phox}$ knock-out mice. If the $p47^{phox}$ deletion only occurs in the kidney, or more specifically in mesangial cells, endothelial cells, or podocytes, without influencing beta-cell survival and function, then they would have the same plasma glucose levels as the Akita mice. This can be done using the Cre/LoxP recombination technique targeting cell-specific expression markers. For example, C57BL/6 transgenic mice expressing Cre recombinase driven by podocin promoter can be generated. Then mate Podocin-Cre mice with C57BL/6 mice in which both $p47^{phox}$ alleles are flanked by loxP sites, denoted $p47^{phoxL/L}$. The resulting podocin-Cre/$p47^{phoxL/+}$ mice will be mated with $p47^{phoxL/L}$ mice to generate podocin-Cre/$p47^{phoxL/L}$ mice. These mice will have $p47^{phox}$ deletion only in their podocytes. This approach also has limitations in that a mesangial cell specific marker has yet to be identified. It is also time and resource-demanding to use this technique. Therefore I did not take this approach. Instead, I measured gene and protein expression levels on glomeruli isolated from 8 week-old mice before the blood glucose difference emerged.

NOX is a protein complex consisting of two membrane subunits: NOX and $p22^{phox}$. There are several NOX isoforms including NOX1, NOX2, and NOX4. The activation of NOX1 or NOX2 is dependent on recruitment of four cytosolic proteins: $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and rac GTPase to the cell membrane. The phosphorylation of $p47^{phox}$ is a critical event in this recruitment. In contrast to NOX1 and NOX2, NOX4 is constitutively active and does not require the cytosolic subunits for activation. All of these subunits are expressed in the kidney, and my findings suggest that $p47^{phox}$-dependent NOXs are important sources of superoxide in the diabetic glomerulus. I also measured the mRNA levels of NOX subunits in isolated glomeruli from the four groups of mice. It has been reported that diabetes is associated with increased expression of both NOX2 and NOX4 in kidney cortex. I found that increased NOX activity was associated with significant increases in the mRNA expression of the cytosolic subunits $p47^{phox}$, $p40^{phox}$, and $p67^{phox}$, and the membrane subunits $p22^{phox}$ and Nox2 in the glomeruli of diabetic mice. NOX2 and $p47^{phox}$ protein levels in isolated glomeruli paralleled the changes in mRNA levels. These effects were attenuated by deletion of $p47^{phox}$ suggesting that NOX-mediated oxidative stress may exert a positive feedback loop on subunit expression in the diabetic glomerulus. Although I did not address the mechanism responsible for this effect,
studies by Bondi et al. provide a possible explanation that reactive oxygen species (ROS)-induced TGF-β1 may function in an autocrine manner to increase NOX subunit expression.

Our fourth major finding was that the early increases in NOX subunit expression and activity were associated with increased expression of the extracellular matrix proteins collagen I and fibronectin, and the pro-fibrotic factors, Tgfβ1 and Pai1 in isolated glomeruli. These early changes preceded significant differences in the mesangial matrix scores. These findings are consistent with in vivo studies of the effect of apocynin on the severity of diabetic kidney injury, although apocynin functions as a general antioxidant as opposed to a specific antagonist of p47phox in non-phagocytic cells. Previous studies of PKC-beta and diabetic nephropathy have also implicated a role for p47phox in the development of kidney injury.

One of the limitations of our study is that we did not measure blood pressure at 16-weeks of age. Although blood pressure values were similar at 8-weeks of age, it remains possible that these differences could have contributed to the attenuation of diabetic nephropathy. Fujita and coworkers showed that an antioxidant, apocynin, reduced albuminuria and mesangial matrix expansion in the Akita mouse at 14 weeks of age, but did not lower blood pressure. They also found that a blood pressure reduction of 13 mmHg with a calcium channel blocker did not attenuate albuminuria or mesangial matrix expansion in the Akita mouse, unless the agents block the renin-angiotensin system.

Glomerular injury in the Akita mouse has been related to effects on both the podocyte and mesangial cell. I did not detect a difference in the number of podocytes in my four groups of mice, I sought to link my in vivo observations to a direct cellular effect of p47phox deletion in the mesangial cell. High glucose-induced activation of NOX was attenuated but not normalized in primary mesangial cells derived from p47phox-null mice. I observed similar changes in expression of the NOX subunits in the in vitro and in vivo studies. Changes in ECM protein expression and pro-fibrotic factors like Tgfβ1 and Pai1 were attenuated but not normalized in mesangial cells from p47phox-null mice. These findings confirm previous work in rat mesangial cells, and together support the hypothesis that deletion of p47phox attenuates the mesangial cell response to high glucose.

Although our studies focused on p47phox-dependent NOX activation, other pathways may also contribute to oxidative stress in diabetic glomeruli in vivo. More recently, Chacko and coworkers...
reported that treatment with a mitochondria-targeted ubiquinone reduced glomerular injury in diabetic Akita mice, implicating a role for mitochondrial-derived superoxide \(^{315}\). In addition, Gorin et al delivered NOX4 antisense in vivo using osmotic mini-pumps, and showed less oxidative stress and reduced glomerular injury in streptozotocin-induced diabetic rats \(^{298}\). Experimental studies of diabetic mice with deletion of the gene for NOX4 have not been reported and recent studies in the heart suggest that NOX4 may play a protective role \(^{325}\).

Although animal studies have demonstrated potent inhibition of oxidative stress with certain antioxidants \(^{442}\) with associated end-organ protection under experimental diabetic conditions, human studies with various antioxidants including α-tocopherol have been generally disappointing \(^{429}\). A number of these antioxidants have proven to play a minimal if any role in the treatment of diabetic nephropathy in humans. Recently, Pergola and coworkers have shown that bardoxolone methyl, an oral antioxidant inflammation modulator, improved GFR in patients with advanced chronic kidney disease and type 2 diabetes after 52 weeks of treatment \(^{356}\). The role of p47\(^{phox}\) in human diabetic nephropathy has not been assessed. In order to relate my findings in the mouse to human diabetic nephropathy, I micro-dissected kidney biopsy samples from humans with type 2 diabetes mellitus to measure p47\(^{phox}\) and collagen I\(\alpha1\) expressions in glomerular and tubulointerstitial compartments. I was unable to obtain enough glomeruli to extract and amplify the RNA so we focused on the tubulointerstitial compartment which yielded sufficient RNA and did not require amplification before real time–PCR analysis. The tubulointerstitium is also a target for progressive injury in humans with diabetes mellitus. In a limited number of samples, there was a numerical increase in p47\(^{phox}\) and collagen I\(\alpha1\) expression in the diabetic samples compared to the control samples but the difference did not reach statistical significance. However, there was a positive correlation between expression levels of the p47\(^{phox}\) and collagen I\(\alpha1\), in the kidney samples from human subjects, mirroring our in vivo and in vitro studies in mice. These findings suggest that p47\(^{phox}\)-dependent activation of NOX may also be a mechanism of kidney injury that is relevant to human diabetic nephropathy.

To extend this finding, the next step in the future should involve immunohistochemistry staining using anti-p47\(^{phox}\) and anti-collagen type I antibodies on paraffin-embedded human biopsy samples and determine if p47\(^{phox}\) protein levels are increased in diabetic patients and if they correlate with the degrees of kidney injury. The role of p47\(^{phox}\) in human diabetic nephropathy still remains to be determined.
In summary, my studies support the hypothesis that p47phox-dependent activation of NADPH oxidase is an important determinant of both glomerular injury and beta-cell dysfunction in the Akita mouse model of type 1 diabetes mellitus. The renoprotective effect of deletion of p47phox on the kidney is dependent in part on improved glycemic control. The NADPH oxidase cytosolic subunit, p47phox, may be a treatment target for humans with diabetic nephropathy.
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