Modifiers of the Kidney Response to Activation of the Renin-Angiotensin System: ACE2 and Adiponectin

By

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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Abstract

The renin-angiotensin system (RAS) and the generation of angiotensin II (Ang II) play a central role in promoting kidney disease, but our understanding of the regulation of RAS in the kidney is incomplete. In this context, I examined the interactions between two factors, ACE2 and adiponectin, and the RAS.

ACE2 is a recently discovered homologue of ACE. I studied the impact of ACE2 gene deletion on the early phase of inflammation in ischemia-reperfusion (I/R) injury when the RAS is activated. ACE2−/− mice exhibited a marked increase in the inflammatory response, cellular apoptosis and oxidative stress to renal I/R injury. These findings show that ACE2 is an important determinant of I/R injury, a model of acute kidney injury.

Adiponectin is produced by adipocytes and its circulating levels decline in obesity. I studied direct interactions between Ang II and adiponectin on cell signaling and activation of NADPH oxidase activity. The rationale for this study is based on the recognition that obesity is an emerging epidemic and it is associated with both increased risk for chronic kidney disease (CKD) and reduced levels of adiponectin. Kidney tubular cells expressed adiponectin receptors and adipoR1-mediated activations of AMPK and
cAMP-Epac signaling attenuated Ang II-induced NADPH oxidase activity, NFκB activation, and fibronectin expression. These findings show that adiponectin can protect kidney cells, and that obesity-associated declines in circulating adiponectin may contribute to the link between CKD progression and obesity.

Diabetic nephropathy (DN) is also associated with activation of the RAS. I therefore studied the impact of adiponectin gene deletion on the development of DN. Adiponectin<sup>−/−</sup> mice with diabetes exhibited an exaggerated renal hypertrophic response and more severe albuminuria. Glomerular injury was also greater in the adiponectin<sup>−/−</sup> mice with diabetes. Deletion of the gene for adiponectin led to increased αSMA expression, collagen IV deposition, and macrophage infiltration in the kidney. In vitro studies showed that adiponectin inhibited high glucose-induced activation of mTOR, TGF-β1, NFκB, and NADPH oxidase in glomerular mesangial cells. These findings show that adiponectin modulates the kidney response to high glucose both in vivo and in vitro.

In summary, my studies show that ACE2 and adiponectin are important determinants of kidney injury, at least in part due to their effects on Ang II and high glucose in the kidney.
Acknowledgement

I would like to first express my gratitude to my supervisor and mentor Dr. James Scholey, who has inspired me into research and has led me through my graduate training with great insights and continuous encouragement. Without the patient guidance from a knowledgeable mentor like Dr. Scholey, I would not have mastered the skills I have now and become an independent researcher.

I would also like to acknowledge my committee members, Dr. Andrew Advani and Dr. Scott Heximer, for their invaluable input. Their questions and advices have constantly reminded me to think critically, and their generous support is the foundation upon which I build my research work.

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I am indebted to the members of Scholey lab. Ms. Joyce Zhou has taught me the different lab techniques since the beginning of my graduate study and has been facilitated my work with her experimental expertise throughout my training. I would like to thank our wonderful students, Crystal Kim, Rana Yassa, Vanessa Williams, Amanda Hu and Janice Pan, for it is most pleasant to work with them.

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Author Contribution and Copyright Acknowledgements

Chapter 2: Loss of ACE2 Exacerbates Murine Renal Ischemia-Reperfusion Injury

A) Contributions:

Fang F designed and carried out the experiments, generated and analyzed the data and produced the report for this chapter. Liu GC helped in experimental design and data collection. Zhou X, Yang S, Williams V, Hu A, Pan J participated in data generation. Reich HN, Konvalinka A, Oudit GY, Scholey JW, John R contributed to the experimental design, discussion of data interpretation and report production.

B) Publications:

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Chapter 3:

A) Contributions:

Fang F designed and performed all the experiments, generated and analyzed the data and wrote the report for this chapter. Liu GC helped in generating and interpreting data. Kim C, Yassa R and Zhou J participated in data collection. Scholey JW contributed in designing the experiments, interpreting the data and producing the report.
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<tbody>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</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>ACEI</td>
<td>Angiotensin-converting enzyme inhibitor</td>
</tr>
<tr>
<td>ADAM17/TACE</td>
<td>ADAM metallopeptidase domain 17/tumor necrosis factor-α-converting enzyme</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation end-product</td>
</tr>
<tr>
<td>AGT</td>
<td>Angiotensinogen</td>
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<tr>
<td>AICAR</td>
<td>5-aminoimidazole-4-carboxamide ribonucleotide</td>
</tr>
<tr>
<td>AKI</td>
<td>Acute kidney injury</td>
</tr>
<tr>
<td>AMPK</td>
<td>5' adenosine monophosphate-activated protein kinase</td>
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<tr>
<td>Ang I</td>
<td>Angiotensin I</td>
</tr>
<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
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<tr>
<td>APA</td>
<td>Aminopeptidase A</td>
</tr>
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<td>APN</td>
<td>Aminopeptidase N</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>APPL-1, -2</td>
<td>Adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1, 2</td>
</tr>
<tr>
<td>ARB</td>
<td>Angiotensin receptor blocker</td>
</tr>
<tr>
<td>ATBP</td>
<td>AT2 receptor binding proteins</td>
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<td>ATIP</td>
<td>AT2 receptor associated protein</td>
</tr>
<tr>
<td>ATRAP</td>
<td>Angiotensin receptor associated protein</td>
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<tr>
<td>BAR</td>
<td>Bin-amphiphysin-Rvs</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<td>BMK1</td>
<td>Big mitogen-activated protein kinase</td>
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<tr>
<td>BUN</td>
<td>Blood urea nitrogen</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>CB₁</td>
<td>Cannabinoid receptor 1</td>
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<td>CKD</td>
<td>Chronic kidney disease</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>COX2</td>
<td>Cytochrome C oxidase subunit 2</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<td>CTGF</td>
<td>Connective tissue growth factor</td>
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<tr>
<td>DAG</td>
<td>Diacyl-glycerol</td>
</tr>
<tr>
<td>DHE</td>
<td>Dihydroethidium</td>
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<tr>
<td>DN</td>
<td>Diabetic nephropathy</td>
</tr>
<tr>
<td>DOCA</td>
<td>Deoxycorticosterone acetate</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
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<tr>
<td>Epac</td>
<td>Exchange proteins activated by cAMP</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
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<td>ESRD</td>
<td>End-stage renal disease</td>
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<tr>
<td>ET-1</td>
<td>Endothelin-1</td>
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<td>fMLP</td>
<td>N-formylmethionine leucyl-phenylalanine</td>
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<tr>
<td>GABARAP</td>
<td>Gamma-aminobutyric acid receptor-associated protein</td>
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<tr>
<td>gAD</td>
<td>Globular adiponectin</td>
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<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>GBM</td>
<td>Glomerular basement membrane</td>
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<td>GFR</td>
<td>Glomerular filtration rate</td>
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<td>GLUT-1, -4</td>
<td>Glucose transporter-1, -4</td>
</tr>
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<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<tr>
<td>GPI</td>
<td>Glycophosphatidylinositol</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HO-1</td>
<td>Heme oxygenase 1</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>Homeostatic model assessment-insulin resistance</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>I/R</td>
<td>Ischemia-reperfusion</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IgA, IgG</td>
<td>Immunoglobulin A, G</td>
</tr>
<tr>
<td>IL-1,-6,-10</td>
<td>Interleukin 1, 6, 10</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>IRAP</td>
<td>Isulin-regulated animopeptidase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MR</td>
<td>Mineralocorticoid receptor</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
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<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NEP</td>
<td>Neutral endopeptidase</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NT-proBNP</td>
<td>N-terminal prohormone of brain natriuretic peptide</td>
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<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
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<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
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<td>PKB</td>
<td>Protein kinase B</td>
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<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>PKI</td>
<td>Protein kinase inhibitor</td>
</tr>
<tr>
<td>PLA2</td>
<td>Phospholipase A2</td>
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<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
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<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PR-5</td>
<td>Pathogenesis related-5</td>
</tr>
<tr>
<td>PRCP</td>
<td>prolylcarboxypeptidase</td>
</tr>
<tr>
<td>PRR</td>
<td>(pro)renin receptor</td>
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<tr>
<td>PTB</td>
<td>Phosphotyrosine-binding domain</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RACK</td>
<td>Receptor for activated protein kinase C</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RAS</td>
<td>Renin-angiotensin system</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>S6K</td>
<td>P70-S6 kinase</td>
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<td>SLE</td>
<td>Systemic lupus erythematosus</td>
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<td>Smad-2,-3</td>
<td>Mothers against decapentaplegic homolog 2</td>
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<td>SNS</td>
<td>Sympathetic nervous system</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>T1DM, T2DM</td>
<td>Type 1 diabetes mellitus, type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>Tissue inhibitor of metalloproteinase 1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TZD</td>
<td>Thiazolidinedione</td>
</tr>
<tr>
<td>UAE</td>
<td>Urine albumin excretion</td>
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<td>UCP-1</td>
<td>Uncoupling protein 1</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>UUO</td>
<td>Unilateral ureteral obstruction</td>
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<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion protein 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
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<td>vWF</td>
<td>von Willebrand factor</td>
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<tr>
<td>ZO-1</td>
<td>Zonula occludens protein 1</td>
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<tr>
<td>αSMA</td>
<td>Alpha smooth muscle actin</td>
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</tbody>
</table>
Chapter 1

Introduction
1.1 Renin-angiotensin system (RAS)

1.1.1 Background on RAS biology

The classic renin-angiotensin system

The renin-angiotensin system (RAS) was first characterized more than a century ago [1] but remains a field of productive research. In the classic RAS, the sole precursor of the angiotensin peptides, angiotensinogen (AGT), is produced and released by liver and gives rise to angiotensin I through enzymatic cleavage by renin secreted by juxtaglomerular apparatus of kidney; angiotensin I (Ang I) is then cleaved by ACE in the pulmonary epithelium produced to generate the main effector of the RAS, angiotensin II (Ang II). Both Ang II receptors, namely AT1 and AT2 receptor, are G-protein coupled receptors. AT1 receptor mediates the major effects of Ang II via activating signaling pathways such as the protein lipase A2 (PLA2), protein kinase C (PKC) and extracellular signal-regulated kinases (ERK) pathways [2]. On the other hand, AT2 receptor activates the inhibitory G proteins and phosphotyrosine phosphatases, which counter the actions of AT1 receptor [2, 3]. The short and long term signaling events following Ang II stimulation have been summarized before [4]. Ang II can also stimulate the production of aldosterone through AT1 receptor in the adrenal cortex and together these two balance the body fluid and salt content as well as modulate the blood pressure. In addition to circulating RAS, this system has also been identified locally in different tissues, including the kidney, heart and adipose tissue, and plays a critical role in modulating the functions and diseases in these organs [5-8].

New arm of RAS: the ACE2-Ang-(1-7)-Mas axis

ACE2

More recently, additional arms of the RAS have been identified besides the classic pathway, among which the ACE2-angiotensin-(1-7)-Mas pathway is the best studied [9-12]. ACE2 share 40% homology with the whole sequence of ACE and is so far the only ACE homolog that retains enzymatic activity [13-15]. Unlike ACE that removes 2 amino acids from the carboxy-terminal of its substrates, ACE2 is a monocarboxypeptidase,
catalyzing the removal of a single C-terminal amino acid [13]. ACE2 has been shown to promote the production of angiotensin-(1-9) from Ang I and also degrade Ang II to angiotensin-(1-7) (Ang-(1-7)) [13, 16]. Although ACE2 is most efficient in processing Ang II into Ang-(1-7), subsequent experiments have discovered many more peptides that can also serve as its substrates [17]. Among these substrates, apelin-13, opioid peptide dynorphin A and des-Arg bradykinin are also promptly hydrolyzed by ACE2. In contrast to ACE, ACE2 does not metabolize bradykinin and its enzymatic activity is not blocked by the classic ACE inhibitors such as captopril [16]. Originally ACE2 was discovered in high abundance in kidney, heart and testes [16], but then its expression was also detected in the lung [18, 19], small intestine [18, 20], brain [21], retina [22], liver [23] and placenta [24]. Within the kidney, ACE2 is widely expressed in the tubular system and peritubular tissue and is mostly concentrated on the brush border of proximal tubular epithelial cells together with ACE [25-27]. ACE2 and ACE showed differential distribution in the glomeruli; with ACE2 primarily located on the parietal and visceral glomerular epithelial cells and lesser so on the mesangial cells while ACE located on the endothelial cells [28]. The expression levels of renal ACE2 are perturbed in animal models and human specimens of several kidney diseases including diabetic nephropathy, hypertensive renal disease and IgA nephropathy [9].

Angiotensin-(1-7)

The main product of ACE2 enzymatic activity is the 7 amino acid peptide Ang-(1-7). However the ways to generate Ang-(1-7) is far from the limited interaction between ACE2 and Ang II. Ang-(1-7) was in fact first thought to be a derivative of Ang-(1-9) by ACE hydrolysis, where Ang-(1-9) was found as the result of ACE2-catalyzed Ang I degradation [13, 29, 30]. Other enzymes can also utilize Ang-(1-7); for example nepriylisin (also known as neutral endopeptidase 24.11), oligopeptidase (alternatively, EC3.4.24.15) and prolyl oligopeptidase (alternatively, EC3.4.21.26) can all generate Ang-(1-7) from Ang I [12, 31, 32]. Prolylcarboxypeptidase (PRCP; or angiotensinase C) is another enzyme detected in kidney that can lead to the yield of Ang-(1-7) from Ang II, and therefore process similar potential as ACE2 in Ang II disposal [12, 33]. Despite the existence of multiple Ang-(1-7) forming enzymes, ACE2 remains the predominant
contributor of this peptide in kidney [34, 35]. Ang-(1-7) can be further broken down into Ang-(1-5) and Ang-(1-4) by ACE2 and neprilysin respectively [30, 36], which inactivate the peptide. The function of Ang-(1-7) is largely opposing those of Ang II, and has been proposed to be beneficial in different organ systems [10, 37].

Mas receptor

Santos and colleagues first proved that Mas is the endogenous receptor for Ang-(1-7) rather then the previously mis-associated Ang II [38]. The expression level is high in brain and testis, but its presence has been also demonstrated in kidney, heart and the vasculature [39]. The distribution of Mas in kidney is quite broad, including the proximal tubules and the afferent arterioles [10, 40, 41]. The Mas receptor like AT1 and AT2 receptors belongs to the GPCR family, but a recent survey on the Ang-(1-7) signaling networks using phosphoproteomics also unveiled positive associations to the Akt and FOXO1 pathways [42]. Ligand-binding of Ang-(1-7) to Mas was capable of inducing arachidonic acid release and calcium influx [38, 43]. Interestingly, Mas has been shown to directly dimerize with the AT1 receptor and regulate its function [44, 45] whereas Ang-(1-7) was reported to have a bi-phase modulatory role in Na⁺-ATPase by interacting with both the AT1 and AT2 receptors in kidney [46, 47]. These data revealed the complexity of RAS networks.

The ACE2-Ang-(1-7)-Mas axis as a counterbalancing mechanism for activation of the ACE-Ang II-AT1 axis

Activation of the ACE2-Ang-(1-7)-Mas axis mainly results in effects contrary to those of ACE-Ang II-AT1 activation, hence it is reasonable to hypothesize that ACE2-Ang-(1-7)-Mas axis is an endogenous counterbalancing mechanism to the ACE-Ang II-AT1 axis. Indeed, experimental evidence consistently demonstrates a beneficial role of ACE2-Ang-(1-7)-Mas axis in the cardiovascular system. Mutant mice without ACE2 developed aged-related cardiomyopathy due to oxidative stress induced by PI3Kγ signaling, which was reversed by the AT1 blocker irbesartan confirming excess Ang II as a disease contributor [48]. In a model of atherosclerosis, deletion of the ACE2 gene in ApoE-null mice caused dysfunction in both endothelial cells and macrophages, leading to increased
expression of adhesion molecules and pro-inflammatory cytokines and further increased atherosclerotic plaque accumulation [49]. In contrast, overexpressing ACE2 protected endothelial cells against atherogenic insults [50] and stabilized the atherosclerotic plaques in rabbits with high-fat diet and balloon-induced vessel injury by providing endothelial protection [51]. Localized ACE2 overexpression also significantly reduced ischemic injuries and preserved cardiac function [52].

Mechanistically part of the cardioprotective effects of ACE2 stems from its function in lowering Ang II levels [53, 54], and others from its function in increasing Ang-(1-7). Ang-(1-7) inhibited thrombus formation by enhancing nitric oxide (NO) release from platelets in a Mas receptor-dependent way [55], and was proven to mediate the anti-thrombotic effect of the ACE inhibitor (ACEI) captopril and the angiotensin receptor blocker losartan [56]. Tallent and colleagues investigated the anti-proliferative effect of Ang-(1-7) on vascular smooth muscle cells (VSMCs) and proved that this effect involved the release of prostacyclin and inhibition of Ang II-induced ERK activation [57]. Neointimal formation after stent implantation was reduced by Ang-(1-7) infusion [58]. Contrary to the beneficial effects of Ang-(1-7) supplementation, deletion of the Ang-(1-7) receptor Mas in mice resulted in a reduction of the NO content and elevated oxidative stress in the endothelial cells, which further led to hypertension [59, 60].

The ACE2-Ang-(1-7)-Mas axis may regulate metabolism as well. ACE2 adenovirus treatment attenuated the early β cell injury in type 2 diabetic db/db mice, which was mediated through Ang-(1-7) [61]. Ang-(1-7) activates the Akt signal transduction pathway, which may be the underlying mechanism for its roles in promoting glucose transporter 4 (GLUT4) membrane translocation, reducing tissue RAS activation, enhancing adiponectin production and overall improving insulin sensitivity and lipid profiles [62-64]. Deficiency in Mas receptor in mice, however, was associated with many metabolic syndrome-like symptoms, including increased circulating insulin, cholesterol, triglycerides, fat mass, as well as impaired glucose tolerance and insulin sensitivity [65].
**Other RAS components**

Recently research has identified new members of the RAS. Experimental results suggest that Ang II can be converted into bioactive peptides other than Ang-(1-7) through enzymatic processing [66, 67]. Ang III is the product of Ang II losing one N-terminal amino acid catalyzed by aminopeptidase A, which can be further hydrolyzed by the same enzyme into Ang-(3-8), or Ang IV. Ang IV is also formed from Ang II and Ang III by aminopeptidase N. Both these peptides can be alternatively derived from Ang I by sequential loss of one or two N-terminal amino acid(s) and then ACE cleavage at the C-terminal. Ang III and Ang IV were shown to contain important regulatory roles in brain and appeared to signal preferentially via AT2 and AT4 receptor [68-70] respectively. The expression of the AT4 receptor was also detected in the nephrons of animal and human kidneys [71-74], and infusion of Ang IV in the renal artery modulated cortical blood flow and sodium reabsorption in kidney [75]. In endothelial cells, Ang IV increased endothelial nitric oxide synthase (eNOS) activity [76], inducing vasorelaxation, and upregulated plasminogen activation inhibitor 1 (PAI-1) expression [77, 78]. In addition to the potential blood pressure regulatory effect of the Ang IV-AT4 signaling, the AT4 receptor has been identified as an insulin-regulated aminopeptidase (IRAP) [79]. Since IRAP promotes the translocation of GLUT4 to the cell membrane, it has been hypothesized that AT4 facilitates brain metabolism, which may be the mechanism underlying the positive effects of Ang IV on learning and memory [80].

**AT1 and AT2 binding proteins**

In the classical RAS, receptor-binding proteins have been found to regulate the function of the Ang II receptors AT1 and AT2 [81, 82]. Daviet and colleagues first discovered the AT1-interacting angiotensin receptor-associated protein ATRAP using a yeast two-hybrid system with the C-terminal of AT1a being the bait [83]. The specificity of ATRAP binding is largely confined to AT1 receptor, showing no detectable association to receptors like the AT2, bradykinin B2 or β2 adrenergic receptor [83]. The expression of ATRAP has been verified in the cardiovascular system [84], and in kidney this protein co-localizes with the AT1 receptor in the tubules [85]. Transfection experiments in cell culture systems indicated that ATRAP reduced the membrane presence of AT1 by
triggering AT1 receptor internalization, which negatively regulated the pro-hypertrophic effect of AT1 signaling by inhibiting pathways like ERK and Akt [86-89]. ATRAP may functionally attenuate many deleterious effects of AT1 receptor activation: in the transgenic mice overexpressing this protein, oxidative stress and hypertrophy in the cardiac tissue after Ang II infusion or aortic banding were ameliorated, and even local increase in ATRAP level protected the heart from hypertrophy after Ang II stimulation [90, 91]. ATRAP gene SNP was associated with essential hypertension in a genome-wide association study (GWAS) and hypertensive Dahl rats showed decreased renal ATRAP levels [92]. In human kidney ATRAP was present in the mesangial cells, podocytes, tubular cells and the renal vasculature [93, 94], suggesting that it may be a relevant player in human renal physiology and kidney disease development [94, 95].

Other AT1-associated proteins have also been discovered. ARAP is another specific binding protein to AT1, which regulates AT1 differently from ATRAP. In HEK-293 cells, ARAP has been found to facilitate AT1 recycling and receptor resensitization after Ang II stimulation [96]. Mice with increased ARAP1 expression specifically in the renal proximal tubules showed increased blood pressure and kidney size, which can be normalized by RAS blockade [97]. Human kidney biopsies showed ARAP1-positive staining in the renal vascular smooth muscle cells and mesangial cells, but not in the tubules [98]. The distribution pattern indicates that ARAP1 may regulate AT1 signaling in a more spatially specific way. The GABA<sub>A</sub> receptor-associated protein (GABARAP) also interacts with AT1 receptor on its intracellular end and increases AT1 receptor membrane traffic [99]. The physiological and pathophysiological importance of GABARAP in relation to AT1 receptor is still yet to be clarified.

For AT2 receptor, associations have been identified between the AT2 receptor-associated proteins ATIPs and ATBPs, which appear to regulate the trafficking of AT2 [100-103]. However more research is necessary to understand the exact functions of these families of proteins.
Figure 1. Schematic diagram of the renin-angiotensin system (RAS)
Angiotensin peptides were shown in blue box, the receptors were shown in green boxes and the enzymes were labeled in orange. PRR, (pro)renin receptor; NEP, neutral endopeptidase; APA, aminopeptidase A; APN, aminopeptidase N.
1.1.2 RAS and kidney disease

**Clinical evidence of RAS activation in kidney disease**

The effects of RAS on the kidney have been extensively studied and reviewed for many types of kidney diseases with both animal models and clinical trials [104, 105], and it is well-established that RAS activation is a crucial contributor to the development and progression of kidney diseases. The renal expression levels of major RAS components are elevated in several types of human kidney diseases such as hypertensive kidney disease, diabetic nephropathy (DN) and IgA nephropathy [106-109]. Clinical evidence has consistently shown that RAS blockade by ACE inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) convey renoprotective functions that may be independent of their blood pressure-lowering effects. In the Bergamo Nephrologic Diabetes Complications Trial (BENEDICT), the ACEI trandolapril delayed the onset of microalbuminuria with or without the calcium channel blocker verapamil in type 2 diabetic patients, while verapamil alone failed to preserve kidney function as compared to placebo [110]. In another clinical study in patients with type 1 diabetes, captopril slowed the progression of kidney disease as measured by doubling of serum creatinine and risk to reach combined end point of dialysis, kidney transplant and death [111]. Similarly for ARBs, the risk of microalbuminuria initiation and the burden of urinary albumin excretion were reduced in diabetic patients with irbesartan treatment (the Irbesartan Microalbuminuria in Hypertensive Patients with Type 2 Diabetes (IRMA-2) study) [112] and the rate of progression to end point of end stage renal disease (ESRD) or death was also lower in the irbesartan group with comparison to placebo or amlodipine group (the Irbesartan Diabetic Nephropathy Trial (IDNT)) [113]. In the Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan (RENAAL) and subsequent studies [114-116], patients with type 2 diabetes mellitus (T2DM) and overt nephropathy were randomized to losartan or placebo, and proteinuria, doubling of serum creatinine and incidence of ESRD and death were monitored. Losartan decreased the risks for all the aforementioned parameters and the benefits were independent of its effect on blood pressure.
ACEI and ARB seems to have comparable effectiveness in renoprotection [117]. The effectiveness of dual RAS blockade with ACEI and ARB, however, is debatable. Combined therapy was found to induce more reduction in proteinuria than single drug treatment [118, 119], but risk of reaching renal end point was not different than either ACEI or ARB treatment alone (the Ongoing Telmisartan Alone and in Combination with Ramipril Global Endpoint Trial (ONTARGET) and Olmesartan Reducing Incidence of ESRD in DN Trial (ORIENT)).

The effects of inhibiting other RAS components on renal outcomes in patients are also being studied. In the Aliskiren in the Evaluation of Proteinuria in Diabetes (AVOID) study where 599 patients with DN and losartan treatment were recruited, direct renin inhibitor aliskiren resulted in a 20% reduction in urinary albumin excretion rate after correcting for blood pressure as compared to placebo [120]. However there was a higher incidence of severe hyperkalemia in the aliskiren group, eliciting caution in using this drug together with other RAS blockade medication. A few short-term studies [121, 122] in diabetic patients suggested that aldosterone receptor antagonist spironolactone conferred additional renoprotective effect on the background of ACEI or ARB therapy, but hyperkalemia is still a concern when combining aldosterone receptor antagonist to ACEI and ARB. Overall, clinical studies support that RAS activation plays a causative role in kidney disease.

**Ang II as a main contributor to kidney injuries**

The main effector of RAS, Ang II, has long been known to circulate in the plasma as a potent vasoconstrictor and strong inducer of water and salt retention in the kidney, which explains its role in raising blood pressure [123]. Besides its physiological effects on hemodynamics, Ang II also function in a paracrine and even intracrine fashion to stimulate cell proliferation and hypertrophy, apoptosis, tissue inflammation, oxidative stress and fibrosis [105]. The concentration of Ang II is much higher in kidney compartments than the plasma level and was found to be regulated in a manner distinct from the systemic Ang II as well [124-126]. Increased levels of Ang II are directly linked to glomerulosclerosis and the breakdown of the filtration barrier [127], as animals infused with Ang II had increased expression of α-smooth muscle actin (αSMA),
transforming growth factor-\( \beta \)1 (TGF-\( \beta \)1) and desmin in mesangial cells and podocytes, in addition to more collagen I deposition in glomeruli [128, 129]. Ang II injection in rats also promoted the production of chemokine RANTES in glomerular endothelial cells which caused more monocyte/macrophage infiltration [130], whereas the pro-hypertrophic effect on Ang II in the glomerulus was signaled by the ERK and c-Jun N terminal kinases (JNK) pathways [131].

In contrast to Ang II infusion, blockade of Ang II signaling is renoprotective. Kim and colleagues showed that blocking Ang II signaling with candesartan or enalapril attenuated kidney fibrosis as measured by tissue mRNA levels of TGF-\( \beta \)1, type I, III, IV collagen and fibronectin in deoxycorticosterone acetate (DOCA)-salt hypertensive rats. A corresponding improvement in urinary albumin excretion and structure change were also observed [132]. Similar beneficial effect of Ang II inhibition was seen in the remnant kidney model, where ARB reversed the elevation of several profibrotic factors such as \( \alpha \)SMA, desmin and TGF-\( \beta \)1, preventing injuries such as glomerular hypertrophy, glomerulosclerosis and albuminuria [133, 134]. ACEI and ARB treatments also inhibited TGF-\( \beta \)1 upregulation after high glucose stimulation and lessened glomerulosclerosis and proteinuria in the streptozotocin (STZ)-induced diabetic rats [135, 136].

**TGF-\( \beta \) as a mediator of Ang II-induced fibrosis in the kidney**

Investigations on kidney diseases suggest that fibrosis is the common final pathway that result in the loss of functional renal tissue and ESRD [137]. As it is listed above, many of the augment effects of Ang II on cellular hypertrophy and proliferation, and renal fibrosis are mediated by the profibrotic cytokine TGF-\( \beta \) [138, 139]. The TGF-\( \beta \) is a family of more than 30 proteins and TGF-\( \beta \)1 is the prototype of these proteins. Classically TGF-\( \beta \) is secreted as homodimer and binds to the type II receptor, which then recruit the type I receptor and activate the Smad proteins. Smad proteins translocate into the nucleus and modulate gene expression [140]. Border and colleagues first proved that TGF-\( \beta \) is a critical component of renal fibrotic pathogenesis [141], and experimental findings show that TGF-\( \beta \) directly stimulates the transcription of extracellular matrix proteins in mesangial cells, endothelial cells and tubular cells, downregulates collagenase but upregulates tissue inhibitor of metalloproteinases, increases other profibrotic cytokines
like connective tissue growth factor (CTGF) and augments fibroblast differentiation through epithelial-mesenchymal transition [142-144]. TGF-β was additionally shown to positively regulate both the size and number of mesangial cells [145, 146].

There is a close interaction between Ang II and the TGF-β signaling pathway. Ang II can activate TGF-β via reactive oxygen species (ROS) [147], but Ang II itself can also stimulate TGF-β synthesis by inducing transcription factors c-fos and c-jun and release through the mitogen-activated protein kinase (MAPK) pathway [148, 149]. TGF-β can in turn induce angiotensinogen mRNA expression in proximal tubular cells, suggesting a potential perpetuating cycle between the RAS and TGF-β [150]. Not only the ligand but the TGF-β receptor type II level rises in response to Ang II in proximal tubular cells [151] and possibly in podocytes via vascular endothelial growth factor (VEGF) [152]. Ang II may even bypass TGF-β and activate downstream effectors of the same pathway: Smad 2 and 3 can be phosphorylated downstream of the ERK1/2 and p38-MAPK pathway following Ang II stimulation [153, 154], and CTGF can be induced by Ang II in a calcineurin-, ERK1/2- or Rho-dependent ways [155-157]. The interaction between Ang II and TGF-β is functionally relevant as neutralizing antibodies against TGF-β abolished the Ang II-induced tubular cell hypertrophy [158]. ACEI and ARB diminished high TGF-β levels in animals undergoing unilateral ureteral obstruction (UUO) or treated with cyclosporine [159-161]. In humans, ACEI and ARB also reduced the urinary TGF-β excretion in patients with type 1 and type 2 diabetes [162-164], hypertension [165, 166] and IgA nephropathy [167, 168].

**NADPH oxidase-generated reactive oxygen species as mediators of Ang II-induced kidney damage**

One of the most important injurious pathways induced by Ang II is the generation of ROS by nicotinamide adenine dinucleotide phosphate-oxidase (NAPDH oxidase) [147]. NADPH oxidase is a multimeric protein complex present ubiquitously in the body and functions to increase cellular ROS. The classic NADPH is composed of the membrane subunits Nox2 and p22phox, as well as the cytosolic subunits p47phox, p67phox and the small G-protein Rac. Activation of the NADPH oxidase involves the membrane recruitment of the cytosolic subunits. In humans, homologues exist for different subunits, namely Nox1,
Nox3, Nox4, Nox5, Duox1, Duox2, NoxO1 and NoxA1. NADPH oxidase is abundant in the kidney, especially Nox4, which was originally named the renal NADPH oxidase (Renox) [169]. It has been demonstrated that Ang II triggers ROS production by NADPH oxidase and then transduces the signals downstream through pathways such as Akt/protein kinase B (PKB) [170], epithelial growth factor receptor (EGFR) and JNK [171], ERK1/2 [172] and cyclooxygenase-2 (COX2) [173]. Superoxide mediates the increase in blood pressure in response to Ang II infusion [174], and the NADPH oxidase components p22phox is upregulated in hypertensive rats [175]. Deletion of the NADPH subunits p47phox or Nox1 significantly blunted the blood pressure elevation in the presence of Ang II [176-178]. On the other hand, overexpression of p22phox or Nox1 potentiated the hypertrophic response to Ang II in smooth muscle cells [179, 180]. The participation of ROS in Ang II responses is further confirmed by the inhibition of free radical scavengers catalase and superoxide dismutase (SOD) on hypertension and hypertrophy after Ang II treatment [181, 182]. Similarly in kidney, as in other tissue, oxidative stress as a result of high level of ROS produced by NADPH oxidase propagate the injurious signal of Ang II, therefore lowering ROS by either antioxidants or RAS blockers delays kidney disease progression. Ang II has been coupled to both Nox4 and Nox2 in the kidney [170, 183]. Lodha and colleagues found that Ang II was able to induce mesangial cell apoptosis via ROS, which also involved TGF-β [184]. Infusion of AngII was associated with an increase in kidney levels of p22phox and Nox1, and a corresponding decrease in extracellular SOD [185]. In models of glomerulonephritis, Ang II raised the level of Nox2 and exacerbated mesangial expansion, which was reversed by the antioxidant probucol [186]. Treatment with the antioxidant tempol prevented inflammatory cell infiltration and albuminuria in eNOS-null mice with hypertensive nephropathy [187]. The overactivation of fibrogenesis by Ang II was also normalized by the antioxidants tempol and apocynin [188]. Various studies have indicated that the beneficial effects of ARBs were at least partially achieved by lowering ROS and oxidative stress in the kidney [187, 189].
Figure 1. 2 Schematic diagram of signaling pathways mediating Ang II-induced damages in kidney. Dashed lines indicates potential interactions.
Other RAS components that contribute to kidney injuries: (pro)renin, mineralocorticoid receptor, aldosterone

Besides Ang II, other RAS components are also involved in the development and progression of kidney disease. A specific receptor for renin and prorenin, the (pro)renin receptor (PRR), was first cloned in the mesangial cells and later detected in additional locations of the nephrons, especially the collecting ducts [190-192]. Ligand-binding of renin and prorenin to the PRR induced activation of several signaling transduction pathways such as ERK1/2, p38 MAPK, phosphoinositide 3-kinase (PI3K), cyclooxygenase-2 (COX-2), and Wnt pathways, and mediated the increase in TGF-β, PAI-1 and fibronectin in collaboration with or independent of Ang II [193-195]. Rather than simple a receptor to transduce signals, PRR is hypothesized to cause conformational changes in renin and (pro)renin after binding. As a consequence, receptor-bound (pro)renin is capable of hydrolyzing Ang I and receptor-bound renin displays higher catalytic efficiency than free floating renin [191, 196]. Due to the lack of a transgenic animal model, the role of the PRR and its ligands in kidney disease is relatively less understood. However PRR was found to positively influence pro-fibrotic factors and was involved in kidney fibrosis in rats with hypertension [197]. PRR level was also elevated in the kidney of diabetic rats [198].

Aldosterone is a mineralocorticoid hormone secreted by the adrenal cortex in response to Ang II to increase sodium reabsorption via the mineralocorticoid receptor (MR). Chronic aldosterone infusion in combination with high salt intake in rats resulted in hypertension, albuminuria and increased oxidative stress in the kidney, and the MR antagonist eplerenone or tempol attenuated kidney injury [199]. In the rat remnant kidney model, plasma aldosterone levels were increased over 10 fold after nephrectomy together with increase in blood pressure and proteinuria, whereas treatment with the MR antagonist spironolactone suppressed the hypertension and proteinuria transiently. MR antagonist also protected the kidney against glomerulosclerosis and interstitial fibrosis in STZ-induced diabetic rats [200], but was reported to exert a major antifibrotic effect in glomeruli rather than tubulointerstitium in the hypertensive Dahl salt-sensitive rats [201]. Aldosterone interacts with Ang II in mediating kidney damage: ACEI and ARB can
attenuate kidney injury associated with high aldosterone levels [202] while MR blocker reduced oxidative stress after Ang II infusion in cardiac tissue [203]. Conversely, administering aldosterone negated the renoprotective effects of captopril in stroke-prone spontaneously hypertensive rats [204].

At the cellular level, aldosterone stimulated mesangial cell hypertrophy and proliferation through the ROS-induced EGFR transactivation then downstream MAPK, PI3K/Akt pathways [205]. Podocytes are other direct targets of aldosterone, as uninephrectomized rat with aldosterone infusion and high-salt diet developed proteinuria and podocyte foot process effacement and loss of the podocyte-associated protein nephrin and podocin [206]. ROS were shown to be a contributor of this pathological process. MR blockade, in contrast, decreased kidney tissue oxidative stress and salvaged the podocyte and renal function in hypertensive Dahl salt-sensitive rats or Ren2 rats [207, 208], and rescued podocytes from apoptosis in STZ-induced diabetic rats [209]. As stated above, one molecular mechanism through which aldosterone contributes to kidney damage is increased ROS production and oxidative stress. Additionally aldosterone can promote inflammation by inducing the expression of pro-inflammatory cytokines including interleukin (IL)-1β, IL-6 and monocyte chemotactic protein-1 (MCP-1, or CCL2) and the inflammatory mediator NFκB, which were inhibited by selective MR inhibitors [210, 211]. In cultured mesangial cells, aldosterone upregulated the mRNA and protein level of the profibrotic factor PAI-1 independent of TGF-β [212]. PAI-1 deficient mice retained the fibrotic and inflammatory response to aldosterone infusion, but showed less mesangial matrix expansion and urine albumin excretion [213]. Aldosterone raised the TGF-β level in mesangial cells, which then increased fibronectin expression via Smad2 [214, 215]. The level of CTGF is also higher after aldosterone stimulation in both mesangial cells and uninephrectomized rat [216].
1.2 ACE2

1.2.1 ACE2-Ang-(1-7)-Mas axis in kidney diseases

Role of ACE2 in kidney injury

Given the essential role of Ang II as a contributor in the development and progression of kidney disease, the negative regulator of Ang II, the ACE2-Ang-(1-7)-Mas system, should logically be protective against insults to the kidney. Indeed, accumulating research data support a beneficial role of ACE2-Ang-(1-7)-Mas axis in the kidney. As introduced previously, ACE2 is abundant in kidney tissue, but its expression is disturbed in disease states: lower renal ACE2 expression level was detected in hypertensive spontaneous hypertensive rats (SHR) [217], rats of two kidney one clip (2K1C) model of hypertension [218], obese Zucker rats on a high salt diet with increased blood pressure [219], rats after subtotal nephrectomy [220] and rats with septic shock-induced renal injury [221]. Decreased ACE2 signal was also detected in biopsy samples from patients with IgA nephropathy without ACEI or ARB treatment [222]. The distribution of ACE2 is more complex in diabetic nephropathy, with higher expression in tubules but lower expression in the glomeruli in STZ-induced diabetic animals or obese db/db mice compared to controls [223-225], and it is accompanied with a higher ACE level in the glomeruli [28]. The high ACE/ACE2 ratio favors the generation of Ang II, which may account for the profound glomerular injury that is a feature of diabetic nephropathy. In human diabetic nephropathy, both ACE2 protein and mRNA were decreased in the glomeruli compared to healthy control subjects [226, 227].

The loss of ACE2 may be due to active shedding from the cell surface through the action of metallopeptidase such as ADAM17 (also known as TACE) [228]. ADAM17 was able to cleave ACE2 from cultured human airway epithelial cells and HEK293 cells and release it from cell membrane [228]. Consistent with this hypothesis, upregulation of ADAM17 has been shown in db/db mice and in human kidney diseases [225, 229], and higher plasma and urine ACE2 content has been reported in diabetic animals and patients with diabetic nephropathy [230-234]. ADAM17 was also associated with fibrosis and
inflammatory cell infiltration in both glomerular and tubulointerstitial compartments in various kidney diseases [229].

Rather than just a marker of injury, ACE2 is involved in the pathogenesis of kidney disease. ACE2 deficiency or inhibition increases renal susceptibility to various insults. Rats fed a high salt diet have increased ACE/ACE2 ratios and oxidative stress in the glomeruli, and mice without ACE2 have more oxidative stress in glomeruli as well [235]. Pharmacological inhibition of ACE2 with MLN-4760 worsened the albuminuria and glomerular mesangial matrix expansion in STZ-induced diabetic mice [236]. Absence of ACE2 in type I diabetic Akita mice caused increased deposition of fibronectin and α-smooth muscle actin (α-SMA), more thickening of the glomerular basement membrane and doubled the urinary albumin excretion rate compared to diabetic mice with ACE2 [237]. In the UUO model, ACE2 knock-out (KO) mice developed worse renal fibrosis, as measured by accumulation of α-SMA and collagen I, and inflammation, as measured by increases in TNF-α, IL-1 MCP-1, infiltrating macrophages and T-cells after surgery. Activation of the TGF-β and NFκB signaling pathways were proposed to be responsible for these changes [238]. Similarly in animals with limb ischemia-reperfusion (LIR), ACE2 deficiency was associated with worse inflammation and oxidative stress in kidneys and compromised survival [239].

ACE2 overexpression or supplementation, on the other hand, helps to defend the kidney from injurious stimuli. Mice overexpressing ACE2 showed lesser degree of inflammation and oxidative stress in kidneys after limb ischemia-reperfusion [239]. Podocyte-specific ACE2 overexpression delayed the onset of albuminuria, preserved the podocyte number, attenuated the decrease in nephrin and the increase in TGF-β, and prevented glomerular hypertrophy in a STZ-induced diabetic mouse model [240]. Supplementing ACE2 to diabetic mice with either human recombinant ACE2 injection or ACE2-expressing adenovirus transfection reversed the extracellular matrix deposition and increased oxidative stress in glomeruli, along with a reduction in blood pressure and albuminuria [241, 242]. Recombinant ACE2 administration prevented the rise in circulating Ang II level as well as the rise in blood pressure after Ang II infusion [243]. Interestingly, Ang II could downregulate ACE2 through activation of the ERK1/2 and p38 MAPK pathways.
while simultaneously enhancing ACE expression, which was blocked by losartan [244]. This implies that a vicious cycle may be established after the initial insults and perpetuate kidney injuries.

**Role of Ang-(1-7) in kidney injuries**

The main product of ACE2 catalytic activity, Ang-(1-7) is a vasodilator, protects endothelial cells and has many functions that counter those of Ang II. In the kidney, Ang-(1-7) is shown to be mostly beneficial with some controversies. Less Ang-(1-7) was detected in the urine of patients with essential hypertension than in healthy subjects, suggesting a potentially reduced level of this peptide [245]. Ang-(1-7) infusion by osmotic minipump inhibited Ang II-induced glomerulosclerosis in rat with anti-Thy-1 antibody-induced glomerulonephritis [246]. In the stroke-prone SHR (SPSHR) rats, Ang-(1-7) reduced fibrotic change in glomeruli, restored nephrin expression and suppressed the renal content of proinflammatory factors IL-6, TNF-α and NFκB. These Ang-(1-7) infused SPSHR rats also had improved blood pressure and proteinuria [247]. Ang-(1-7) prevented kidney damage in diabetic animals [248, 249]. In wild-type and SHR rats with STZ-induced diabetes, Ang-(1-7) attenuated proteinuria, which may be at least partially mediated by reduced oxidative stress [249, 250]. The renoprotective effects of Ang-(1-7) have been observed in other disease models as well. Chronic infusion of Ang-(1-7) reduced blood pressure and proteinuria in SHR rats with L-NAME treatment [251], while Ang-(1-7) inhibitor A779 further exacerbated hypertension and inhibited the effects of captopril in this disease model [252].

Ang-(1-7) directly inhibits many injurious effects caused by elevated Ang II: High glucose-induced TGF-β upregulation, protein synthesis, and NADPH oxidase activation were inhibited by Ang-(1-7) [241, 253]. Ang-(1-7) blocked the activation of ERK1/2 and p38 MAPK in cultured rat proximal tubular cells and mouse mesangial cells with AngII stimulation, which translate into inhibition of TGF-β pathway activation [248, 254]. Ang-(1-7) also inhibited Ang II-induced mesangial matrix expansion, increased fibronectin and NADPH oxidase subunit expression, and albuminuria, which was reversed by A779 co-treatment [248].
However, some studies suggest there are no, or even detrimental effects in the kidney with Ang-(1-7) treatment. Inconsistent results have been published on the role of Ang-(1-7) in epithelial-to-mesenchymal transformation (EMT), as it may positively [255] or negatively [256] regulate EMT. There are also debates about its effect in the nephrectomy model, where Ang-(1-7) was reported to either improve or exacerbate cardiac damage after kidney tissue removal [257, 258]. Ang-(1-7) showed no beneficial effects on proteinuria or blood pressure in animals with adriamycin-induced acute kidney injury [258]. Shao and colleagues found that Ang-(1-7) infusion led to aggravated proteinuria, increased TGF-β and AT1 receptor levels and decreased AT2 and Mas levels in STZ-induced diabetic rats [259]. Contrary to expectation, infusion of Ang-(1-7) exacerbated kidney inflammation via activating the NFκB pathway and promoting production of IL-6, MCP-1 and macrophage infiltration in a mouse model of UUO, and mutations in the AT1A, AT1B or AT2 receptors did not change the detrimental effect of Ang-(1-7) infusion, indicating these may be mediated through the Mas receptor [260].

Role of Mas receptor in kidney injury

Mas is the endogenous receptor for Ang-(1-7) and mediates many of its functions in kidney. A few studies have investigated specifically the function of Mas in kidney injury. Increased Mas expression in the kidney was detected in rats with renal ischemia/reperfusion (I/R) and uninephrectomy accompanying a decrease in renal Ang-(1-7) level, suggesting Mas might be upregulated in this disease model as a compensatory mechanism [261]. Mas-null mice had a comparable degree of renal damage after I/R. However, Mas agonist, AVE0991, did reduce serum creatinine level and kidney neutrophil infiltration, as well as improving both glomerular and tubular injury index in mice with I/R [262]. Deletion of the gene for Mas in mice was associated with severe renal dysfunction as hyperfiltration, microalbuminuria, and reduced renal blood flow. These transgenic mice also developed glomerular atrophy, had increased fibrosis in both glomerular and tubulointerstitial compartments, and more TGF-β mRNA expression [263]. Absence of Mas was associated with worse hypertension in animals with 2K1C surgery, and antioxidants tempol and apocynin partially corrected blood pressure [264]. In contrast to the above cited studies, Esteban and colleagues reported better, rather than
worse, kidney outcomes in Mas KO mice after UUO and I/R surgeries [260]: Mas KO mice had lower blood urea nitrogen (BUN) level, less glomerular fibrosis and macrophage infiltration after UUO, while these animals also attenuated leukocyte infiltration, production of IL-6 and MCP-1 and activation of NFκB in response to I/R injury. Reasons for the seemingly inconsistent effects of the Ang-(1-7) and Mas in different renal injury models are not clear. Possible explanations include dose, route and duration of administration, different degree of local RAS activity and other unidentified receptor/ligand for Ang-(1-7)/Mas and the variations in model animals [31].

In Chapter 2, I will describe the effect of ACE2 gene deletion on renal ischemia-reperfusion injury, with a focus on the early inflammatory response, oxidative stress and cell apoptosis in the kidney. The rationale for this part of my work is based on the recognition that the RAS is activated and contributes to renal I/R injury.
Figure 1. The differential effects of activation in the ACE-Ang II-AT1 axis and the ACE2-Ang-(1-7)-Mas axis

The ACE2-Ang-(1-7)-Mas axis has been proposed as a counterbalancing mechanism to the ACE-Ang II-AT1 axis.
1.3 Adiponectin

1.3.1 Obesity and chronic kidney disease: epidemiology and mechanisms

**Epidemiology of obesity and CKD**

Many risk factors have been identified for CKD development, and it is recognized that obesity is a critical one among them. The definition of obesity is based on body mass index (BMI), where BMI between 18.5-24.9 kg/m² is defined as normal, 25 – 29.9 kg/m² as overweight and over 30 kg/m² as obese. Unfortunately the rate of obesity is rising and has become an epidemic worldwide. In the United States, age-adjusted prevalence of obesity has reached 35.5% and 35.8% among adult men and women respectively in the 2009-2010 National Health and Nutrition Examination Survey, and the 12-year statistics showed a significant linear trend of increase among men and certain ethnicities of women [265]. In Canada the obesity rate is staggeringly high as well, with nearly one quarter of the adult population qualified as obese in the 2007-2008 Canadian Health Measures Survey [266]. An even more troubling issue is childhood obesity. The 2009-2010 U.S. survey identified that 16.9% of children and adolescents aged 2 to 19 years were obese, and 12-year trend analyses also indicated a significant increase of obesity among males of this age group [267].

The prevalence of chronic kidney disease is rising parallel to that of obesity. It has been reported that there are over 50 million CKD patients worldwide who are susceptible to cardiovascular events, and may deteriorate to end-stage renal failure (ESRD) when they would require costly therapies like chronic dialysis or kidney transplantation [268]. Obesity is associated with risk factors for CKD such as hypertension and diabetes. Obesity contributes to the development of hypertension: In the Framingham offspring study, adiposity is associated with increased blood pressure in both men and women, and obese middle-aged women were at 7-times increased risk to develop hypertension [269]. In the Coronary Artery Risk Development in Young Adults (CARDIA) study, BMI is positively correlated with blood pressure in both men and women, regardless of their race [270]. Besides, obesity is also strongly associated with diabetes mellitus, another main cause of CKD [271]. Furthermore, research evidence indicates that obesity at younger
Age predisposes individuals to kidney disease late in life: a review of the CARDIA data found that higher BMI was linked to more rapid decline in eGFR after adjustment for age, race, sex, hyperlipidemia, smoking and physical activity [272].

**Obesity is a risk factor of CKD development: epidemiological evidence**

A series of epidemiological studies have been performed in an effort to determine the relationship between obesity and kidney disease, and a positive association emerges between increased BMI and the likelihood of developing CKD and ESRD. From a vast record database of a health care delivery system in northern California, Hsu and colleagues analyzed the relative risk (RR) of ESRD in individuals from different weight groups, and identified an increasing susceptibility to renal failure with higher BMI. The adjusted RR was 1.87 for subjects with BMI between 25 and 29.9 kg/m² compared to normal weight controls, 3.57 for subjects with BMI between 30 and 34.9 kg/m², 6.12 for subjects with BMI between 35 and 39.9 kg/m², and 7.07 for subjects with BMI over 40 kg/m² [273]. Using the data from the Framingham Heart Study, Foster and colleagues calculated that there was a 68% higher odds of developing stage 3 CKD (as defined by estimated glomerular filtration rate (eGFR) < 59 ml/min/1.73m²) in obese participants than normal weight participants. Overweight individuals showed no additional risk of developing stage 3 CKD compared to normal weight individuals in this study [274]. Increased BMI was also reported to be positively associated with risk of ESRD in a Japanese study, in which subjects in the highest weight group (BMI ≥ 25 kg/m²) had almost double the incidence of ESRD than subjects in the lowest weight group (BMI < 21.0 kg/m²) [275]. A Swedish study that recruited 926 chronic renal failure patients and 998 control subjects found that individuals who were overweight at age 20 had 3-fold increased risk for chronic renal failure compared to those with BMI less than 25 kg/m², whereas men with obesity and women with morbid obesity (BMI > 35 kg/m²) any time in life were 3 to 4 times more likely to develop chronic renal failure. The study also confirmed that the increased risk in obese subjects could be observed in all major types of CKD, with a strongest association with diabetic nephropathy [276]. Bonnet and colleagues examined the effect of obesity in a group of patients with IgA nephritis and observed that individuals with BMI ≥ 25 kg/m² had more severe kidney lesion.
Overweight IgA patients were also at increased risk of developing arterial hypertension and chronic renal failure [277]. In kidney transplant patients, a 5% or more increase in BMI 1 year post-transplantation was associated with a significantly higher rate of graft loss, suggesting that excessive weight gain may impair graft survival [278]. Also in patients with kidney transplant, incidence of cardiac events, including congestive heart failure, atrial fibrillation and myocardial infarction, was much higher in the highest BMI quartile (29.35%) compared to lowest BMI quartile (8.67%) [279].

In contrast, weight loss improves kidney function. A follow up study of extreme obese (BMI ≥ 40 kg/m²) patients after bariatric surgery demonstrated that better 24-hour albuminuria measurement correlated with reduced body weight [280]. Similarly in overweight patients with proteinuric nephropathies, a low-calorie diet lowered proteinuria and stabilized renal function together with decreasing body weight [281].

**Higher body weight is associated with better survival in CKD patients**

However, higher body weight is associated the higher survival rates in CKD patients and dialysis patients. In a cohort of unselected chronic renal failure patients in Sweden, an epidemiological study discovered a correlation between high mortality rate with low BMI [282]. An Austrian group of researchers investigated the relationship between body weight and cardiovascular mortality in patients with mild and moderate CKD, reporting that hazard ratio (HR) was highest in leaner patients with moderate CKD (GFR = 45 ml/min/1.73 m²) and decreased as BMI rose (HR = 1.28 for BMI = 20 kg/m² vs 25 kg/m²; HR = 0.76 for BMI = 30 kg/m² vs 25 kg/m²; HR = 0.58 for BMI = 35 kg/m² vs 25 kg/m²). The association became even steeper in proteinuric patients with moderate CKD, where HR lowered from 9.43 in patients at BMI = 25 kg/m² to 3.74 for those at BMI = 30 kg/m² and 1.95 for patients at BMI = 35 kg/m² [283]. Negative correlation was reported for body weight and all-cause mortality in a group of male US veterans with CKD, in which individuals with BMI above the 90th percentile had an adjusted HR of 0.39 compared to individuals with BMI below the 10th percentile [284]. Similar results have been reported in dialysis patients. Among 1,453 hemodialysis patients from French dialysis centers, individuals with low rather than high BMI and cholesterol levels had increased risk of overall mortality and cardiovascular mortality [285]. In a large observational study using
data from 418,055 hemodialysis patients in the US Renal Data System, Johansen and colleagues examined a wide range of BMI and survival over 2-year follow up. Risks of hospitalization and mortality were reduced in individuals with increased BMI, and held true even for individuals with extreme high BMI. However, the trend did not apply to patients of Asian ethnicity [286]. For peritoneal dialysis (PD) patients, a small scale study indicated that overweight (BMI > 27.5 kg/m\(^2\)) PD patients had an overall survival advantage compared with normal weight (BMI between 20 – 27.5 kg/m\(^2\)) patients, but were not more protected against cardiovascular events [287]. Even in patients post renal transplantation, some epidemiological studies found no increased risk of graft loss and obesity [288, 289].

The paradoxical survival benefit of increased BMI in patients with kidney disease is not completely understood. It may be that higher body weight reflects better nutrition so that heavier patients can adapt better to the chronic illness and protein restricted diets. An alternative hypothesis is that BMI is not a good measurement of obesity since it does not distinguish between fat and muscle content. Indeed, it is suggested that within a group of healthy male subjects, increased BMI was an independent risk factor for CKD [290]. In a cohort study of 13,324 patients, an increase in the waist-to-hip ratio, but not BMI, was related to higher risk of CKD and worse combined outcome of CKD and all-cause mortality in both male and female participants [291]. Whereas BMI was not found to be predictive of all-cause mortality, a group of researchers did reveal a positive link between increased waist circumference and mortality in post-transplant patients even after adjusting for BMI [289]. These studies suggest that a better way to quantify obesity will be helpful to clarify its effects on kidney health.

**Mechanism linking obesity and CKD: hyperfiltration**

One potential mechanism linking obesity and CKD is hyperfiltration. The diagnosis of CKD is based on the reduction of kidney function, which is commonly measured by glomerular filtration rat (GFR). CKD is presented as having decreased GFR, with or without albuminuria [292]. In obesity, the tissue blood flow, arterial pressure and cardiac output all rise [293], and since the number of nephrons in humans does not change after birth, the increasing volume load demands an increase in single-nephron GFR. This fixed
nephron number also indicates that people who have fewer nephrons at birth, which has been shown to have some dependency on prenatal nutritional availability and birth weight, are more susceptible to develop hypertension and kidney disease with obesity [294]. The contributing role of hyperfiltration to CKD development is confirmed in animal models of subtotal nephrectomy, where rats with renal ablation experienced an increase in single-nephron GFR and suffered an array of glomerular damages, including accumulated intracellular protein reabsorption droplets in podocytes, podocyte foot process effacement and mesangial expansion. These pathological changes were resolved in nephrectomized rats on a low protein diet which did not experience the raised ΔP or initial glomerular plasma flow rate [295].

That hyperfiltration occurs in obesity has been verified by Chagnac and colleagues, when they compared the GFR, effective renal plasma flow (ERPF) and filtration fraction (GFR to ERPF ratio) and dextran sieving coefficient (an approximation of ultrafiltration coefficient $K_f$) profiles of obese individuals (mean BMI = 35 kg/m$^2$) without diabetes and control individuals at normal weight (mean BMI = 22 kg/m$^2$) [296]. GFR is calculated as: $GFR = K_f \times (\Delta P - \Delta \Pi)$, where $\Delta P$ and $\Delta \Pi$ are the respective difference in hydrostatic pressure and oncotic pressure within the glomerular capillary and Bowman’s space. In Chagnac’s study, $\Delta P$ was determined as the best fit to the dextran sieving coefficient profile calculation model among different values (35, 40 and 45 mm Hg), on the basis of which GFR, ERPF and filtration fraction were estimated. The obese group in this study had a $\Delta P$ closer to 40 mm Hg, whereas the intraglomerular hydrostatic pressure difference in non-obese group was closer to 35 mm Hg. The GFR, ERPF and filtration fraction were all higher in the obese group as well. In another interesting study on BMI and renal function, it was found that BMI was positively associated with both GFR and filtration fraction in healthy young adult male subjects (BMI 23.0 ± 2.5 kg/m$^2$) on a high-salt diet, further demonstrating that adiposity is a modifier of kidney function not only in overt obesity [297].

**Mechanism linking obesity and CKD: podocyte injury**

The renal histopathological phenotype of obese kidney disease patients is recognized as “obesity-related glomerulopathy” that is observed in biopsy specimens alone or with
focal segmental glomerulosclerosis [298]. The common lesions include glomerulomegaly, mesangial matrix expansion, podocyte hypertrophy, podocyte foot process effacement, glomerular basement membrane thickening, and fibrosis in the renal interstitium [298]. It has been hypothesized that the attempts of podocytes to adapt to increased glomerular size, which was shown to be proportional to body size, may lead to glomerular injury and consequently proteinuria [299]. The need to cover a larger area may prompt podocytes to hypertrophy but podocyte exhaustion and inadequate coverage may cause foot processes to become detached from the basement membrane. A study by Wiggins and colleagues elegantly described this progress in aging Fischer 344 rats that spontaneously developed proteinuria and glomerulosclerosis when provided with free access to food [300]. Podocytes in these rats firstly adapted to the increasing glomerular size with body weight growth by both hypertrophy and hyperplasia, which was followed by a stage when hypertrophy continued but podocyte number stopped to change. With the growing trend uncurbed, glomerulosclerosis and proteinuria finally happened when podocytes could no longer increase their size in proportion to glomerular enlargement. With human renal biopsy samples, lower podocyte density and fattening of the podocyte foot processes were also observed in patients of obesity-related glomerulopathy [301].

**Mechanism linking obesity and CKD: RAS activation**

As detailed in the previous section, RAS activation is perhaps the most important underlying cause of CKD. Adipose tissue possesses a complete local RAS system and RAS activation is shown in obesity. Groups of investigators confirmed that AGT, renin, ACE, ACE2, AT1 and AT2 receptors, and the renin receptor were expressed to varying extents by adipocytes and stromal cells [302-305]. Non-classical processing enzymes for AGT, the chymase and cathepsins, and enzymes for Ang II degradation, endopeptidase and aminopeptidase, were also detected in adipose tissue, revealing that there is a complex and tightly controlled local RAS which may be a vital part of normal functional regulation in adipose tissue [306, 307]. The adipose RAS seems to be independently maintained from the systemic RAS, for example, renin levels in fat tissue are different from that in the circulation although tissue AGT level does show some correlation with plasma levels [308]. Adipose RAS responds differently to high salt intake than systemic
RAS and does not seem to be part of the feedback loop in maintaining sodium and blood pressure homeostasis [309]. The expression of AGT in adipose tissue is in turn sensitive to changes in nutrients such as fatty acids, hormones like insulin and androgens, as well as the pro-inflammatory cytokine TNF-α [310-312].

Although gaps still exist in our understanding of the effects of local RAS, it has been implicated in regulating energy storage in fat tissue. Feeding upregulated AGT in adipose tissue, which resulted in higher local Ang II concentration [313]. Ang II inhibited lipolysis both directly by engaging the AT1 receptor on adipocytes and by causing vasoconstriction again through the AT1 receptor [314, 315]. In addition, Ang II increases lipid content in adipose tissue further by promoting membrane translocation of the lipid transporter, facilitating glucose uptake through interactions with insulin signaling, and upregulating the adipocyte expression of lipogenic enzymes glycerol-3-phosphate dehydrogenase and fatty acid synthase [316-318]. Besides being involved in lipid metabolism in adipose tissue, RAS modulates cell proliferation of adipocytes. Expression of AGT is initiated as preadipocytes differentiate into mature adipocytes, and absence of AGT impedes preadipocyte differentiation and lipid accumulation [319]. In contrast, Ang II functions as a negative regulator of adipogenesis, which was reported to be dependent on AT1 receptor in preadipocytes but on AT2 receptor in mesenchymal stem cells [320, 321]. The anti-adipogenic effect of Ang II was also studied in the context of obesity. The differentiation of primary isolated preadipocytes from obese individuals was suppressed to a larger degree by Ang II incubation than the preadipocytes from normal weight individuals, and this effect was reversed by the AT1 receptor blocker losartan [322]. The ability of adipocytes to proliferate is critical for their function and overall metabolism, as insufficient adipogenesis in proportion to energy influx represents a form of lipodystrophy, which impairs the endocrine functions in adipose tissue, increases metabolic burden in other organs and may initiate type 2 diabetes mellitus [323].

Excess energy stimulates expression of RAS in adipose tissue, which can in turn contribute the increase in RAS activity at a systematic level. A transcriptome study in healthy adult male subjects who were overfed with a lipid-enriched diet for 2 months showed a time-dependent increase in gene expressions of AGT, ACE and aminopeptidase
A in subcutaneous adipose tissue [324]. On the contrary, a 5% reduction in body weight was associated with a 20% decrease in AGT level in fat tissue [325]. The circulatory level of RAS components such as AGT, renin and ACE rose with increase in BMI and were lowered by weight loss, and this indicated that systemic RAS is overactivated in obesity [325, 326]. Previous experimental data suggest that adipose tissue is an important contributor of circulating Ang II: The AngII concentration in the venous drainage of abdominal subcutaneous fat tissue is 23% higher than that in the arterial vessel, an evidence that fat tissue does produce a significant amount of Ang II [310]. Organ-specific deletion of the AGT gene with an aP2 promoter-driven Cre recombinant system resulted in a 24 – 28% reduction in AGT level in plasma and hypotension in normal chow-fed transgenic mice as compared to wild-type control animals [327]. Conversely, adipose tissue-specific overexpression of AGT in wild-type mice raised plasma levels by 30% and also caused hypertension, while localized adipose tissue overexpression of AGT in whole body AGT knock-out mice restored 20 – 30% of its circulation level and more importantly, normalized the blood pressure in these transgenic mice [328].

Additional to its role as a potential driving force for high systemic angiotensinogen concentration and inducing hypertension, adipose RAS overactivation may be linked to adipose tissue inflammation in association with obesity [329]. Overproduction of AGT in adipose tissue upregulated the local expression of pro-inflammatory cytokines MCP-1 but suppressed the expression of the anti-inflammatory cytokine IL-10, which was accompanied with glucose intolerance and insulin resistance in transgenic mice [330]. In cultured adipocytes, MCP-1 and resistin secretion were also higher after Ang II stimulation, and this was prevented by inhibiting NFκB or NADH oxidase signaling [330]. On the other hand, RAS blockade with either ACEI or ARB attenuated adipose tissue inflammation induced by high fat-diet and improved insulin sensitivity in peripheral tissue, further supporting that the adipose RAS is a critical mediator of pathogenesis in this organ [331, 332].
**Mechanism linking obesity and CKD: adipokines**

**Leptin**

It is now well recognized that adipose tissue has important endocrine functions and secretes a variety of bioactive factors that are known as adipokines. Amounting research data have emerged in support of the notion that adipokine dysregulation is a critical part of obesity related kidney disease development. Among these adipokines, leptin and adiponectin are the best characterized. (The biology of adiponectin will be discussed in detail in the next section.) Leptin is primarily an appetite suppression hormone secreted by the white adipose tissue, as mice with genetic mutation in either leptin (the ob/ob mice) or leptin receptor (the db/db mice) become obese due to hyperphagia and suffer diabetic nephropathy [333]. The leptin receptor belongs to the class I cytokine-receptor family and mainly signals through the janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway and the MAPK pathways [334]. Many factors have been implicated in leptin expression regulation, including insulin, glucocorticoids, TNF-α and IL-1, which increase its circulating level, and coldness, fasting, exercise, growth hormone, somatostatin, smoking and androgens, which decrease leptin levels [335].

Epidemiological studies support a positive association between both obesity and kidney injury and leptin levels. Due to its role on appetite regulation, epidemiological studies have been done to measure plasma leptin level in obese subjects, and found that leptin resistance but not deficiency was the major pathological issue in these individuals [336]. Compared to normal weight subjects, serum leptin levels are 5 – 10 times higher in obese individuals. Higher serum leptin level were detected in renal failure patients as well, which was likely due to inefficient clearance via megalin and was not attenuated by hemodialysis or peritoneal dialysis [337-339]. Moreover, observational studies suggested that leptin levels increased more steeply per unit increase of BMI in patients with ESRD than control subjects with normal kidney function, which further jeopardized the health of ESRD patients by causing anorexia and cachexia, and predisposed them to infection by disrupting neutrophil chemotaxis in the presence of uremia [340, 341]. Leptin levels were rapidly restored to normal level after successful renal transplantation, but the more prolonged effect was not reported [342].
Several mechanisms have been proposed to explain the role of leptin in mediating obesity-associated kidney injury, and one important mechanism is hypertension and sympathetic nervous system (SNS) overactivation. In obesity, the effects of leptin on appetite suppression and SNS activation in the central nervous system are dissociated from each other [343], and leptin contributes to hypertension because of its action in enhancing SNS activation. The SNS overactivation associated with obesity can negatively impact the kidney even independent of hypertension, as rats with subtotal nephrectomy show amelioration of glomerulosclerosis and proteinuria with SNS output blockade [344]. Skinny mice exposed to chronic leptin infusion or genetically manipulated to overexpress leptin show SNS activation in kidney, more plasma and urine catecholamines and higher blood pressure [345, 346]. In humans, leptin is positively correlated to blood pressure in men after correcting for age, BMI, and insulin resistance [347].

In addition to its link to hypertension, leptin is a potential regulator of renal fibrosis and inflammation as well. Leptin was able to upregulate the expression of TGF-β in glomerular endothelial cells and the expression of TGF-β type II receptor in neighboring glomerular mesangial cells [348, 349]. Infusion of leptin in animals caused increased levels of type I and type IV collagen expression and deposition in the glomeruli parallel to the increased TGF-β signal, as well as proteinuria. Hsu and colleagues also showed that long-term treatment with leptin rendered renal proximal tubular cells susceptible to apoptosis, which was likely to be mediated through COX-2 and prostaglandin E(2) (PGE(2)) [350]. Leptin plays an important role in regulating immune function, as it promotes chemotaxis and oxygen radical release in neutrophils and phenotypic shifting biased to Th1 versus Th2 in T lymphocytes [351, 352]. Because of its role in immunity, leptin may affect disease progress in acute kidney injury and immunity mediated nephropathy. Indeed leptin deficient ob/ob mice had reduced kidney damage and albuminuria compared to wild-type control animals in a model of nephrotoxic serum-induced nephritis [353].

**Resistin, visfatin and TNF-α**
Other adipokines have also been implicated in kidney diseases. Resistin is a low molecular weight protein (molecular weight: 12.5kDa) secreted mainly by adipose macrophages and by adipocytes to a smaller extent [354]. Plasma levels of this protein rise with adiposity, and this increased level of resistin has been related to increased inflammatory markers and reduced GFR in CKD patients [355]. Resistin negatively correlated with eGFR but positively to the albumin-to-creatinine ratio in a family of non-diabetic, untreated individuals, suggesting that it may play a role in kidney function modulation [356]. Given that the major sources of resistin are macrophages, its role in regulating tissue inflammation has been investigated. Bokarewa and colleagues reported that resistin had strong inflammation inducing effects in rheumatoid arthritis [357]. An in vitro study found that recombinant resistin could activate cultured endothelial cells and promote the production of pro-inflammatory cytokines MCP-1 and adhesion molecule VCAM-1, where endothelin-1 (ET-1) was potentially involved [358]. Resistin derived from infiltrating macrophages in atherosclerotic aneurysms has been proposed to impact on endothelial cells by upregulated ET-1 and PAI-1, and on vascular smooth muscle cells (VSMCs) by promoting cell migration [359]. In patients with essential hypertension, plasma resistin levels were positively associated with circulating levels of ET-1 and inflammatory markers such as TNF-\( \alpha \), IL-6 and von Willebrand factor (vWF) [360]. ET-1 is important in kidney function besides its effect on blood pressure. Transgenic mice overexpressing ET-1 manifested glomerulosclerosis, tubulointerstitial fibrosis, decreased GFR and renal cysts in an age-dependent but hypertension-independent manner [361]. Therefore it is possible that resistin may contribute to kidney injury through the action of ET-1 and pro-inflammatory cytokines.

Visfatin, also identified as pre-B cell colony-enhancing factor, is a 52-kDa adipokine the expression of which has been correlated with obesity [362]. Higher circulating levels of visfatin have also been observed in T2DM patients than in control subjects [363]. Visfatin is closely associated with inflammation and endothelial dysfunction in CKD, as it was higher in kidney allograft recipients and was correlated with VCAM and high sensitive C-reactive protein (CRP) [364]. In glomerular endothelial cells, visfatin enhanced the assembly of NADPH oxidase in the lipid raft and led to further superoxide generation, which resulted in increased permeability of cultured glomerular endothelial
cell layers [365]. Glucose stimulated the expression of visfatin in glomerular mesangial cells, where it in turn promoted glucose influx via increasing the expression and membrane translocation of GLUT-1 receptors [366]. Treatment with visfatin also significantly increased production of the pro-fibrotic factors TGF-β, PAI-1 and type I collagen in mesangial cells. In addition to acting as a profibrotic factor, Huang and colleagues demonstrated that the various RAS components, renin, AGT and AT1 receptor, were upregulated in cultured rat mesangial cells after visfatin incubation, which suggested that visfatin may contribute to the development of diabetic nephropathy at least in part by activating the intraglomerular RAS [367]. However further research to characterize the in vivo role of visfatin in kidney disease is necessary.

Another cytokine secreted by fat worth mentioning is TNF-α, a well-known pro-inflammatory cytokine involved in many types of tissue damage. Adipose tissue macrophages are significant sources of adipose TNF-α, and in obesity, this cytokine may be produced indigenously in kidney through the actions of Ang II, advanced glycation end products (AGEs) and oxidized low-density lipoprotein (LDL) [368]. For instance, a group of investigators showed that neutralization of TNF-α using a soluble TNF type 1 receptor suppressed the activation of NFκB signaling and expression of inflammatory markers and TGF-β in the remnant kidney in rats after subtotal nephrectomy [369]. The hypertension and albuminuria associated with renal failure were also ameliorated. This suggests that TNF-α may be a risk factor in obesity related kidney injury.

With the understanding that dysregulation of adipokines is one of the most important pathogenic mechanisms underlying obesity-associated kidney injury, Chapter 3 and 4 are devoted to the study of the adipokine adiponectin in kidney injury.
Figure 1. 4 Summary of the major mechanisms by which obesity contributes to kidney injury
1.3.2 Diabetic nephropathy (DN)

Pathological features of DN

There is a strong association between obesity and diabetes. Kidney disease is a common complication of diabetes with almost one third of T2DM and T1DM patients developing renal dysfunction [370]. Diabetic nephropathy is probably the most prevalent form of CKD and is the leading cause of ESRD globally [371]. The most preeminent pathological feature of DN is the structural changes in glomeruli, including glomerular hypertrophy, thickening of the glomerular basement membrane (GBM), mesangial matrix expansion and glomerulosclerosis [372]. Ultrastructural changes such as podocyte foot process effacement, and podocyte apoptosis are also detected in DN, which cause defects in the filtration barrier and contribute to albuminuria. Hyalinosis of the afferent and efferent arteriole may additionally accompany the glomerular changes.

The tubulointerstitium is not spared in DN, and it has been proposed that tubular cell atrophy and apoptosis in the early stage of DN may be more severe than previously assumed. By overexpressing catalase specifically in the proximal tubular cells, Brezniceanu and colleagues demonstrated that there was profound angiotensinogen upregulation and ROS overproduction in the tubular compartment in STZ-induced diabetic mice [373]. Tubulointerstitial inflammation and fibrosis are also observed in later stages of DN. In a cohort of T1DM patients with nephropathy, Najafian and colleagues described injuries at the glomerulotubular junction that resulted in atubular glomeruli [374]. This may represent atrophy of the tubules, which may be involved in disease progression. In addition, Kumar and colleagues reported that apoptotic cells were detected in the tubules, endothelium or interstitium, but interestingly not in the glomeruli, in a small group of DN patients, again suggesting that serious tubulointerstitial damage is indeed associated with DN [375]. A rare form of tubular cell vacuolization, the Armanni-Ebsterin cells, has been observed in DN as well.
**Pathogenic mechanism of DN: polyl and hexosamine hypotheses**

Pathogenesis of DN is complex and much research efforts have been put into understanding the molecular mechanisms of kidney injury in the context of diabetes. The most obvious abnormality in diabetes is the increase in blood glucose level. The many effects that hyperglycemia can cause cells have been thoroughly reviewed by Brownlee [376]. It has been shown that exceedingly large amount of glucose influx lead to glucose shunting away from the glycolysis pathway into the polyl and hexosamine pathways, both of which further interact with other factors implicated in DN pathogenesis that may cause tissue damage.

Through the polyl pathway, glucose is first converted into sorbitol then to fructose, which consumes NADPH in this process. Decreased intracellular NADPH content contributes to oxidative stress. Involvement of this pathway in DN has been proved by studies with pharmacological inhibitors of aldose reductase, the first enzyme in polyl pathway, zenarestat, which showed beneficial effects in patients with diabetic neuropathy [377]. Transfection with aldose reductase expressing vectors in cultured human mesangial cells enhanced the expression of the profibrotic factor fibronectin through activation of the JNK and Akt signaling pathways [378].

In the hexosamine pathway, glucose is metabolized to generate UDP-N-acetylglucosamine, which is used as a substrate for proteoglycan and O-linked glycoprotein synthesis. In bovine aortic endothelial cells, hyperglycemia was associated with increased activity in the hexosamine pathway, which in turn upregulated the expression of TGF-β1 and PAI-1 through glycosylating and activating the transcription factor Sp-1 [379]. Similarly in glomerular mesangial cells, activation of the hexosamine pathway enhanced the expression of PAI-1 via Sp-1, as well as the conversion of latent TGF-β into its functional form, which was implicated in mesangial cell proliferation and matrix protein accumulation [380, 381].

**Pathogenic mechanism of DN: AGEs**

Advanced glycation end-products (AGEs) are thought to be the products of non-enzymatic glycosylation of proteins. It is now recognized that the reactive intracellular
Dicarbonyls generated from influxed glucose play key roles in the initiation of AGEs [382]. AGEs can cause cellular damage in several ways: by modifying intracellular proteins and disrupting the proper functions of these proteins, by modifying extracellular proteins so that the interactions between the matrix components and that of matrix to cells are altered, and by activating AGE receptors and inducing oxidative stress and inflammation [376].

Experimental results indicate that enhanced production of AGE is associated with tissue damage while inhibition of these molecules ameliorates injury. In diabetes, AGE accumulates because of increased production and decreased clearance, and has been shown to predict the development of diabetic complications such retinopathy and nephropathy [383, 384]. AGE modification of type IV collagen and laminin impairs the cross-linking of these matrix proteins, and AGE-induced glycosylated type IV collagen was found to be more resistant to matrix metalloproteinase (MMP) degradation [385-387]. Hence altered matrix-matrix interactions due to hyperglycemia-stimulated increase in AGE may contribute to the development of GBM thickening and glomerulosclerosis. Besides, AGE modified circulating and matrix proteins can interact with cells through AGE receptors. In endothelial cells, binding of AGE to its receptor RAGE promoted inflammation through NFκB and upregulated the adhesion molecule VCAM-1 [388]. Altered albumin was shown to stimulate the secretion of macrophage-colony stimulating factor (M-CSF) by monocytes in vitro, which may be involved in diabetic complications in vivo [389]. Glomerular mesangial cells express AGE receptors and stimulation with AGEs raised levels of fibronectin and type IV collagen in a platelet-derived growth factor (PDGF)-dependent way [390]. Conversely, treatment with the AGE inhibitor aminoguanidine in rats with STZ-induced diabetes ameliorated albuminuria, reduced the deposition of collagen in isolated glomeruli and tubules and prevented mesangial matrix expansion [391]. In the spontaneous diabetic Otsuka-Long-Evans-Tokushima-Fatty (OLETF) rats, a thiazolidine derivative OPB-9195 was able to inhibit the formation and accumulation of AGEs in kidney, which further slowed the progression of glomerulosclerosis and alleviated the albuminuria without affecting blood glucose levels [392].
Enhanced presentation of AGEs and lipoxidation products that were co-localized with area of glomerulosclerosis and nodular lesions have been well documented in biopsy samples of patients with DN [393]. In a randomized trial in T1DM patients with renal complications, treatment with an investigative AGE inhibitor pimagedine appeared to slightly retard the progression of kidney disease above the effects of ACEI and ARB therapies [394]. This indicated that blocking the formation of AGE may prevent the development and progression of diabetes complications.

**Pathogenic mechanism of DN: PKC pathway**

Increased glycolysis leads to higher level of diacylglycerol (DAG), an intermediate byproduct, which in turn activates the protein kinase C (PKC) pathway. Increased ROS and AGEs interacting with RAGE receptors have also been shown to upregulate PKC activity which then can feed back to the process and further enhance the oxidative and carbonyl stress [395]. There are more than 10 isoforms of PKC and PKC-β seems to the predominant isoform activated in diabetic glomeruli. Hyperglycemia in STZ-induced diabetic rats activated PKC in vivo in glomeruli, which was prevented by oral feeding of the animals with a PKC-β inhibitor LY333531. Increased expression of TGF-β, fibronectin and type IV collagen were also inhibited by LY333531 [396]. In a following study, Koya and colleagues studied the effect of LY333531 in a mouse model of T2DM [397]. In db/db mice, PKC activation was involved in the impaired glomerular response to nitric oxide (NO), which may contribute to diabetes-associated kidney damage. In contrast, PKC inhibitor treatment reduced glomerular mesangial matrix expansion, glomerular levels of TGF-β, fibronectin and type IV collagen, as well as albuminuria, which suggested that PKC inhibitors may be effective therapies in DN treatment [398].

The specific effects of PKC have been explored in glomerular mesangial cells and endothelial cells. In glomerular mesangial cells, ET-1 induced activation of MAPK was further enhanced by high glucose incubation. This effect was blocked by inhibition of PKC with phorbol ester [399]. Hence PKC may mediate the mesangial cell hypertrophy after high glucose stimulation. Both PKC activation and increased fibronectin expression were demonstrated in cultured rat mesangial cells with high glucose treatment, and PKC agonist mimicking the effect of high glucose on fibronectin overproduction [400].
endothelial cells, PKC may play an important role in promoting inflammation, as the selective PKC inhibitor calphostin C was shown to prevent the activation of NFκB signaling in response to high glucose treatment [401].

**Pathogenic mechanism of DN: ROS**

The seemingly independent pathways described above may have a common downstream effector in mediating diabetic injury through increased generation of ROS. On the other hand, Nishikawa and colleagues showed that preventing the rise in intracellular ROS concentration inhibited the activation of polyol, AGE and PKC pathways, which then reduced the inflammation via suppressing the NFκB signaling [402]. In the same study, the investigators also demonstrated that mitochondria were a major source of ROS in diabetes, as overexpression of either the mitochondrial superoxide dismutase manganese superoxide dismutase (MnSOD) or the uncoupling protein-1 (UCP-1) was able to inhibit the hyperglycemia-stimulated ROS signaling. The molecular mechanism underlying the effect of ROS may rely on the diminished GAPDH activity, which leads to accumulation of glucose metabolic products and contribute to the activation of the aforementioned pathways [379]. More specifically in the kidney, overexpression of either the MnSOD (SOD-2) or the Cu²⁺/Zn²⁺ SOD (SOD-1) reduced superoxide contents and the expression of collagen in mesangial cells incubated with high glucose [403, 404]. In vivo, overexpressing human SOD in mice normalized the various injury markers measured in kidneys after STZ-induced diabetes, including mesangial matrix expansion and accumulation of TGF-β, type IV collagen and the oxidative marker malondialdehyde (MDA) in kidney. Albuminuria and increased plasma creatinine levels were also lower in transgenic mice compared to wild-type control animals [404]. Similar attenuations of glomerular mesangial matrix expansion, deposition of TGF-β and type IV collagen, and oxidative stress as measured by MDA and nitrotyrosine were observed in T2DM db/db mice transgenic for human SOD1 [405]. Additionally, the investigators demonstrated that increased SOD1 activity could improve the renal filtration function via a NO-dependent manner.
1.3.3 Biology of adiponectin

Adiponectin molecule and oligomers

Adiponectin was originally independently identified by 4 groups, and was aliased Acrp30 [406], AdipoQ [407], apM1 [408] and GBP28 [409]. The 30 kDa protein is maintained at a physiological concentration of 0.5 – 30 μg/ml in plasma, making it one of the most abundant proteins in circulation. On average females have higher plasma adiponectin levels than males, and testosterone suppresses the secretion of adiponectin [410, 411]. The physiological roles of adiponectin are yet to be fully elucidated, but it is an important metabolic regulator as adiponectin increases fatty acid beta oxidation and glucose uptake in skeletal muscles and inhibits gluconeogenesis in liver through the AMP-activated protein kinase (AMPK) [412]. The structure of adiponectin is roughly divided into a signal segment that designates the protein for secretion, a C-term globular domain that shares similarity with the Complement factor C1q and TNF-α, and a N-term tail domain that is homologous to collagen [413]. The globular domain is functionally important in signaling and the tail domain is involved in building the secondary and tertiary structures.

Different forms of adiponectin complexes have been found at physiological conditions in the body. Adiponectin oligomerizes to form a trimer, hexamer and high molecular weight (HMW) protein complexes that are stabilized by disulfide bonds [411]. The formation of adiponectin multimers requires a pair of endoplasmic reticulum resident proteins ERp44 and Ero1-Lα, and knock-down of either protein disrupt the secretion of the HMW oligomer [410]. ERp44 directly interacts with adiponectin and retains the HMW complex in the ER. Ero1-Lα functions to displace the HMW adiponectin from ERp44 and facilitates its release which has been proposed as the mechanism of PPARγ agonist-induced HMW adiponectin upregulation. Adiponectin may be cleaved in vivo to generate fragments of the globular domain (gAD), which has been shown to circulate in serum and exhibits the same enhancing effect on fatty acid oxidation in muscle [414]. The enzymatic process of gAD generation in the body is unclear, but in vitro experiments demonstrated that monocyte derived leukocyte elastase was able to produce the globular fragment from full-length adiponectin [415].
The proportions of full-length, low molecular weight (LMW) and HMW oligomers are relatively constant under normal conditions even with dietary change, however the different forms of adiponectin are not functionally equivalent, with the HMW complexes being the most bioactive [410, 411]. The various oligomers may also have differential effects in disease, for example HMW adiponectin was able to suppress the activation of NFκB in response to TNF-α stimulation whereas globular adiponectin strongly activated NFκB and enhanced the expression of proinflammatory cytokines in vascular endothelial cells, suggesting that degradation of adiponectin could mediate inflammation in vivo [416]. HMW multimer levels were better correlated with glucose tolerance, thiazolidinedione (TZD)-induced improvement in hepatic insulin sensitivity, and high-density lipoprotein (HDL) concentration, and were more sensitive to weight loss and insulin treatment than total adiponectin level [417-419]. Kobayashi and colleagues also showed in vitro that the HMW adiponectin complex prevented human umbilical vein endothelial cell (HUVEC) apoptosis induced by serum starvation via activating the AMPK signaling pathway, which was not seen with other adiponectin forms [420]. Considering these data, it seems that the formation of HMW multimers is more important in the context of metabolic derangement and pathological processes and it has even been proposed that LMW may compete and antagonize the effect of HMW complexes [419]. Further studies are warranted to shed more light on the roles of the various adiponectin oligomers.

**Adiponectin receptors: adipoR1, adipoR2, T-cadherin**

Two major receptors have been discovered for adiponectin: the adiponectin receptor 1 (AdipoR1) and receptor 2 (AdipoR2). Specificities lie in the N-terminal end of the receptors, with the rest of the protein sequences sharing much homology between the two receptors [421, 422]. Distribution of AdipoR1 is ubiquitous in the body with the highest abundance in skeletal muscle, while the distribution of AdipoR2 is more concentrated in the liver. Both the adipoR1 and adipoR2 are seven-transmembrane proteins that have a unique reversed topology compared to the G protein coupled receptors, as the N-terminal end of adiponectin receptors are intracellular. AdipoR1 binds preferentially to globular adiponectin than to full-length adiponectin, and adipoR2 has intermediate
affinity for both adiponectin molecules. Homodimers and heterodimers have been found for the adiponectin receptors as well [423]. Several signaling pathways have been reported for the adiponectin receptors, but the primary signal transduction cascade is the AMPK pathway downstream of adipoR1 and the peroxisome proliferator-activated receptor (PPAR)-α pathway downstream of adipoR2. Adiponectin has been shown to stimulate MAPK and Akt signaling activities as well. AdipoR1 and adiponectin have been detected in the cytoplasm suggesting that the receptors may be internalized together with the ligand [424]. The endocytosis of adipoR1 is dependent on clathrin, since disrupting the clathrin endocytosis process by mutant Eps15 overexpression, potassium depletion and dominant-negative Rab5 expression all impaired the internalization of adipoR1. Mutant Eps15 and potassium depletion-caused adipoR1 endocytosis disruption enhanced phosphorylation of AMPK and ACC after adiponectin treatment, suggesting that internalization of AdipoR1 may be a way to downregulate its signaling.

More recently another binding partner for the hexamer and HMW adiponectin complexes, T-cadherin (T-cad), has been identified [425]. T-cad is structurally different from the classic cadherin molecules as it lacks a cytoplasmic domain and is anchored on the cell surface via a glycosyl phosphatidyl inositol (GPI) moiety [426]. The special protein structure suggesting weak homophilic binding ability and the less restricted cell surface presence suggests that T-cad and may also be able to mediate intracellular signaling [427]. First discovered in nervous tissue to guide axon growth, the expression of T-cad is strongly detected in the cardiovascular system as well. Using mutant mice that lacked T-cad, Denzel and colleagues demonstrated that T-cad was essential in mediating the cardioprotective effect of adiponectin [428]. T-cad knock-out mice suffered more severe cardiac hypertrophy following pressure overload challenge and larger infarction area following ischemia-reperfusion than wild-type control animals, which was similar to that seen in adiponectin knock-out mice. T-cad null mice also showed diminished AMPK phosphorylation after cardiac injury induction, and unlike the adiponectin null mice, the phenotype could not be saved by administering recombinant adenovirus-expressed adiponectin. Additional to the cardiovascular system, T-cad was upregulated together with HMW adiponectin oligomers in a rat model of diet-induced liver fibrosis [429]. These data suggest that T-cad may be a critical mediator for the effect of adiponectin in
physiological and pathophysiological conditions, and a more thorough understanding of the signaling pathways downstream of T-cad is highlighted.

**Adiponectin receptor binding proteins**

The exact mechanism of signal transduction in response to adiponectin binding to its receptors is yet to be fully understood; however several adiponectin receptor associated proteins have been discovered. The adaptor protein containing the pleckstrin homology domain, phosphotyrosine domain and leucine zipper motif (APPL1) interacts with the N-term of adipoR1 via its phosphotyrosine binding domain (PTB), where interaction is enhanced by adiponectin binding [430]. Phosphorylation and activation of AMPK and p38 MAPK caused by adiponectin stimulation were increased by overexpression of and decreased by siRNA knock-down of APPL1, respectively. APPL1 facilitates the adiponectin-induced activation of AMPK by interacting with the upstream AMPK kinase LKB-1 and promoting its cytosolic translocation from the nucleus and anchoring the kinase in the cytosol [431]. The binding of APPL1 to adiponectin receptors may be negatively regulated by its homolog APPL2, which competes with APPL1 for adipoR1 association and directly binds to APPL1 to block the interaction between APPL1 and adipoR1 [432]. Treatment with adiponectin or metformin frees APPL1 and adipoR1 from APPL2 binding and allows the adiponectin signal to be conveyed through.

APPL1 is functionally relevant in mediating the effect of adiponectin. The increase in GLUT4 membrane translocation with adiponectin treatment is impaired by mutant APPL1 with a deletion in the PTB or the Bin-amphiphysin-Rvs (BAR) domain [431]. In endothelial cells, APPL1 together with AMPK are involved in the inhibitory effects of adiponectin on IL-18-induced NFkB activation and endothelial apoptosis [433] and the stimulatory effects of adiponectin on eNOS activation [434]. In primary cultured cardiac fibroblasts, siRNA knock-down of APPL1 blocked the phosphorylation of AMPK after adiponectin treatment, therefore association of APPL1 to adiponectin receptors may be an underlying mechanism of adiponectin-induced MMP activation, matrix elaboration and fibroblast migration in cardiac tissue [435]. Consistent with this, Park and colleagues showed that transgenic mice overexpressing APPL1 were protected from developing
cardiac dysfunction after 16 weeks of high fat diet, confirming the importance of this protein in the heart [436].

Several other binding partners have been described for adipoR1 as well. The protein kinase casein kinase 2 (CK2) was shown to interact with adipoR1 in the C2C12 myocyte cell [437]. Inhibition of CK2 by 2-dimethylamino-4,5,6,7-tetrabromo-1H-benz-imidazole (DMAT) blocked the phosphorylation of acetyl-CoA carboxylase (ACC), which is the kinase target of AMPK and mediates adiponectin induced fatty acid oxidation, suggesting that the protein may participate in adiponectin signaling. The receptor for activated protein kinase C (RACK) 1 was co-immunoprecipitated with adipoR1 in a co-expression experiment in COS-7 cells, and membrane co-localization of RACK1 with adipoR1 was enhanced by adiponectin treatment [438]. Downregulation of RACK1 reduced the glucose uptake in HepG2 cells incubated with adiponectin. Nonetheless the motif responsible for the RACK1-adipoR1 interaction and the details on recruitment of RACK1 to adipoR1 are unknown, highlighting the need for further research. The endoplasmic reticulum protein 46 (ERp46) binds exclusively to the N-terminus of adipoR1 but not to adipoR2 [439]. It has been proposed that ERp46 may function to sequester adipoR1 in the endoplasmic reticulum and reduce the availability of this receptor to the cell membrane. Knock-down of ERp46 in HeLa cells positively influenced the phosphorylation of AMPK but negatively modulated that of p38 MAPK in response to adiponectin stimulation, suggesting that ERp46 may take part in the fine-tuning of adiponectin’s effects in cells.
Figure 1. Schematic diagram of the full-length adiponectin molecule, circulating adiponectin fragments and oligomers and the adiponectin receptors

Full length adiponectin: 30kDa; HMW adiponectin: oligomers of 12 -18 adiponectin molecules.
Figure 1. 6 Signaling pathways activated and inhibited downstream of adiponectin and its receptors.
Green lines: activation; red lines: inhibition; dashed blue lines: potential interactions.
Figure 1. 7 Summary of adiponectin functions

- Anti-atherogenic
- Anti-inflammatory
- $\uparrow$ FA oxidation
- $\uparrow$ glucose uptake and utilization
- $\downarrow$ gluconeogenesis
- $\downarrow$ ROS generation
1.3.4 Adiponectin in diseases

Decline of circulating adiponectin concentration in diseases

In contrast to other adipokines introduced previously, the level of adiponectin falls paradoxically in obese individuals as compared to healthy volunteers [440]. Circulating adiponectin concentration is also lower in patients with T2DM, coronary artery disease or essential hypertension [441-443]. The lower serum adiponectin level in T2DM is accompanied with a reduction of adipoR1 expression in subcutaneous and omental adipose tissue, the levels of which were largely restored after aggressive weight loss [444]. Thus it is speculated that the absence of adiponectin may contribute to the disease process. Interestingly, polymorphisms in the promoter region, exon and intron and a rare mutation in the adiponectin gene have been associated with expression levels of adiponectin and the risk of developing several disease conditions, including obesity, insulin resistance, diabetes and coronary artery disease in different ethnicities [445].

The mechanism for this decrease in adiponectin level in obesity has not been fully elucidated, but some studies suggest that adipose macrophage-derived TNF-α is able to suppress the production and secretion of adiponectin from adipocytes. Differentiated 3T3-L1 adipocytes enhanced the release of pro-inflammatory cytokines from co-cultured RAW264 macrophages possibly through the release of free fatty acids, and the macrophages in the co-culture system downregulated the expression of adiponectin via TNF-α, as a neutralizing antibody against TNF-α prevented this effect. Further, this inflammatory feedback loop was stronger when hypertrophied 3T3-L1 adipocytes or adipose tissue from obese ob/ob mice were used in the culturing system [446]. Besides, oxidative stress in adipose tissue could also decrease the expression of adiponectin, as cultured 3T3-L1 cells decreased the expression of adiponectin when exposed to high oxidative stress after fatty acid incubation [447]. Treatment with the antioxidant NAC reversed the suppression on adiponectin expression.

Adiponectin as a protective factor in the cardiovascular system

Current research results indicate that adiponectin is protective in the cardiovascular system owing to its anti-atherogenic and anti-inflammatory properties. Adiponectin
protects endothelial cells from injurious stimuli. Adiponectin was able to inhibit TNF-α induced upregulation of adhesion molecules such as VCAM-1, ICAM-1 and endothelial-leukocyte adhesion modulcure-1 (E-selectin), and hence prevented monocyte attachment to the vascular wall [448]. It also reduced the secretion of proinflammatory cytokines like TNF-α itself from macrophage [449]. Adiponectin has been shown to protect endothelial cells from LDL toxicity by inhibiting cell proliferation and generation of superoxides as well as enhancing eNOS activity [450, 451]. In vivo experiments using adiponectin gene knock-out mice further confirmed the importance of this adipokine in endothelial cells, as adiponectin null mice on a western atherogenic diet showed impaired endothelial cell dependent vasorelaxation [452].

In vivo and in vitro experiments demonstrated that adiponectin is able to prevent the formation and promote the stability of atherosclerotic plaques. Overexpressing adiponectin in the ApoE null mice with an adenovirus vector rendered the animals more resistant to atherosclerosis formation, as these animals developed smaller lesions and had lower levels of inflammation [453]. The accumulation of lipids in macrophages is a critical step in atherosclerotic plaque formation, and adiponectin was shown to attenuate this process by reducing the expression of the macrophage scavenger receptors (MSR) without affecting the expression of CD36 or apolipoprotein E [454]. Superoxide generation in neutrophils after stimulation by PMA, DAG or N-formyl-methionyl-leucyl-phenylalanine (fMLP) was also suppressed by adiponectin [455]. In addition to suppressing the initiation of atherosclerosis, adiponectin stabilized the atherosclerotic plaque structure by upregulating the tissue inhibitor of metalloproteinase-1 (TIMP-1) possibly through promoting the release of IL-10, which may prevent the plaque from rupturing [456].

The protective effect of adiponectin on the cardiovascular system extends beyond atherosclerosis. Shibata and colleagues found that adiponectin null mice had larger infarct size after ischemia-reperfusion induced myocardial injury, and replenishing adiponectin reduced the infarct size in both adiponectin KO and wild-type mice [457]. The same study also showed that adiponectin inhibited cardiomyocyte apoptosis via AMPK signaling, while it suppressed an increase in TNF-α levels via a COX-2
dependent pathway. That adiponectin positively influences healing after ischemic injuries was confirmed in a mouse model of hind limb ischemia, as low capillary density in the recovering area in adiponectin knock-out mice was rescued by adiponectin administration. Injection of a dominant-negative (DN) AMPK construct completely abrogated the effect of adiponectin on ischemic injuries in the hind limb in wild-type mice [458]. Overexpressing adiponectin using an adenovirus-vector improved the angiogenesis in wild-type animals with hind limb ischemia as well. The mechanism underlying the pro-angiogenic effect of adiponectin may rely on its role in stimulating endothelial differentiation from the circulating CD14 positive monocytes [459]. In another model of heart disease, absence of adiponectin in mice resulted in worse left ventricular hypertrophy and increased mortality after cardiac pressure overload, and restoring adiponectin prevented the downregulation of AMPK signaling and the activation of ERK signaling, which might then in turn attenuate cardiac remodeling [460].

In humans, epidemiological studies have identified a negative correlation between the plasma adiponectin levels and the risks of cardiac events and mortality in various populations, including a general healthy cohort, patients with T2DM, and patients who had undergone coronary angiography because of angina [461-463]. In patients with ischemic heart disease, low circulating adiponectin levels were independently associated with the complexity, acuteness and plurality of coronary artery atherosclerosis [464]. The serum adiponectin concentration may be an indicator of susceptibility to develop atherosclerosis as well, since Nilsson and colleagues showed that male participants in the lowest quartile of plasma adiponectin level had significantly increased intima-media thickness in the carotid artery and higher insulin resistance score as compared to individuals in the highest quartile [465].

**Adiponectin as an insulin-sensitizing hormone**

Research evidence suggests that the beneficial effects of adiponectin extend beyond the cardiovascular system. As briefly described previously, it increases glucose utilization and fatty acid oxidation in skeletal muscle and liver, and decreases gluconeogenesis in the liver, and studies have well supported the role of adiponectin as an endogenous insulin-sensitizing agent. In non-human primates, obese rhesus monkeys showed lower
plasma adiponectin levels in parallel with decreased insulin sensitivity, prior to the onset of diabetes, which indicated a potential causal role of this adipokine in insulin resistance [466]. Adiponectin has been shown to reverse insulin resistance, which may depend on its role in facilitating glucose uptake in the peripheral organs via upregulating the GLUT4 receptor expression and membrane translocation [467, 468]. Mice with a 3 fold increase in circulating adiponectin level due to a mutation in the collagenous domain exhibited enhanced insulin sensitivity, much like the effect of long-term PPARγ treatment [469]. In obese KKAŷ mice, plasma adiponectin levels fell when mice were fed on high-fat diet and administration of exogenous adiponectin alleviated the insulin resistance and dyslipidemia in these mice [467]. Treating obese mice with adiponectin also improved hyperglycemia and fatty acid utilization in skeletal muscle, decreased gluconeogenesis in liver and promoted weight loss [414, 470]. As discussed previously, dysregulation of several adipokines has been observed in obesity-linked metabolic disorders where adiponectin may play an essential part. Higher than physiological level of adiponectin expression completely rescued the symptoms of the metabolic syndrome such as hyperglycemia, hyperinsulinemia and dyslipidemia in obese leptin-deficient mice whereas double mutant mice lacking adiponectin and leptin showed aggravated fat accumulation in the liver [471, 472]. In addition to adiponectin, expression of adipoR1 and adipoR2 was also lower in muscle and adipose tissue of obese ob/ob mice, which were inversely correlated to plasma insulin level [473].

Results from human epidemiological studies are consistent with the findings from animal experiments. Decreased serum adiponectin concentration correlates with risk of developing T2DM in humans even after adjustment for WTH ratio, BMI and glycosylated hemoglobin A (1c) [474]. In cohorts of Pima Indians and Japanese men, hypoadiponectinemia was identified as a better indicator of increased risk for T2DM or metabolic syndrome development than inflammatory markers such as TNF-α, IL-6 and CRP [475, 476]. A study by Bayes and colleagues also showed that risk of developing new onset diabetes after renal transplantation was higher in patients with lower pre-transplant circulating adiponectin levels [477]. Some studies have shown that insulin and the insulin signaling pathway can in turn influence the plasma levels of adiponectin, suggesting that severe insulin resistance may be paralleled by an adiponectin insensitivity
as well [478]. For adiponectin receptors, Civitarese and colleagues also found that a family history of T2DM was associated with decreased expression of both the adiponectin receptors in skeletal muscle of non-diabetic Mexican Americans [479].

**Adiponectin and inflammation**

The inhibitory effect of adiponectin on the activation of inflammatory signaling pathways and expression of pro-inflammatory cytokine and adhesion molecules has been shown with different stimuli, and an inverse relationship between circulating adiponectin levels and of pro-inflammatory markers TNF-α and CRP has been shown in several clinical studies [480-483]. Despite a large amount of evidence support an anti-inflammatory role of adiponectin, its effect in autoimmune disease is less well defined. TNF-α neutralizing therapy increased plasma adiponectin concentration in patients with rheumatoid arthritis, which was associated with better prognosis [483]. However, complicated results have been reported on the tissue and systemic adiponectin levels in patients related to the severity of rheumatoid arthritis (RA), with increased synovial adiponectin levels associated with RA and increased plasma adiponectin after anti-TNF-α treatment in RA patients [484, 485]. Plasma adiponectin levels were higher in the hypertrophied mesenteric fat of patients with Crohn’s disease [486], while studies using adiponectin mutant mice showed completely different effects of adiponectin on inflammation in models of chemical-induced inflammatory bowel disease (IBD) [487, 488], highlighting that further research is necessary in this field. In patients with systemic lupus erythematosus (SLE), systemic and urine adiponectin levels were higher in SLE patient with renal presentation when compared to control individuals or non-renal flare patients [489], and even more interestingly a high TNF-α level was detected simultaneously with high adiponectin levels in SLE patients. Overall, studies on the effect of adiponectin in autoimmune diseases are far from conclusive, and more researche is needed.

**Adiponectin signaling as a therapeutic target**

Considering the various benefits of adiponectin such as being cardiovascular protective and insulin sensitizing, it is reasonable to target this hormone in therapeutic regimens. In fact, several types of medication exert their effects at least partially through upregulating
adiponectin. The PPARγ agonists TZDs increase the secretion of adiponectin by post-transcriptional modifications [490], which have been proposed to be the main mediator of their anti-diabetic effects. Nawrocki and colleagues demonstrated that the insulin-sensitizing effect of pioglitazone, a TZD, was blunted in obese ob/ob mice with adiponectin gene deletion [491]. Clinical studies also identified that specific SNPs in the adiponectin gene correlated with less responsiveness to TZD treatment in patients [492]. Adiponectin levels also rose with ACEI and ARB treatment in patients with essential hypertension, which was positively associated with improvements in insulin sensitivity [493]. This effect of RAS blockade is possibly mediated by increased preadipose differentiation. Tian and colleagues reported in human subjects more omental fat preadipocyte differentiation tissue after ACEI and or ARB treatment than after TZD treatment, which occurred in parallel with a dramatic increase in plasma adiponectin levels [494]. Adiponectin has been found to be an underlying active agent partially mediating the pharmacological effects of the endocannabinoid receptor (CB₁) antagonists type of weight loss medication [495].

Efforts have also been devoted to direct activation of adiponectin signaling. Directly delivering adiponectin as a therapeutic agent can be complicated, as the plasma concentration of this adipokine is high and the different isoforms of adiponectin (HMW vs. gAD molecule) may not be equally bioactive. Currently, the synthesis of commercial quantity of full-length adiponectin with proper post-transcriptional modification is still difficult. Hence commercial manufacturing molecules that can activate the adiponectin receptors or their downstream signaling pathways may be more approachable. Osmotin is a pathogenesis related-5 (PR-5) family plant protein that shares similar 3D structure with globular adiponectin [496]. In vitro experiments confirmed that the protein was capable of activating AMPK through the adiponectin receptors in C2C12 myocytes [496]. Since PR-5 family proteins are stable enough to retain their activities after passing through the human digestive and respiratory system, osmotin may be potentially useful as a therapeutic adiponectin receptor agonist [497]. Recently a group of investigators led by Okada-Iwabu reported that a small molecule adiponectin receptor agonist AdipoRon was able to induce similar insulin sensitizing effects in skeletal muscle and liver as adiponectin, and reduced oxidative stress in adipose tissue [498]. AdipoRon also
improved glucose tolerance, enhanced response to insulin in peripheral tissues and corrected dyslipidemia in T2DM db/db mice. Therefore adipoRon appears to a promising adiponectin mimicking therapeutic candidate for treating insulin resistance and diabetes.

**Complex relation between adiponectin and kidney injury: epidemiological evidence**

Although it is now clear that adiponectin exerts many beneficial effects on the cardiovascular system and against metabolic dysfunction, epidemiological studies have generated perplexing results about the role of adiponectin in kidney disease. Within a group of T1DM patients with diabetic nephropathy from the Finnish Diabetic Nephropathy Study, Saraheimo and colleagues showed that serum adiponectin concentration was positively correlated to the degree of albuminuria, and negatively to GFR, two indicators of impaired renal function. The same study however did confirm the inverse relationship between adiponectin and waist-to-hip ratio [499]. Follow-up analyses from the Finnish Diabetic Nephropathy Study further identified an association between higher adiponectin levels with diabetic nephropathy progression to ESRD in patients with macroalbuminuria, but not with DN progression in patients with microalbuminuria or normoalbuminuria [500]. As cited before, serum adiponectin levels were lower in Pima Indians with T2DM for less than 10 years as compared to normoglycemic control subjects or patients had stable DM for over 10 years. However participants in the same study who had macroalbuminuria also had the highest level of serum adiponectin and participants with normoalbuminuria had the lowest adiponectin concentration [501]. In agreement with this, Koshimura and colleagues confirmed that higher serum adiponectin levels were found in T2DM patients with overt nephropathy, and they also detected higher urinary adiponectin excretion in these patients in comparison with patients with incipient nephropathy or no nephropathy [502].

In non-diabetic patients with mild to moderate kidney disease, lower serum adiponectin level was associated with higher insulin resistance score (HOMO-IR score) as well as increased risk of cardiovascular events [503]. In the report by Iwashima and colleagues, serum adiponectin level in CKD patients showed a step-wise elevation with increasing kidney disease severity (from stage 1 to stage 5); however it was negatively correlated with risk of developing cardiovascular disease in this cohort, as hypoadiponectinemia
was found to associate with lower cardiac event-free survival rate in patients with previous ischemic heart disease [504]. On the contrary, the analysis of the Modification of Diet in Renal Disease Study indicated that each 1 μg/ml increase in adiponectin increased the risk of all-cause mortality rate by 3% and cardiovascular mortality by 6% in patients with stage 3 to 4 CKD [505].

In dialysis patients, Zoccali and colleagues found serum adiponectin was increased as compared to healthy control subjects, but higher adiponectin levels in ESRD patients correlated with reduced risk of adverse cardiovascular events [506]. In contrast another group of investigators reported that higher baseline and longitudinal adiponectin levels were correlated with increased risk of cardiovascular events, including stroke and sudden death but not myocardial infarction, in ESRD patients undergoing hemodialysis due to T2DM-associated nephropathy [507]. This negative association between adiponectin and the survival of hemodialysis patients was similarly observed by Ohashi and colleagues, as their calculation suggested a 10.3% increase in risk for all-cause mortality with each 1 μg/ml increase in adiponectin level [508].

In summary, conflicting results exist regarding the relationship between plasma adiponectin level and the progression and mortality in CKD, but a positive correlation has been reported by several research groups and in different forms of kidney disease.

Mechanisms for the elevated serum adiponectin level detected in patients with CKD and ESRD and the positive correlation with mortality are not yet fully elucidated. Several possibilities have been proposed: Impaired renal function in CKD and ESRD patients may disrupt the excretion of adiponectin. In the Tanno and Sobetsu study where alteration in serum adiponectin levels in relation to aging was analyzed, adiponectin level rose in a pattern mirroring that of the BUN, hence the investigators suggested that reduced kidney clearance might be responsible for high serum adiponectin concentration [509]. However liver has been determined as the main site for adiponectin biodegradation so the extent of kidney clearance is unclear.

High adiponectin levels may reflect the underlying metabolic derangement in CKD patients, where adiponectin levels increase to counteract this condition. In the study by
Becker and colleagues [503], plasma adiponectin levels were inversely related to insulin resistance and plasma triglycerides concentration independently of diabetes. In the previously cited study where adiponectin level positively correlated to cardiovascular mortality in dialysis patients, N-terminal prohormone of brain natriuretic peptide (NT-proBNP), a marker of cardiac tissue damage, was also higher [507]. Increased adiponectin in this study was also paralleled by lower CRP levels; hence it has been proposed that the high serum adiponectin in these patients may reflect a compensatory mechanism in attempt to attenuate inflammation. Some clinical studies suggested that increased circulating adiponectin might be related to the severe systemic wasting in ESRD patients, which would impair survival. Lee and colleagues found negative correlations between adiponectin level and BMI, triglyceride and glucose levels, but a positive association was established between adiponectin and subjective global assessment score and malnutrition-inflammation score in hemodialysis patients, confirming that adiponectin levels were increased in malnourished dialysis patients when compared to well-nourished dialysis patients [510].

In a reflective review on vascular injury and CKD, Shen and colleagues argued that the reactivity of adiponectin receptors to their ligand may be altered in ESRD patients, and resistance at the receptor level may explain the profound inflammation that persists in hemodialysis patients even when circulating adiponectin level was dramatically elevated [511].

**Complex relationship between adiponectin and kidney injury: experimental evidence**

Despite the controversial epidemiological findings, experimental results from in vitro cell culture and in vivo animal work shows adiponectin as primarily a renoprotective hormone. *In vitro* experiments further demonstrate that adiponectin specifically modulates different types of cells in the kidney. In proximal tubular HK2 cells, both adipoR1 and adipoR2 were present, and incubation with adiponectin suppressed the secretion of MCP-1 via AMPK activation [512]. Adiponectin has been shown to inhibit migration of cultured rat glomerular mesangial cells induced by PDGF, which was dependent on its suppression of the big mitogen-activated protein kinase 1(BMK1) and
p38 MAPK signaling [513]. PDGF-induced PDGF-receptor autophosphorylation or Akt signaling activation in rat mesangial cells was not affected by adiponectin incubation. Therefore adiponectin may suppress the excessive mesangial cell turn-over in CKD.

Adiponectin has also been found to be a critical regulator of podocyte function. In a fundamental study done by Sharma and colleagues [514], adiponectin gene deletion in mice caused albuminuria and podocyte abnormalities at baseline, which were exacerbated after STZ-induced diabetes. Treatment with either gAD or full-length adiponectin attenuated albuminuria and podocyte foot process effacement, as well as increased expression of tight junction protein ZO-1. The adipoR1 was found to be the dominant adiponectin receptor on podocytes, where AMPK activation was essential for the observed effect of adiponectin and gAD was found to be more effective. Oxidative stress caused by NOX4 NADPH oxidase activation was likely involved in the podocyte damage in adiponectin knock-out mice, as oxidative markers and NOX4 expression were increased in the glomeruli of mutant mice and the urinary hydrogen peroxide level was also higher. Treatment with adiponectin and the AMPK agonist AICAR alleviated oxidative stress. Rutkowski and colleagues elaborated the essential role of adiponectin on podocyte health using a transgenic mouse model (POD-ATTAC) of inducible podocyte damage that was reversible after withdrawing the toxin [515]. The podocyte foot injuries became permanent and hence albuminuria could not be recovered when an adiponectin deficient mutation was crossed into the POD-ATTAC mice, whereas the double mutant mice deteriorated to renal failure. In contrast, overexpressing adiponectin accelerated recovery after induction of podocyte injury and showed less fibrosis in the interstitium.

In vivo experimental work has demonstrated that adiponectin is able to prevent kidney damages in several injury models. Mutant mice without adiponectin were more susceptible to injury caused by renal tissue ablation, and showed more inflammatory cell infiltration, proinflammatory cytokines and adhesion molecule expression, profibrotic factor accumulation, and NADPH oxidase subunits expression when compared to wild-type control animals [516]. Consequently exacerbated glomerulosclerosis, tubulointerstitial fibrosis and albuminuria were also evident in the remnant kidney of adiponectin KO mice. Adiponectin overexpression was shown to be renoprotective
against diabetic nephropathy in animal models [517]. Adenovirus-transferred adiponectin in Wistar rats with STZ-induced diabetes rescued albuminuria and loss of nephrin expression in glomeruli, and decreased TGF-β, ET-1 and PAI-1 levels in the renal cortex. Adiponectin also increased inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) in the kidney. C57Bl/6 mice exhibited higher urinary MCP-1 and H2O2 excretion as rapidly as 1 week on high-fat diet, which proceeded albuminuria [518]. An AMPK agonist inhibited the rise in MCP-1 and H2O2 in urine and kidney tissue and renal hypertrophy, implying that adiponectin may play a role in ameliorating inflammation and hypertrophy in kidney. Serum adiponectin levels were decreased in mice with renal ischemia/reperfusion injury, whereas treatment with adiponectin improved kidney filtration and reduced inflammatory cell infiltration and cell apoptosis in the ischemic mice [519]. The protective effect of adiponectin against renal I/R injury was found to be mediated by the prostacyclin-PPARα-heme oxygenase-1 (HO-1) pathway, which was supported by the inadequate response to adiponectin-induced HO-1 upregulation in PPARα knock-out mice.

However, the beneficial role of adiponectin is not universally recognized for all types of kidney injury. Jin and colleagues found there was surprisingly less tubular damage, tubular cell apoptosis, activation of the NFκB signaling pathway and expression of pro-inflammatory cytokines in adiponectin knock-out mice after renal I/R injury [520]. The alleviated tubular injury in adiponectin deficient mice appeared to be immune-mediated, as wild-type mice with bone marrow transplantation from adiponectin null mice also showed lessened injury and vice versa. In vitro experiments suggested that adiponectin promoted macrophage migration via the PI3 kinase pathway. Another group of investigators also demonstrated a positive role of adiponectin in monocyte-to-fibroblast transition in mouse models of renal fibrosis, which may explain the reduced fibrosis in the obstructed or ischemic kidney in the adiponectin null mice [521]. This pro-fibrotic effect of adiponectin in monocytes was found to be AMPK-dependent. The exact role of adiponectin in immune-mediated nephropathy is also under debate. In homozygous lymphoproliferation spontaneous mutation mice (MRL mice) on a C57BL/6 background, adiponectin worsened lupus-like kidney damage, with exacerbation of mesangial expansion, crescent formation and IgG and C3 deposition [522], where mutation to the
adiponectin gene alone in C57BL/6 mice did not convey susceptibility to autoimmune diseases. In humans, aberrantly glycosylated IgA-1 was shown to suppress the level of adiponectin in vivo and also in vitro in the glomerular mesangial cells [523]. Reasons underlying these inconsistent reports remain unclear.

Current research evidence suggests that the effect of adiponectin in kidney injury may be stimuli-specific. Therefore I sought to explore the interaction between adiponectin and RAS-related kidney damage. Chapter 3 depicts the in vitro study on the effects of adiponectin on the Ang II-induced NADPH oxidase activation in renal tubular cells. The role of adiponectin in kidney in vivo will be described in Chapter 4. Alterations in kidney damage due to adiponectin gene deletion were assessed in a mouse model of type 1 diabetes mellitus, the Akita mouse.
1.4 Experimental design and hypothesis

The renin-angiotensin system is essential for the normal function of the kidney and activation of the RAS has been shown to mediate many of the injurious responses in the kidney under disease conditions. Therefore, I sought to study the modifiers of RAS, in particular the ACE analogue ACE2 and the adipokine adiponectin, and their role in kidney injury.

Increased RAS activity has been shown in acute kidney injury (AKI), which contributes to the early inflammation in AKI. However the effect of ACE2 in this pathogenic process was not clear. To explore this, I examined the effect of ACE2 gene deletion on ischemia-reperfusion injury in kidney in Chapter 2. To test my hypothesis that ACE2 conveys protection against AKI, wild-type and global ACE2 knock-out mice were subjected to renal ischemia-reperfusion injury and markers of kidney damage were examined. Due to the acute nature of the injury model, outcome measures were focused on inflammation, which was determined as the amount of infiltrating inflammatory cells and productions of pro-inflammatory cytokines. Tissue oxidative stress and cell apoptosis were measured as the potential mechanistic link and consequence of inflammation, respectively.

Another potential modifier of the RAS that I am interested in is adiponectin. Many of the known effects of Ang II and adiponectin in kidney seem to be opposing each other, but the interactions between these 2 important bioactive factors in kidney have not been fully elucidated. The impact of adiponectin on kidney was first studied in an in vitro cell culture system. In Chapter 3 of my thesis, the effect of adiponectin on Ang II-induced NADPH oxidase activation was investigated in vitro in cultured renal proximal tubular cells. Previous experimental evidence suggested that adiponectin could attenuate oxidative stress in endothelial cells and kidney podocytes, so I proposed that adiponectin could inhibit the increase in NADPH oxidase activation in response to Ang II stimulation. In addition, I also studied which signal transduction pathway(s) might be responsible for mediating the possible antioxidative function of adiponectin against Ang II stimulation in renal tubular cells. Pharmacological activators and inhibitors of the AMPK and cAMP signaling pathways were used to verify whether they were involved.
Lastly in order to better define the role of adiponectin in kidney disease in vivo, I crossed adiponectin KO mice with T1DM Akita mice to generate a line of diabetic mice without adiponectin. My hypothesis in this part of my project was that adiponectin null mice would show exacerbated renal injury in response to diabetes. General structure and function changes, such as kidney hypertrophy and albuminuria, and degree of fibrosis, inflammation and oxidative stress were assessed. The corresponding molecular mechanisms underlying these kidney tissue injuries were further tested in the cultured human mesangial cells.

In summary, I will test the following three hypotheses:

1. Deletion of the gene for ACE2 will exacerbate I/R injury in the kidney.
2. Adiponectin will attenuate Ang II-induced NADPH oxidase activation in kidney cells.
3. Deletion of the gene for adiponectin will exacerbate diabetes-induced kidney injury.

The figures presented below illustrate the rationale and design for the experiments testing these hypotheses.
Figure 1. 8 Rationale for assessing the role of ACE2 in acute kidney injury.
RAS activation has been demonstrated in ischemia-reperfusion (I/R), a model of AKI, which may promote oxidative stress and inflammation in damaged kidney. Because of its role in Ang II degradation, I propose that ACE2 may protect the kidney from I/R injury.
Figure 1. Scheme of experiments for assessing the role of ACE2 in acute kidney injury (Chapter 2)
Figure 1. 10 Rationale for examining the interaction between adiponectin and Ang II on ROS generation.
Reactive oxygen species (ROS) generated by NADPH oxidase is an important mediator of Ang II-caused kidney injury. Adiponectin has been shown to prevent HG- and LDL-caused oxidative stress in endothelial cells. My hypothesis is that adiponectin can inhibit activation of NADPH oxidase following Ang II stimulation in renal tubular cells.
Figure 1.11 Experimental design for examining the interaction between adiponectin and Ang II on ROS generation (Chapter 3)
The possible involvement of the AMPK and cAMP signaling pathways on the effects of adiponectin were also studied.
Figure 1. 12 Rationale for studies on the role of adiponectin on diabetic nephropathy.
To test whether adiponectin may influence pathological processes in the kidney, the effect of adiponectin on diabetic nephropathy is tested \textit{in vivo} in a mouse model of type I diabetes mellitus. A high glucose (HG) environment has been shown to increase RAS activity, which in turn contributes to the development and progression of diabetic nephropathy. Hence I hypothesize that adiponectin can benefit the kidney in the diabetic environment.
Figure 1. 13 Generation of the $\text{Ins}2^{+/C96Y}\text{Apn}^{+/+}$ (Akita/APN) mouse line
(A) Pedigree of the Akita/APN mouse line. (B) Representative images for Akita and adiponectin genotyping (separate reaction for WT and APN).
Figure 1. 14 Experimental design for studies on the role of adiponectin in diabetic nephropathy
Experiments were done in vivo in mice from the Akita/APN line and in vitro in cultured human mesangial cells.
Chapter 2

Loss of ace2 Exacerbates Murine Renal Ischemia-Reperfusion Injury

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

Ischemia-reperfusion (I/R) is a model of acute kidney injury (AKI) that is characterized by vasoconstriction, oxidative stress, apoptosis and inflammation. Previous studies have shown that activation of the renin-angiotensin system (RAS) may contribute to these processes. Angiotensin converting enzyme 2 (ACE2) metabolizes angiotensin II (Ang II) to angiotensin-(1-7), and recent studies support a beneficial role for ACE2 in models of chronic kidney disease. However, the role of ACE2 in models of AKI has not been fully elucidated. In order to test the hypothesis that ACE2 plays a protective role in AKI we assessed I/R injury in wild-type (WT) mice and ACE2 knock-out (ACE2 KO) mice. ACE2 KO and WT mice exhibited similar histologic injury scores and measures of kidney function at 48 hours after reperfusion. Loss of ACE2 was associated with increased neutrophil, macrophage, and T cell infiltration in the kidney. mRNA levels for pro-inflammatory cytokines, interleukin-1β, interleukin-6 and tumour necrosis factor-α, as well as chemokines macrophage inflammatory protein 2 and monocyte chemotactic protein-1, were increased in ACE2 KO mice compared to WT mice. Changes in inflammatory cell infiltrate and cytokine expression were also associated with greater apoptosis and oxidative stress in ACE2 KO mice compared to WT mice. These data demonstrate a protective effect of ACE2 in I/R AKI.
2.2 Introduction

Ischemia-reperfusion (I/R) is an important cause of acute kidney injury (AKI) and a common occurrence in volume depleted or septic patients and in the setting of organ procurement for transplant. I/R often leads to significant kidney damage including progressive chronic kidney disease (CKD) [524-526]. The pathophysiology of I/R injury is complex and includes the effects of hypoxia and cell death on various renal tubulointerstitial cells, vascular or hemodynamic factors, and inflammatory processes [527, 528]. Recent evidence suggests that the evolution and persistence of inflammation triggered by the initial non-immune insult plays a critical role in the outcome of I/R [529]. Activation of the renin-angiotensin system (RAS) is implicated in most forms of kidney injury, and inhibiting its main effector, angiotensin II (Ang II), remains a cornerstone of therapy for progressive CKD [530, 531]. The sequential action of renin and then angiotensin converting enzyme (ACE) on angiotensinogen and angiotensin I respectively produces Ang II, which contributes to vasoconstriction, local tissue oxidative stress, inflammation, and fibrosis in CKD [532, 533]. In a similar manner, the actions of Ang II may also contribute to I/R kidney injury. In previous studies of I/R injury in rats, the RAS was found to be activated and kidney Ang II levels increased after I/R [261, 534, 535].

The discoveries of ACE2, a homolog of ACE, and the Mas receptor, which binds angiotensin-(1-7) (Ang-(1-7)), have generated new interest in the RAS [536, 537]. ACE2 converts Ang II to Ang-(1-7), and the effects of Ang-(1-7) may oppose those of Ang II [538, 539]. In support of this concept, we and others have shown that genetic deletion or pharmacologic inhibition of ACE2 worsens experimental kidney disease including diabetic nephropathy and unilateral ureteral obstruction, while administration of recombinant ACE2 or over-expression of ACE2 improves kidney injury [236-238, 241, 540, 541].

In the current study, we hypothesized that ACE2 would also be protective in kidney I/R. In order to test this hypothesis, we compared I/R-induced histopathologic injury and
inflammation, apoptosis, and oxidative stress in wild-type mice and mice with a deletion in the ace2 gene.
2.3 Materials and Methods

**Ethics Statement:** All experiments were conducted following the guidelines of the University of Toronto Animal Care Committee.

**Animals:** Wild-type (WT) and Ace2\(^{−/−}\) (ACE2 KO) mice on C57BL/6J background were generated as previously described [217], housed at the Division of Comparative Medicine at the University of Toronto, and fed standard mouse chow with free access to water. Only male mice were used in this study.

**Ischemia-Reperfusion:** We first employed the unilateral model of I/R. 8-week old mice were administered analgesic (ketoprofen, 0.1ml/10g body weight) followed by anesthesia with inhaled isoflurane mixed with oxygen. Using a back incision, the left renal pedicle was exposed and the left renal artery constricted with a 4-0 suture for 45 minutes and subsequently released. Vascular occlusion and release was confirmed by observing corresponding changes in kidney color. During the entire surgical procedure, animals were placed on a 37 °C heating pad to maintain body temperature, and then allowed to recover under a warming light. Sham animals received an identical procedure without ligature. Mice were sacrificed 48 hours after surgery for tissue harvest, with body and kidney weights recorded. In order to obtain a better functional measure of injury, we studied the bilateral model of I/R. Using flank incisions, both renal pedicles were exposed and occluded with microaneurysm clamps (Roboz Surgical Instrument, Gaithersburg, MD, USA) for 25 minutes. Blood was collected at 48 hours after surgery for measurement of plasma BUN and creatinine.

**Blood biochemistry:** Blood samples were collected from the carotid artery with Microvette® (Sarstedt Inc., Montreal, QC, CA) at time of sacrifice. Plasma was isolated by centrifuging blood samples at 2000g for 5 min at room temperature, and stored at -80°C until use. Plasma blood urea nitrogen (BUN) and creatinine assessments were done at the Toronto Centre for Phenogenomics (Toronto, ON, CA).

**Histology and Immunohistochemistry:** The left kidney (sham or I/R operated) was harvested and transversely sectioned into 3 approximately equal portions. The two polar
portions were snap-frozen and the middle portion placed into 10% neutral buffered formalin (Sigma Aldrich, St Louis, MO, USA) for histology and immunohistochemistry analyses. Fixed kidney tissue was paraffin-embedded, sectioned, stained and scanned. 3-µm periodic acid-Schiff (PAS) stained sections were used to score histopathologic injury, which was done blinded to the experimental group. Tubular injury was assessed on a scale of 0 to 4 (0 being no injury and 4 being the most severely injured).

The following primary antibodies were used for immunohistochemistry: ACE (Santa Cruz Biotechnology, Santa Cruz, CA, USA), ACE2 (R&D, Minneapolis, MN, USA), neutrophil (AbD Serotec, Raleigh, NC, USA), F4/80 (AbD Serotec, Raleigh, NC, USA), CD3 (Dako Canada, Inc., Burlington, ON, CA), caspase-3 (Cell Signaling Technology, Inc., Danvers, MA, USA), Ki-67 (Dako Canada, Inc., Burlington, ON, CA) and nitrotyrosine (Millipore Biosciences Research Reagents (Chemicon), Temecula, CA, USA). TUNEL staining was performed according to a published protocol [542]. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide. Quantitation of neutrophils, CD3, TUNEL and Ki-67 positive cells was based on counting of positively staining nuclei by a Nuclear algorithm, and quantitation of macrophages and nitrotyrosine on positively stained area measured by a Positive Pixel Count algorithm of Aperio ImageScope software (Aperio Technologies, Inc., Vista, CA, USA). Caspase-3 positive cells were manually counted.

**Quantitative real-time PCR:** Snap-frozen mouse kidney tissue was homogenized in liquid nitrogen with pre-cooled pestle and mortar on dry ice, and total RNA extracted using RNeasy® Mini kit (Qiagen Inc., Mississauga, ON, Canada) following the manufacturer’s protocol. 1µg of the extracted RNA was reverse transcribed into cDNA with QuantiTech® Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany) and used for quantitative real-time PCR. The TaqMan® Gene Expression Assay system (Applied Biosystems, Foster City, CA, USA) was used to perform real-time PCR with the following primers: *il1b* (Catalogue number (Cat#): Mm01336189_m1), *il6* (Cat#: Mm00446190_m1), *tnf* (Cat#: Mm99999068_m1), *cxcl2* (MIP-2, Cat#: Mm00436450_m1), *ccl2* (MCP-1, Cat#: Mm00441242_m1), *bax* (Cat#: Mm00432051_m1), *bcl2* (Cat#: Mm00477631_m1), *ace* (Cat#: Mm00802048_m1), *ace2*
(Cat#: Mm01159006_m1), *agt* (angiotensinogen, Cat#: Mm00599662_m1), *ren1* (renin, Cat#: Mm02342884_g1), *agtr1a* (angiotensin II receptor type 1 Cat#: Mm00616371_m1), *mas1* (Cat#: Mm00434823_s1). 18s (Cat#: Hs99999901_s1) was used as internal control.

**Western blot:** The following antibodies were used: anti-Bcl-2 antibody (#3498) (Cell Signaling Technology, Inc., Danvers, MA, USA); anti-Bax antibody (ab10813) (Abcam Inc., Cambridge, MA, USA); β-Actin (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA). Snap-frozen kidney tissue was homogenized by sonification in lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA). Proteins in tissue lysates were separated by 10% SDS-PAGE gel, blotted onto PVDF membrane and detected with an enhanced chemiluminescence system (ECL) kit (Millipore Corp., Billerica, MA, USA). Densitometry measurement was calculated by Scion Image software (Scion Corp. Frederick, MD, USA).

**Angiotensin II peptide measurement:** The concentration of renal parenchymal Ang II was determined by an Angiotensin II EIA kit (Peninsula Laboratories, LLC, San Carlos, CA, USA). According to the manufacturer, the Ang II-binding antibody does not cross-react with Ang 1 or Ang (1-7). Tissue was prepared as follows: snap-frozen mouse kidney tissue was homogenized in ice-cold methanol on ice and centrifuged at 12000g, 4°C for 10 minutes. The supernatant was collected and dried by centrifugal evaporation. Dried samples were reconstituted with the EIA buffer supplied by the manufacturer and used for Ang II measurement. Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) from the reconstituted samples and used for normalization.

**Statistical analysis:** Unless specified otherwise, results are expressed as mean ± SE. One-way ANOVA with Bonferroni post-hoc test was used for comparison of multiple groups. All statistical analyses were done with GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA), and statistical significance defined as p < 0.05.
2.4 Results

2.4.1 Whole animal, macroscopic and microscopic kidney examination: Compared to corresponding sham animals, there was no significant change in body weight after I/R in either WT mice or ACE2 KO mice (Table 2.1). Kidney weight to body weight ratio increased in both groups after I/R (Table 2.1). BUN and creatinine levels were measured to assess kidney function. Plasma BUN was modestly elevated to a similar extent in both WT and ACE2 KO animals after I/R, while plasma creatinine values were unchanged (Table 2.1).

Histopathologic injury after I/R was assessed in PAS-stained sections. Injury was confined to the tubulointerstitial compartment and most pronounced in the outer medullary region, and included areas of tubular necrosis (Figure 2.1 A). The inner medulla was the area next most affected, albeit to a much lesser extent, followed by the renal cortex. The mean value for tubular injury score after I/R tended to be higher in the ACE2 KO mice compared to the WT mice, but the difference did not reach statistical significance (Figure 2.1 B). There was also no difference between the groups when injury scores were limited to the outer medulla (data not shown).
### Table 2.1 Whole Animal Data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT sham</th>
<th>ACE2 sham</th>
<th>WT IR</th>
<th>ACE2 IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>23.71±0.55</td>
<td>21.83±1.27</td>
<td>22.98±0.45</td>
<td>21.06±0.29*</td>
</tr>
<tr>
<td>LKW (g)</td>
<td>0.1422±0.0040</td>
<td>0.1350±0.0110</td>
<td>0.1741±0.0076*</td>
<td>0.1723±0.0066</td>
</tr>
<tr>
<td>LKW/BW</td>
<td>0.0060±0.0001</td>
<td>0.0061±0.0001</td>
<td>0.0076±0.0004*</td>
<td>0.0082±0.0004*</td>
</tr>
<tr>
<td>BUN (mmol/L)</td>
<td>8.4±0.52</td>
<td>9.3±0.64</td>
<td>11.4±0.47*</td>
<td>12.6±0.80*</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>21±0.8</td>
<td>21±1.4</td>
<td>22±1.1</td>
<td>22±1.4</td>
</tr>
</tbody>
</table>

BW (Body weight) and LKW (left kidney weight) were recorded at time of sacrifice 48 hours after surgery for all experimental groups. BUN and creatinine levels were measured with frozen plasma samples. Results are shown as mean ± SE. For BW, LKW AND LKW/BW, n = 8 for WT sham; n = 8 for ACE2 sham; n = 12 for WT IR; n = 11 for ACE2 IR. For BUN and creatinine, n = 6 for all groups. * p<0.05 compared to WT sham.
Figure 2. 1 Histopathologic changes after renal I/R

(A) Representative images of PAS stained kidney sections from wild-type (WT) and ACE2 knock-out mice (ACE2 KO) after sham or I/R surgery, magnification: 50x; insert: high power field of the cortico-medullary junction, magnification: 200x. (B) Tubular injury score based on PAS sections for each experimental group, on a scale of 0 to 4. Results are presented as mean ± SE. n = 8 for WT sham and ACE2 sham; n = 12 for WT IR; n = 11 for ACE2 IR. * p < 0.0001 vs. WT sham.
2.4.2 Inflammatory cell infiltration and pro-inflammatory cytokine expression:
Neutrophils, F4/80 positive cells (macrophages) and CD3 positive cells (T cells) were assessed as indicators of renal parenchymal immune cell infiltration. Figures 2.2, 2.3 and 2.4 show the marked neutrophil, macrophage, and T cell infiltration within the kidneys of mice after I/R. Mean values for neutrophil infiltration were significantly higher in the ACE2 KO mice subjected to I/R compared to WT mice (p<0.05) (Figure 2.2B). A similar trend was seen for the numbers of macrophages (Figure 2.3B) and CD3-positive cells (Figure 2.4B), although the differences did not reach statistical significance.

Measurement of mRNA levels of pro-inflammatory cytokines, IL-1β, IL-6, and TNFα, and chemokines, MIP-2 and MCP-1 showed dramatic induction of all genes after I/R (Figure 2.5). Additionally, mean mRNA levels of all cytokines after I/R were higher in the kidneys of the ACE2 KO mice compared to the kidneys of WT mice with a statistically significant difference for IL-1β and MCP-1 (Figure 2.5D, E).
Figure 2. 2 Kidney neutrophil infiltration following I/R
(A) Representative images of anti-neutrophil staining of kidney sections from WT sham, ACE2 sham, WT IR and ACE2 IR mice; magnification: 200x. (B) Quantitation of neutrophil infiltration using ImageScope Nuclear algorithm. Results are presented as mean ± SE. n = 5 for WT sham; n = 8 for ACE2 sham; n = 8 for WT IR; n = 9 for ACE2 IR. * p < 0.05 vs. WT sham.
Figure 2. 3 Macrophage (F4/80 positive cell) infiltration after I/R
(A) Representative images of F4/80 staining of kidney sections from WT and ACE KO mice after sham or I/R surgery; magnification: 200x. (B) Percentage of F4/80 positive area in cortico-medullary region calculated by ImageScope Positive Pixel Count algorithm. Results are presented as mean ± SE. n = 4 for WT sham; n = 7 for ACE2 sham; n = 8 for WT IR; n = 9 for ACE2 IR. ** p < 0.05 vs. ACE2 sham.
Figure 2. 4 T cell (CD3 positive cell) infiltration following I/R
(A) Representative images of CD3 staining of kidney sections from WT sham, ACE2 sham, WT IR and ACE2 IR mice; magnification: 200x. (B) Quantitation of T cell infiltration using ImageScope Nuclear algorithm. Results are presented as mean ± SE. n = 8 for WT sham and ACE2 sham; n = 9 for WT IR; n = 11 for ACE2 IR. * p < 0.05 vs. WT sham.
Figure 2. 5 Pro-inflammatory cytokine and chemokine levels after I/R
mRNA expression levels of IL-1β (A), IL-6 (B), TNFα (C), MIP-2 (D) and MCP-1 (E), in WT and ACE2 KO mouse kidney after sham or I/R surgery. 18s was used as internal control. Results are normalized to WT sham and presented as mean ± SE. n = 5 for WT sham; n = 6 for ACE2 sham; n = 6 for WT IR; n = 5 for ACE2 IR. * p < 0.05 vs. WT sham. † p < 0.05 vs. WT IR.
2.4.3 Detection of apoptosis, cell proliferation and oxidative stress: There was an almost five-fold increase in the number of apoptotic cells (caspase 3-positive cells) in the WT mice after I/R compared to the sham-operated mice (Figure 2.6). Furthermore, the mean number of caspase 3-positive cells was significantly higher in ACE2 KO mice after I/R compared to WT mice. Compared to caspase-3, TUNEL staining showed greater heterogeneity within groups in the numbers of positive cells. Mean values of TUNEL-positive cells appeared similarly increased after I/R in the WT and ACE2 KO mice (Figure 2.7). This may be due to a decrease in specificity of the TUNEL stain when there is both apoptosis and necrosis as occurs in I/R injury [543].

mRNA levels of Bax and Bcl-2 were increased after I/R (Figure 2.8A, B). A pro-apoptotic phenotype was more evident in the ACE-2 KO group at the protein level with a significant increase in Bax in the ACE-2 KO mice after I/R and no difference between the groups in Bcl-2 expression (Figure 2.8C).

As a measure of recovery from I/R injury, we assessed cellular proliferation by immunohistochemistry for Ki-67. As expected, the numbers of proliferating cells were markedly increased after I/R (Figure 2.9). Numbers of Ki-67 cells were slightly lower in the ACE-2 KO compared to WT mice, but mean values were not statistically different between the groups (Figure 2.9B).

In order to assess oxidative stress, we measured nitrotyrosine staining in kidney tissue. There was an increase in nitrotyrosine staining after I/R in both groups of mice with significantly greater staining in ACE2 KO mice compared to WT mice (Figure 2.10).
Figure 2.6 Immunohistochemistry for cleaved caspase-3 following I/R
(A) Representative images of caspase-3 staining in kidney sections from WT sham, ACE2 sham, WT IR and ACE2 IR mice; magnification: 200x. Positively staining cells were counted in randomly chosen areas, and the number of positive cells and size of each area were recorded. (B) Quantitation of caspase-3 staining by numbers of positive cells per mm² of tissue. Results are presented as mean ± SE. n = 8 for WT sham; n = 7 for ACE2 sham; n = 12 for WT IR; n = 10 for ACE2 IR. * p < 0.05 vs. WT sham. † p < 0.05 vs. WT IR.
Figure 2. 7 Immunohistochemical TUNEL staining after I/R
(A) Representative images of TUNEL staining on kidney sections in WT and ACE2 group after sham or I/R operation; magnification: 200x. (B) Quantification of positively stained cells as determined by ImageScope Nuclear algorithm. Results are presented as mean ± SE. n = 5 for WT sham; n = 6 for ACE2 sham; n = 7 for WT IR; n = 9 for ACE2 IR.
Figure 2. 8 Bcl-2 and Bax expression following I/R
mRNA levels of Bax (A) and Bcl-2 (B) were determined by real-time PCR. 18s was used as internal control. Results were normalized to WT sham. (C) Protein expressions of Bax and Bcl-2 were measured by western blot. Representative images and densitometries are provided. β-Actin was used as loading control. Results are presented as mean ± SE. For real-time PCR, n = 5 for WT sham; n = 5 for ACE2 sham; n = 6 for WT IR; n = 5 for ACE2 IR. For western blot, experiments were performed in triplicates. * p < 0.05 vs. WT sham. † p < 0.05 vs. WT IR.
Figure 2. 9 Cellular proliferation after I/R
(A) Representative images of Ki-67 staining on kidney sections from WT sham, ACE2 sham, WT IR and ACE2 IR mice. Magnification: 200x. (B) Percentage of positively stained cells (positive cells/total cells) as determined by ImageScope Nuclear algorithm. Results are shown as mean ± SE. n = 5 for WT sham; n = 7 for ACE2 sham; n = 8 for WT IR; n = 9 for ACE2 IR. * p < 0.05 vs. WT sham.
Figure 2. Induction of oxidative stress following I/R

(A) Representative images of nitrotyrosine immunostaining in kidney sections from WT and ACE2 KO mice after sham or I/R surgery; magnification: 200x. (B) Quantitation of nitrotyrosine staining by positive area per total area using Image Scope Positive Pixel Count algorithm. Results are presented as mean ± SE. n = 5 for WT sham; n = 7 for ACE2 sham; n = 8 for WT IR; n = 11 for ACE2 IR. * p < 0.05 vs. WT sham. † p < 0.05 vs. WT IR.
2.4.4 Expression of RAS components and renal AngII level: In order to relate I/R-induced kidney injury to activation of the RAS, we measured the intra-renal expression of RAS components. In sham mice, immunohistochemistry for ACE showed strong staining along the apical border of the proximal tubules (Figure 2.11A). Staining was strongest in the corticomedullary junction area and slightly weaker and patchy in the outer cortex. Following I/R, staining for ACE was decreased in both WT and ACE2 KO mice. The decrease was most pronounced in the corticomedullary area corresponding with severe tubular injury. Immunohistochemistry for ACE2 showed similar staining as for ACE in the WT sham mice (Figure 2.11B). After I/R, WT mice showed decreased ACE2 staining in the corticomedullary area but slightly increased staining in the outer cortex.

As expected, ACE2 was not detected in the two groups of ACE2 KO mice by immunohistochemistry or mRNA measurements. There was no significant difference in the expression of any of the other RAS components between the WT and ACE2 KO sham groups (Table 2.2). As illustrated in Figure 2.12, WT mice showed a trend towards increased expression of all RAS components after I/R. In the ACE2 KO mice, I/R produced little or no change in mRNA levels of angiotensinogen, renin and Mas receptor, a small decrease in ACE and a small increase in AT1 receptor.

We then measured Ang II peptide levels in kidney tissues of the four groups of mice. Tissue Ang II levels were similar between sham WT and ACE2 KO mice, but after I/R, were significantly greater in the kidneys of ACE2 KO mice (n = 7) than WT mice (n = 8) (Figure 2.13).
Figure 2. Immunohistochemical staining for ACE and ACE2
Representative images of ACE (A) and ACE2 (B) stained kidney sections in WT and ACE2 KO mice after sham or I/R surgery. Magnification: 50X.
Table 2. 2 mRNA levels of components of the renin-angiotensin system in WT and ACE2 KO mice after sham operation of I/R

<table>
<thead>
<tr>
<th>Components</th>
<th>WT sham (n = 5)</th>
<th>ACE2 sham (n = 5)</th>
<th>WT IR (n = 7)</th>
<th>ACE2 IR (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>0.70 ± 0.06</td>
<td>0.89 ± 0.09</td>
<td>1.06 ± 0.35</td>
<td>0.36 ± 0.12</td>
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<tr>
<td>ACE2</td>
<td>0.74 ± 0.04</td>
<td>N.D.</td>
<td>2.13 ± 0.85</td>
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<tr>
<td>Angiotensinogen</td>
<td>0.69 ± 0.06</td>
<td>1.07 ± 0.23</td>
<td>2.37 ± 0.69</td>
<td>0.85 ± 0.18</td>
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<td>Renin</td>
<td>0.68 ± 0.08</td>
<td>1.01 ± 0.19</td>
<td>2.31 ± 1.00</td>
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<tr>
<td>AT1R</td>
<td>0.73 ± 0.06</td>
<td>0.67 ± 0.07</td>
<td>2.31 ± 0.71</td>
<td>1.21 ± 0.17</td>
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<tr>
<td>Mas</td>
<td>0.68 ± 0.12</td>
<td>0.84 ± 0.10</td>
<td>1.14 ± 0.33</td>
<td>0.78 ± 0.30</td>
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</table>

Values are expressed as fold change versus WT sham and presented as mean ± SE. N.D., non-detectable.
Figure 2. Changes in expression levels of RAS components after renal I/R

Expression of ace (A), ace2 (B), angiotensinogen (C), renin (D), agtr1 (angiotensin II receptor, type I) (E), and mas (F) after I/R were compared to corresponding control sham animals in WT and ACE2 KO groups. mRNA levels were determined by real-time PCR and 18s used as internal control. Results are presented as mean ± SE. n = 6 in WT group; n = 4 in ACE2 group.
Figure 2. 13 Levels of Ang II in kidney tissue in WT and ACE2 KO mice after sham or I/R surgery
Peptide levels were determined by ELISA and normalized to total protein. Results are presented as mean ± SE. n = 7 in WT sham and IR group; n = 8 in ACE2 sham and IR group. * p < 0.05 vs. WT sham.
2.4.5 Evaluation of renal function change after bilateral I/R injury: To obtain a more robust measure of kidney function after I/R, we utilized a bilateral model of injury. There were no differences in plasma BUN and creatinine values between WT and ACE2 KO mice at 48 hours after reperfusion (Figure 2.14).
Figure 2. Plasma BUN and creatinine levels in WT and ACE2 KO mice following bilateral I/R

Plasma samples were obtained at sacrifice 48 hours after surgery. Frozen plasma samples were used for BUN and creatinine measurement. Results are presented as mean ± SE. n = 7 in WT group; n = 4 in ACE2 group.
2.5 Discussion

It has been reported that the intrarenal RAS is activated in the I/R model of AKI but the effect of loss of the gene for ace2 on the kidney’s response to I/R has not been studied. We observed that deletion of the ace2 gene significantly increases cellular inflammation, pro-inflammatory cytokine expression, apoptosis and oxidative stress following I/R. These data are the first to demonstrate a potentially protective effect of ACE2 on AKI.

I/R is a major cause of AKI with considerable morbidity and often leading to chronic kidney disease [544, 545]. The mechanisms of injury in AKI are complex and include ATP depletion with consequent cellular injury including necrosis or apoptosis [546], inflammatory cell recruitment and oxidative stress [527-529, 546]. The cortico-medullary junction including the S3 proximal tubule segment is especially vulnerable to ischemia because of intrinsic low oxygen tension coupled with elevated metabolic demand [527]. Besides tubular cell injury, diffuse endothelial cell damage has also been demonstrated in I/R, and both tubular and endothelial cells contribute to the recruitment of inflammatory cells [547-549]. Inflammation has become recognized as being a critical component of I/R injury. Various studies have shown important roles for neutrophils, T cells, B cells, macrophages and the complement pathway, although whether a dominant immune mechanism exists is not entirely clear [550-552]. Similarly, a variety of cytokines/chemokines secreted by injured kidney and infiltrating cells have been shown to be important to maintain the local inflammatory environment, while also causing direct cellular injury as with TNF-mediated apoptosis [553].

Most research in AKI-I/R has focused on these effectors of injury, namely, inflammatory cells, cytokines/chemokines, apoptosis or oxidative stress [554-556], but much less is known about potential upstream events including the formation of Ang II. The actions of Ang II overlap with mechanisms of I/R injury, namely inflammation and oxidative stress [557]. Moreover, because Ang II is elevated as early as 4 hours after I/R, mediators of I/R injury may at least in part be up-regulated by Ang II. While renin is known to be rate limiting for Ang II formation, the degradation to Ang-(1-7) by ACE2 is also a determinant of tissue Ang II concentrations. In this regard, a salutary effect of ACE2 has
been shown in several models of CKD, including diabetic nephropathy, renal ablation, and most recently, unilateral ureteral obstruction, where a role for Ang II in potentiating injury is well established [237, 238, 241, 541, 558]. A few early studies examining I/R have shown benefit by blocking Ang II, supporting the hypothesis that Ang II mediates at least some of the kidney’s responses in this form of injury [559, 560]. Thus ACE2 could similarly affect the outcome of AKI.

Forty-eight hours after I/R, we found that the kidneys from ACE2 KO mice showed greater numbers of neutrophils, macrophages and T cells compared to the kidneys of WT mice. Although histologic injury scores tended to be higher in ACE2 KO mice, the differences were not statistically significant. Besides the dominant injury at the cortico-medullary junction area which included tubular necrosis, we also saw some injury in the cortex and medulla. Increased inflammatory cell infiltration was accompanied by higher levels of major pro-inflammatory cytokines and chemokines in the kidneys of ACE2 KO mice. IL-1β, IL-6, TNFα, MIP-2 and MCP-1 play important roles in immune function, including cell recruitment, maturation and activation [561, 562]. Resident cells are likely the main source of these cytokines/chemokines early after injury, whereas infiltrating cells may have a significant role at later time-points [563]. In addition, we found both apoptosis and oxidative stress, two processes that are associated with I/R and influenced by Ang II, to be exacerbated by the loss of ace2 gene [554, 564]. The increase in oxidative stress may be a unifying aspect of the greater injury seen in the ACE2 KO mice, since hypoxia, infiltrating neutrophils and macrophages, and Ang II can each result in the generation of reactive oxygen species. Despite the greater inflammation and oxidative stress in the ACE2 KO mice, kidney function after 48 hours of reperfusion were similar in ACE2 KO and WT mice.

In evaluating the expression of RAS components, we found no differences in mRNA expression levels at baseline between WT and ACE2 KO mice. After I/R, there was a uniform trend towards increased mRNA expression levels in WT mice, but little to no change in ACE2 KO mice. In previous studies of I/R, Ang II levels were consistently elevated between 4 and 24 hours after reperfusion, and returned to baseline levels by 72 hours [261, 534, 535]. We confirmed increased Ang II levels in ACE KO compared to
WT mice at 48 hours after I/R, although by this time, Ang II levels had also decreased compared to baseline. The decrease in Ang II levels is consistent with decreased ACE staining that was seen at this time-point.

We did not explore the mechanism(s) responsible for the observed effect of loss of ACE2 on I/R, which is a limitation of our study. It is tempting to speculate that increased Ang II concentrations played a role, especially since Ang II levels were elevated compared to WT mice even at 48 hours after I/R. As regards Ang (1-7), it is notable that a recent study showed that administration of Ang-(1-7) worsened kidney I/R injury in vivo and activated NF-κB in cultured tubular cells in vitro [260, 565]. On the other hand, there is also evidence that Ang-(1-7) counters the effect of Ang II in cultured tubular cells and mesangial cells [254, 566]. Another mechanism might include des-Arg bradykinin, because absent ACE2 would potentially cause an increase in this active bradykinin metabolite [567]. Additional experiments will be required to better define the mechanism responsible for the protective effect of ACE2 in I/R.

Another limitation of our study is the lack of detailed assessment of recovery from I/R injury between the two groups of mice. We surmised that increased inflammation and oxidative stress seen in the ACE2 KO mice might translate into less complete recovery compared to the WT mice. We used Ki-67 as an indicator of tissue recovery and found the degree of cellular proliferation to be similar between the ACE2 KO and WT mice at 48 hours after I/R.

In conclusion, our data show that ACE2 is a determinant of the renal response to I/R. Pathologic examination of kidneys showed increased inflammation, apoptosis and oxidative stress in ACE2 KO mice compared to WT mice. Administration of recombinant human ACE2 has been shown to mitigate injury in various disease models of elevated Ang II, and may also hold promise in the treatment of I/R-induced acute kidney injury [568, 569].
Chapter 3

Adiponectin Attenuates Angiotensin II-Induced Oxidative Stress in Renal Tubular Cells through AMPK and cAMP-Epac Signal Transduction Pathways

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

Obesity is a risk factor for chronic kidney disease (CKD) progression. Circulating levels of adiponectin, an adipokine, decrease with obesity and play a protective role in the cardiovascular system. We hypothesized that adiponectin might also protect the kidney. Because activation of the renin-angiotensin system (RAS) is a contributor to CKD progression, we tested our hypothesis by studying the interactions between adiponectin and angiotensin II (AngII) in renal tubular cells. Primary human renal proximal tubule cells expressed both adiponectin receptor 1 and 2 (adipoR1, R2). AngII-induced NADPH oxidase activation and oxidative stress were attenuated by adiponectin and dependent on adipoR1. Activation of AMPK with AICAR mimicked, while inhibition of AMPK with compound C abrogated the effect of adiponectin on AngII-induced activation of NADPH oxidase. Similarly the effect of adiponectin was recapitulated by the stable cAMP analogues pCPT-cAMP and db-cAMP and blocked by the adenylate cyclase inhibitor SQ22356. Adiponectin did not activate PKA in renal tubular cells and the specific PKA inhibitor, myristoylated PKI (14-22) amide failed to block the inhibitory effect of adiponectin on AngII-induced NADPH oxidase activation. In contrast the specific Epac activator 8-CPT-2-o-Me-cAMP blocked AngII-induced activation of NADPH oxidase, an effect that was reversed by co-incubation with the AMPK inhibitor, compound C. Finally, adiponectin attenuated AngII-induced NFκB activation and fibronectin protein expression. These in vitro findings support the hypothesis that adiponectin may attenuate the deleterious effects of AngII in the kidney and play a protective role in CKD.
3.2 Introduction

Obesity is an important risk factor for the progression of CKD independent of blood pressure and diabetes mellitus though the mechanisms responsible for this relationship have not been fully elucidated [570-572]. Circulating levels of a cytokine secreted by adipose tissue, adiponectin, are reversely-related to obesity [573-576], and recent studies have shown that adiponectin attenuates cardiovascular injury [577-579]. Adiponectin may play a role in maintaining normal podocyte structure [514, 580, 581] but the effect of adiponectin on the kidney is not well understood. We hypothesized that adiponectin would also play a protective role in the kidney and regulate kidney cells’ response to injury.

Activation of the renin-angiotensin system (RAS) is a key contributor in the progression of CKD [582-586] and blockade of the RAS limits kidney injury [582, 584, 586, 587]. One important mechanism linking the main effector of RAS, angiotensin II (AngII), to kidney injury is the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and increased production of reactive oxygen species (ROS) [588-592]. Interestingly, adiponectin has been shown to reduce high glucose-induced oxidative stress by suppressing the activity of NADPH oxidase in endothelial cells and this effect is through the activation of AMP-activated protein kinase (AMPK) and cAMP pathways [514, 593-596]. In order to determine if adiponectin plays a protective role in renal tubular epithelial cells, we first studied adiponectin receptor expression in our primary human tubular epithelial cells and the signal transduction pathways downstream of adiponectin receptors including AMPK and cAMP pathways. We then sought to determine if adiponectin would prevent AngII-induced activation of NADPH oxidase in kidney tubular cells and to elucidate the signal transduction pathways responsible for any effect.
3.3 Materials and Methods

**Cell:** Human renal proximal tubule epithelial cells (RPTECs, Clonetics® Cells, Lonza) were cultured in DMEM/F-12 HAM medium (Sigma) with 20% fetal bovine serum, epidermal growth factor (10 ng/ml), transferrin (5 μg/ml), insulin (5 ng/ml), hydrocortisone (0.05 μM), penicillin (50 units/ml) and streptomycin (50 μg/ml). Cells were maintained at 37°C, with 5% CO₂ and 95% air. All studies were done with cells between passages 4 -7 as previously described [597, 598].

**Real-time RT PCR:** After reaching full confluence, tubular cells were treated with or without AngII (0.1 μM) for 18 hours. Cells were collected and total RNA was isolated with RNeasy® Mini Kit (Qiagen) following the protocol provided by the manufacturer. cDNA was obtained by reverse transcription with QuantiTech® Reverse Transcription Kit (Qiagen), then used for real time PCR. Real-time PCR was performed with the TaqMan® system (Applied Biosystems) according to the manufacturer’s guideline.

**Western blot:** Antibodies used were as following: anti-adiponectin receptor 1 (AdipoR1, ab70362) and 2 (AdipoR2, ab53399) were from Abcam; anti-Na⁺/K⁺ ATPase antibody was from Santa Cruz (sc-58628); antibody against phospho-AMPKα (Thr172) (#2535) was from Cell Signaling; antibody against phospho-acetyl CoA carboxylase (ph-ACC, 07-303) was from Millipore; antibody against β-actin (A5441) was from Sigma; anti-fibronectin (EP5, sc-8422) antibody was from Santa Cruz; and antibody against α-tubulin (302 011) was from Synaptic Systems. For membrane protein isolation, tubular cells were sub-cultured in 60 mm dishes to full confluence and harvested. Cells were lysed with Lysis buffer A (1 mM NaHCO₃, 5 mM MgCl₂, 50 mMTris-HCl, 10 mM EGTA, 2 mM EDTA, 0.86 mM PMSF, 25 μg/ml leupeptin, 10 mM benzamidine) on ice for 40 min, then centrifuged at 100,000 g, 4°C for 1 hour. The supernatant was discarded. Pellets were incubated with Lysis buffer B (Lysis buffer A supplemented with 1% Triton X-100) on ice for 1 hour then centrifuged at 100,000 g, 4°C for 1 hour. The supernatant was kept for Western blot.

**Lucigenin enhanced chemiluminescence assay:** The final concentrations and the manufacturers of the reagents used were as following: NADPH, 100 μM or 1 mM, Calbiochem; lucigenin, 5 μM, Sigma Aldrich; human angiotensin II (AngII), 0.1 μM,
Sigma Aldrich; recombinant full-length human adiponectin, 50 or 100 ng/ml, BioVision; diphenyleneiodonium (DPI), 10 µM, Sigma Aldrich; apocynin, 100 µM, Calbiochem; anti-adipoR1 antibody (ab70362), 10 µg/ml, Abcam; anti-adipoR2 antibody (ab77612), 10 µg/ml, Abcam; goat IgG (sc-3697), 10 µg/ml, Santa Cruz; 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), 1 mM, Toronto Research Chemicals Inc.; compound C, 1 µM, Sigma Aldrich; pCPT-cAMP, 20 µM, Sigma Aldrich; db-cAMP, 25 µM, Sigma Aldrich; SQ22536, 0.1 mM, Sigma Aldrich; myristoylated PKI (14-22) amide (mPKI), 10 µM, Calbiochem; 8-CPT-2-o-Me-cAMP, 50 µM, Calbiochem. Renal tubular cells were grown to full confluence and then incubated with the appropriate agents as indicated for 18 hours before activity assay. Cells were then washed twice with ice-cold PBS, collected and centrifuged at 18,000 g, at 4°C, for 1 min. Supernatant was discarded and 200 µl of PBS was added to each tube. After 15 seconds of sonication on ice, samples were centrifuged at 18,000 g, 4°C, for 20 min. 100 µl supernatant from each sample was mixed with NADPH and lucigenin, from which the light emission was measured by FB12 Luminometer (Bethold detection systems). Protein concentration was determined by Bradford assay (Bio-Rad) and used for normalization.

**Dihydroethidium (DHE) stain:** Renal tubular cells were seeded onto glass cover slides and cultured to 90% confluence. Cells were then treated for 18 hours with AngII (0.1 µM) and/or adiponectin (100 ng/ml). After washing with PBS, cells were incubated with DHE (2 µM, Invitrogen) at 37°C for 1 hour in dark. At the end of incubation, cells were washed twice with PBS then once with distilled water. The cover slides were mounted onto glass slides using Dako Faramount Aqueous Mounting Medium (S3025, Dako Canada, Inc.). Fluorescent images were captured with Zeiss confocal laser-scanning microscope (LSM510, Carl Zeiss Inc.). At least 6 fields (magnification: 400x) were selected for each experimental group and the fluorescence intensity for each field was scored semi-quantitatively on a scale of 0 to 4 (0 being dark and 4 being the strongest fluorescence) by a trained nephrologist blinded to the experimental conditions.

**PKA activity assay:** Cellular PKA activity was measure with PKA kinase activity kit (Enzo Life Science) following manufacturer’s guide. Whole cell lysates were used and prepared following the manufacturer’s protocol. Briefly, renal tubular cells cultured to 90%
confluence in 60 mm dishes were treated with adiponectin (100 ng/ml, for 10 min, 2 hours or 16 hours) and pCPT-cAMP (20 μM, 10 min). After washed with ice-cold PBS once, cells were lysed with lysis buffer on ice for 10 min (20 mM MOPS, 50 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM sodium vanadate, 5 mM EGTA, 2 mM EDTA, 1% Nonidet P-40, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF and 10 μg/ml leupeptin and aprotinin). Afterwards cells were collected and centrifuged at 15,000 g, 4°C, for 15 min. Light absorbance at 450 nm was measured with the Thermomaxmicroplate reader (Molecular Devices, Inc.). Protein concentrations were determined by Bradford assay (Bio-Rad) and used for normalization.

**NFκB activity assay:** pNFκB-Luc plasmid was purchased from Stratagene, and pRL-TK reporter vector was from Promega. RPTECs were subcultured in 6-well plates for 24 hours and then transfected with 0.4µg pNFκB-Luc plasmid/well and 3.9ng/well pRL-TK vector/well using Effectene Transfection Reagent (Qiagen). 24 hours after transfection, cells were treated with AngII (0.1μM) and/or adiponectin (100ng/ml) for 18 hours, and measured for luciferase activity with Dual-Luciferase Reporter Assay System (Promega). Light emission was read by FB12 Luminometer (Bethold detection systems). Total protein was determined by Bradford assay (Bio-Rad) and used for normalization.

**Statistical analysis:** All results were presented as mean±SE. Statistical analyses were performed using GraphPad Prism4 (GraphPad Software, Inc). One-way ANOVA with Bonferroni post-test was performed for multi-group comparison, and unpaired t-test for comparison between two groups. Statistical significance was defined at a p value of less than 0.05.
3.4 Results

3.4.1 Adiponectin receptors are expressed in human renal tubular cells: To investigate the effect of adiponectin on tubular cells, we first examined adiponectin receptor expression. Real-time RT PCR results indicated that both the AdipoR1 and AdipoR2 were transcribed in our human renal tubular cells and stimulation by AngII for 18 hour did not significantly alter the mRNA levels of the receptors. Western blot analysis also confirmed protein expression in the cell membrane fraction (Figure 3.1).
Figure 3. 1 Adiponectin receptor 1 and 2 were expressed at both mRNA (A, B) and protein level (C) in renal tubular cells

Cells were incubated with or without AngII for 18 hours before harvest. Human GAPDH was used as internal control. Results are presented as mean±SE, n = 3 for each group. For western blot, cell membrane was separated with ultra-speed centrifugation as specified in the Materials and Methods section. Na⁺/K⁺ ATPase served as loading control for the Western blot analysis of the membrane fraction. Experiments were performed in duplicate.
3.4.2 Adiponectin inhibits AngII-induced superoxide generation in renal tubular cells: Cellular levels of superoxide were first determined by measuring NADPH oxidase activity using the lucigenin-enhanced chemiluminescence assay. As expected, AngII increased the NADPH oxidase activity as compared to control conditions (Figure 3.2). Adiponectin attenuated the effect of AngII on NADPH oxidase at a dose of 50 ng/ml, and reduced it further at 100 ng/ml (Figure 3.2A). Treatment by adiponectin alone did not affect the NADPH oxidase activity in renal tubular cells. The sensitivity of the assay for NADPH oxidase activity was not affected by the concentration of NADPH (Figure 3.2B). The specificity of the assay was confirmed with the NADPH oxidase inhibitors diphenyleneiodonium (DPI) and apocynin (Figure 3.2C and D). The inhibition by adiponectin on AngII-induced oxidative stress was also examined by DHE fluorescent stain (Figure 3.3). In agreement with the result from NADPH oxidase activity assay, adiponectin completely normalized the increase in reactive oxygen species (ROS) following AngII treatment.
Figure 3. 2 Adiponectin inhibited Ang II-induced NADPH oxidase activity in a dose-dependent manner in renal tubular cells
A. After treatment with AngII (0.1 μM) and/or adiponectin (50 ng/ml or 100 ng/ml) for 18 hours, NADPH oxidase activity was measured by lucigenin enhanced chemiluminescence assay. B. After incubating with AngII (0.1 μM) for 18 hours, renal tubular cells were collected and sonicated. Samples were then mixed with low (100 μM) or high (1 mM) NADPH and NADPH oxidase activity was measured with a lucigenin chemiluminescence assay. C. The increase in NADPH oxidase activity caused by AngII was attenuated by diphenyleneiodonium (DPI, 10 μM). D. The increase in NADPH oxidase activity caused by AngII was attenuated by apocynin (100 μM). Cells with no treatment were used as the control condition. Results are presented as mean±SE, and at least 3 separate experiments were done in each group. * p< 0.05 vs. Control; # p < 0.05 vs. AngII treated.
Figure 3. 3 Adiponectin reduced the generation of ROS in renal tubular cells after Ang II stimulation

After incubating cells with angiotensin II (AngII) and/or adiponectin (APN) for 18 hours, cellular levels of ROS were detected with DHE staining (2 µM, 1 hour incubation at 37°C in dark) and the intensity of fluorescence was determined semi-quantitatively. AngII = 0.1 µM; APN = 100 ng/ml. Representative fluorescent images were captured with a Zeiss LSM510 confocal laser-scanning microscope at the magnification of 400x. Results are presented as mean±SE, and at least 6 images were scored for each experimental group. * p< 0.001 vs. control; # p < 0.001 vs. AngII with adiponectin treated.
3.4.3 Inhibition by adiponectin on AngII-induced NADPH oxidase activation is adipoR1-dependent: To investigate which receptor for adiponectin was responsible for its anti-oxidative effect in renal tubular cells, blocking antibodies against adipoR1 and R2 were used in combination with AngII and adiponectin. Co-treatment with anti-adipoR1 but not anti-adipoR2 antibody reversed the effect of adiponectin on NADPH oxidase activation following AngII stimulation (Figure 3.4). Treatment with a non-specific IgG did not alter the baseline NADPH oxidase activity.
**Figure 3. 4 Blocking adipor1 but not adipor2 reversed the effect of adiponectin on NADPH oxidase activation after Ang II treatment**

Renal tubular cells were collected for lucigenin-enhanced chemiluminescence assay after 18-hour incubation with AngII (0.1 µM), adiponectin (100 ng/ml), anti-adipoR1 antibody (10 µg/ml, pre-treat 1 hour) or anti-adipoR2 (10 µg/ml, pre-treat 1 hour). Cells treated with goat IgG (10 µg/ml, 18 hour) were used as negative controls. Results were shown as mean±SE, n = 4 for each group. * p< 0.05 vs. control; # p < 0.0001 vs. AngII with adiponectin treated group; ! p< 0.05 vs. AngII treated group; !! p< 0.0001 vs. AngII treated group.

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3.4.4 The inhibitory effect of adiponectin on AngII-induced NADPH oxidase activation in renal tubular cells is mediated by AMPK signaling pathway: Adiponectin attenuates oxidative stress in podocytes and endothelial cells through the AMPK signal transduction pathway [514, 593]. In order to test whether AMPK also mediated the effect of adiponectin on AngII-induced NADPH oxidase, we first treated the renal tubular cells with the AMPK agonist AICAR [514, 593]. Treatment with AICAR alone had no effect on basal NADPH oxidase activity, but AICAR prevented AngII-induced NADPH oxidase activation (Figure 3.5A). We then studied the effect of an AMPK antagonist compound C. The antagonist completely blocked the inhibitory effect of adiponectin on AngII-induced NADPH oxidase activation (Figure 3.5B). In addition, adiponectin increased phosphorylation of AMPK at Thr-172 as well as phosphorylation of the AMPK target protein acetyl CoA carboxylase (ACC) at 1 hour (Figure 3.5C).
Figure 3.5 The effect of adiponectin on Ang II-induced NADPH oxidase activation was mediated by AMPK pathway in tubular cells
The AMPK agonist AICAR reduced the activity of NADPH oxidase after AngII stimulation similarly as adiponectin (A), while the AMPK antagonist compound C completely blocked the inhibition by adiponectin on AngII-induced superoxide generation (B). Activation of the AMPK pathway by adiponectin was confirmed by phosphorylation of AMPK at Thr-172 and its target protein ACC (C). AngII = 0.1 µM;
adiponectin = 100 ng/ml; AICAR = 1 mM; compound C = 1 µM. For A and B, cells were incubated with appropriate agents for 18 hours. For C, cells were treated as indicated for 1 hour, with 30 min pre-incubation for compound C treated groups. β-Actin was blotted as a control. Results are presented as mean±SE. For A, n = 3 in each group; * p < 0.05 vs. control; # p < 0.001 vs. AngII treated. For B, n = 3 in each group; * p < 0.05 vs. control; # p < 0.001 vs. AngII and adiponectin treated. For C, n = 4 for phosphor-AMPK and n = 5 for phosphor-ACC; * p < 0.05 vs. control; ! p < 0.05 vs. adiponectin treated.
3.4.5 The cAMP/Epac pathway is also involved in the inhibition of adiponectin on AngII-induced NADPH oxidase activation: The cAMP pathway was also reported to mediate the anti-oxidative effect of adiponectin in endothelial cells [593, 595]. We therefore investigated if the cAMP pathway played a role in reducing AngII-caused increase in ROS production in renal tubular cells. Treatment with either of the stable cAMP analogues, pCPT-cAMP or db-cAMP, recapitulated the effect of adiponectin on AngII-induced NADPH oxidase (Figure 3.6A). We then studied the effect of an adenylate cyclase inhibitor, SQ22536. This compound blocked the inhibitory effect of adiponectin on AngII-induced NADPH oxidase activation (Figure 3.6B). Increased cellular cAMP levels can signal through PKA and through AMP-activated guanine nucleotide exchange factors (cAMP-GEFs, also known as Epacs) [566, 599]. Using a non-radioactive assay, we measured the level of PKA activity after 10 min, 2 hours or 16 hours of adiponectin treatment. Neither short nor long time stimulation increased PKA activity in the renal tubular cells (Figure 3.7A). We further tested for a role for PKA by treating the cells with a specific cell-permeable PKA inhibitor, myristoylated PKI (14-22) amide (mPKI). mPKI did not inhibit the effect of adiponectin on AngII-induced NADPH oxidase activation (Figure 3.7B). In contrast, the specific Epac activator 8-CPT-2-o-Me-cAMP reduced the activity of NADPH oxidase following AngII treatment (Figure 3.8A). Crosstalk between cAMP and AMPK signaling pathways has been demonstrated in hepatocytes [600]. We therefore studied the effect of the AMPK inhibitor, compound C, on the inhibition of AngII-induced NADPH oxidase by the Epac activator 8-CPT-2-o-Me-cAMP (Figure 3.8B). Activation of Epac did not inhibit AngII-induced NADPH oxidase activation in the presence of compound C.
Figure 3. 6 cAMP signal transduction pathway was also involved in the effect of adiponectin on Ang II-induced NADPH oxidase activation

Renal tubular cells were treated as indicated for 18 hours before collected for lucigenin-enhanced chemiluminescence assay. Stable cAMP analogues (pCPT-cAMP and db-cAMP) mimicked (A), while adenylyl cyclase inhibitor (SQ22536) reversed (B) the antioxidative effect of adiponectin. AngII = 0.1 μM; adiponectin = 100 ng/ml; pCPT-cAMP = 20 μM; db-cAMP = 25 μM; SQ22536 = 0.1 mM. Results are presented as mean±SE, and at least 3 replicates were done for each experimental group. For A, * p < 0.05 vs. control; # p < 0.001 vs. AngII treated. For B, * p < 0.05 vs. control; # p < 0.001 vs. AngII and adiponectin treated.
Figure 3. PKA did not mediate the anti-oxidative effect of adiponectin against Ang II stimulation in human renal tubular cells

Neither short nor long time adiponectin treatment increased PKA activity in human renal tubular epithelial cells (A), and the specific cell-permeable PKA inhibitor, myristoylated PKI (14-22) amide (mPKI), did not affect the inhibition by adiponectin on AngII-induced NADPH oxidase activation (B). pCPT-cAMP was used as a positive control for PKA activity assay. Cells were incubated with appropriate agents for 18 hours before
collected for NADPH oxidase activity measurement. AngII = 0.1 μM; adiponectin = 100 ng/ml; pCPT-cAMP = 20 μM; mPKI = 10 μM (30 min pre-treatment). Results are presented as mean±SE. For A, n = 5 for each group; * p < 0.05 vs. control. For B, n = 3 for control and AngII with adiponectin treated; n = 4 for AngII treated, mPKI treated and AngII with adiponectin and mPKI treated; * p < 0.05 vs. control; # p < 0.001 vs. AngII and adiponectin treated.
Figure 3. Epac was responsible for the effect of adiponectin on Ang II-induced NADPH oxidase activation

Specific Epac activator 8-CPT-2-o-Me-cAMP reduced the activity level of NADPH oxidase following AngII treatment (A), which was blocked by the AMPK antagonist compound C (B). Renal tubular cells were treated for 18 hours before activity measurement. AngII = 0.1 μM; 8-CPT-2-o-Me-cAMP = 50 μM; compound C = 1 μM (30 min pre-treatment). Results are presented as mean±SE, n = 3 for each group. For A, * p < 0.05 vs. control; # p < 0.001 vs. AngII treated. For B, * p < 0.05 vs. control; # p < 0.001 vs. AngII and adiponectin treated; !p < 0.05 vs AngII treated.
3.4.6 Adiponectin attenuated the inflammatory and fibrotic response induced by AngII in renal proximal tubular cells: In order to extend the protective effect of adiponectin in kidney cells beyond ROS production, we studied AngII-induced activation of NFκB, a major mediator of the cellular inflammatory response, and the production of fibronectin, an extracellular matrix protein that accumulates in kidney injury. We transfected cells with an NFκB-reporter construct, and activity was determined by measuring luciferase activity. As expected, AngII caused a robust increase in NFκB activity in renal tubular cells transfected with the NFκB reporter construct. This effect was attenuated by adiponectin (Figure 3.9). The expression of fibronectin was measured by Western blot analysis of protein lysates from our renal tubular cells. Stimulation with AngII was associated with increased fibronectin protein generation, and this effect was also inhibited by adiponectin (Figure 3.10).
Figure 3. 9 Adiponectin reduced NFκB activity induced by Ang II
Human renal tubular cells were transfected with pNFκB-Luc plasmid as described previously. After 18 hours of incubation with or without AngII (0.1 μM) and APN (100ng/ml), cells were lysed and the lysates were used for luciferase activity assay. n = 3 for all groups. * p< 0.001 vs. transfection control; # p < 0.001 vs. AngII-treated group.
Figure 3. Adiponectin attenuated the increase in fibronectin expression after Ang II stimulation in renal tubular cells
Renal proximal tubular cells were incubated with AngII (0.1 µM) and/or adiponectin (100 ng/ml) for 18 hours before harvested for fibronectin immunostaining. α-Tubulin was used as loading control. Results are shown as mean±SE, and n = 4 for each group. * p < 0.05 vs. control; # p < 0.05 vs. AngII and adiponectin treated.
3.5 Discussion

The mechanism(s) responsible for the relationship between obesity and progression of CKD have not been fully elucidated, although recent evidence suggests that adipose tissue-derived hormones and cytokines may play a role [570-572]. Circulating levels of leptin rise with obesity and may play a direct role in kidney injury [586, 601]. In contrast, circulating levels of adiponectin are negatively correlated with body mass index [573-576]. Although adiponectin impacts on cardiovascular injury, it is unclear if the kidney is also a target organ for adiponectin. In the current study, we focused on the interaction between adiponectin and AngII, a well-known contributing factor to CKD progression, and specifically on the effect of adiponectin on AngII-induced production of reactive oxygen species (ROS).

There are two receptors for adiponectin, adipoR1 and adipoR2 [602-605], and our first major observation was that both adiponectin receptor subtypes were expressed in our primary human kidney tubular cells. They co-localized with Na\(^+\)/K\(^+\) ATPase indicating that they were present on the cell membrane. AdipoR1 and adipoR2 are 7-transmembrane spanning proteins distinctive from G protein coupled receptors in that the membrane orientation of the N- and C-terminals is reversed [602, 603, 605]. AdipoR1 is widely expressed in human tissues, most abundantly in the liver tissue and skeletal muscle [604]. AdipoR2 expression is usually considered to be more restricted to liver and muscle [604]. The ligand-binding sites are slightly different for adipoR1 and R2: adipoR1 has higher affinity for globular adiponectin while adipoR2 has intermediate affinity for both globular and full-length adiponectin [602]. In vitro studies have also shown that the receptor subtypes are expressed in podocytes [580]. The regulation of expression of AdipoR1 and AdipoR2 has not been fully characterized [604], and therefore we studied the effect of AngII on expression: there was no effect of AngII on mRNA expression of either receptor in our short term studies.

We next chose to look at interactions between adiponectin and AngII because activation of the renin-angiotensin system and increased generation of AngII contribute to the development and progression of kidney disease [582-585, 587, 606], and we focused on AngII-induced activation of NADPH oxidase because oxidative stress links the
bioactivity of AngII to tissue fibrosis and inflammation [588-592]. Our second major observation in this study was that adiponectin inhibited AngII-induced NADPH oxidase activation and oxidative stress in renal tubular cells in a dose-dependent manner. Adiponectin has been shown to suppress ROS generation and reduce high glucose-induced oxidative stress in endothelial cells and glomerular podocytes [580, 593-596] though an effect on AngII-induced oxidative stress in kidney tubular cells has not been reported previously. Additionally, adiponectin inhibited AngII-induced activation of NFκB, an important regulator of cellular inflammation, and inhibited AngII-induced increases in fibronectin expression. These findings suggest that adiponectin may play a protective role in the kidney by modifying oxidative stress, inflammation and fibrosis when the RAS is activated. Obesity-associated declines in adiponectin might then render the kidney more vulnerable to oxidative stress and account at least in part for the relationship between obesity and CKD.

The signal transduction pathways linking adiponectin to inhibition of NADPH oxidase may be stimulus- and cell-specific, at least to the extent that they have been studied [580, 593, 595]. With blocking antibodies, we found differential roles for the adiponectin receptors on the anti-oxidative effect against AngII in renal tubular cells. Our third major observation was that adipoR1 mediated most if not all of the inhibition on NADPH oxidase activation by adiponectin in renal tubular cells. Binding of adiponectin to its cognate receptors has been shown to modulate several signal transduction pathways, including AMPK, cAMP, PPARα, and p38 MAPK [602, 605]. We utilized full-length adiponectin and focused on AMPK and cAMP because each of these pathways has been related to the effect of adiponectin on oxidative stress. For example, Sharma and coworkers demonstrated that high glucose (HG)-induced NADPH oxidase activation by adiponectin in podocytes was dependent on AMPK activation [580]. In contrast HG-induced NADPH oxidase activation was cAMP-dependent in endothelial cells [595]. Interestingly, Kim and coworkers showed that adiponectin suppressed ROS generation after palmitate stimulation, and that the inhibition was dependent on both cAMP and AMPK signaling [593]. Accordingly, we then sought to define the signal transduction pathways linking adiponectin/adipoR1 to inhibition of AngII-induced NADPH oxidase activation.
Our next major observation was that the inhibitory effect of adiponectin was dependent on both AMPK and cAMP signal transduction pathways in renal tubular cells. In order to establish this we looked at the effect of both stimulation and blockade of AMPK and cAMP signalling pathways with pharmacological activators and inhibitors. We used AICAR, an AMPK agonist, and compound C, an AMPK antagonist, to test the AMPK pathway, and we used two stable cAMP analogues, pCPT-cAMP and db-cAMP, and the adenylate cyclase inhibitor, SQ22536, to test the cAMP pathway. The activation of each pathway in the renal tubular cells was sufficient to inhibit AngII-induced NADPH oxidase activation, as was inhibition of each pathway sufficient to block the inhibitory effect of adiponectin on AngII-induced NADPH oxidase activation. Adiponectin treatment also led to the phosphorylation of AMPK at Thr-172 and an increase in its activity which was assessed by measuring the phosphorylation of a recognized substrate, acetyl CoA carboxylase (ACC), an effect that was abrogated by co-treatment with the AMPK inhibitor, compound C.

Interestingly, treatment with adiponectin failed to activate PKA, the classic downstream effector of cAMP in our cells. In accord with this finding the PKA specific inhibitor, myristoylated PKI (14-22) amide, showed no influence on the effect of adiponectin on AngII-induced NADPH oxidase activation in renal tubular cells. Taken together these findings suggest that the cAMP/PKA is not linked to the inhibitory effect of adiponectin on AngII-induced NADPH oxidase activation. This prompted us to examine the effect of another molecule downstream of cAMP, Epac. Epacs, or exchange proteins directly activated by cAMP, are enriched in renal proximal tubular cells [607], and have been shown to protect proximal tubular cells from cisplatin induced apoptosis [608] and the kidney from ischemia-reperfusion injury [609]. The specific Epac activator 8-CPT-2-o-Me-cAMP inhibited the increase in NADPH oxidase activity after AngII-stimulation, suggesting that Epac is an important molecule for transducing the anti-oxidative signal from adiponectin and cAMP in renal tubular cells.

The involvement of both the AMPK and the cAMP-Epac pathways in the inhibitory effect of adiponectin on AngII-induced NADPH oxidase activation suggests that there may be crosstalk between the two signal transduction pathways. Previously Fu and
colleagues found that activation of the cAMP-Epac pathway led to AMPK activation through LKB1 in liver cells [600]. Omar et al. also reported that a specific Epac activator increased AMPK phosphorylation in adipocytes [610]. We therefore studied the ability of the specific Epac activator, 8-CPT-2-o-Me-cAMP, to block AngII-induced NADPH oxidase activity when AMPK was inhibited by compound C. We found that the inhibitory effect of 8-CPT-2-o-Me-cAMP on AngII-induced NADPH oxidase activation was completely reversed by compound C. This finding suggests that there is crosstalk between cAMP signaling and AMPK signaling in our cells and that Epac plays a key role in this crosstalk.

The mechanisms responsible for the inhibition of NADPH oxidase by activation of AMPK and cAMP-Epac have not been fully elucidated, though some studies suggest that inhibition may be mediated by an effect on the expression levels of NADPH oxidase subunits. For example, deletion of the gene for adiponectin is associated with up-regulation of expression of NOX4 in podocytes [580]. NOX2 is increased in cardiac tissue in adiponectin knockout mice [596]. In addition, Wang and coworkers discovered that the NADPH oxidase subunits p47^{phox}, p67^{phox} and NOX2 increased in mice with deletion in the gene for AMPKα2, an effect that may also involve NFκB [611, 612]. Taken together, these studies suggest that adiponectin negatively regulates NADPH oxidase subunits expression and that this effect may account, at least in part, for the reduction in agonist-induced NADPH oxidase activity by adiponectin.

In summary, our data show that adiponectin receptors are expressed in primary human kidney tubule epithelial cells and that AMPK and cAMP signal transduction pathways are activated by adiponectin in these cells. Adiponectin inhibits AngII-induced NADPH oxidase activation in these cells, an effect that is dependent on AdipoR1. Activation of either AMPK or cAMP by adiponectin is sufficient to exert this inhibitory effect. Crosstalk between these pathways is mediated in part by Epac. Adiponectin also attenuated the increase in NFκB activity and fibronectin expression caused by AngII stimulation. Together, these in vitro findings support the hypothesis that adiponectin may impact progression of CKD by limiting AngII-induced oxidative stress, inflammation and fibrosis in the kidney.
Chapter 4

Loss of adiponectin in $\text{Ins2}^{+/C96Y}$ mutant mice exacerbates diabetic nephropathy
4.1 Abstract

Diabetic nephropathy (DN) is one of the most common forms of chronic kidney disease which may progress to end stage renal disease. Mechanisms of DN development and progression are complicated and have yet to be completely elucidated. Adiponectin is an adipokine that is normally present in abundance in circulation and levels decline in diabetic patients. The role of adiponectin in the development of diabetic nephropathy has not been fully understood, and our hypothesis is that adiponectin plays a beneficial role in kidney against diabetic insults. To test our hypothesis, we studied the kidney injuries in the diabetic $\text{Ins2}^{+/C96Y}$ adiponectin$^{-/-}$ (Akita/APN) mice. Compared to wild-type controls, both diabetic mice with and without adiponectin developed kidney hypertrophy as measured by kidney-to-body weight ratio, glomerular enlargement and albuminuria. The Akita/APN double mutant mice showed exacerbated kidney hypertrophy, while adiponectin treatment prevented the activation of mTOR and S6K in cultured mesangial cells. Immunohistochemical staining for collagen IV and $\alpha$-smooth muscle actin suggested that diabetic mice lacking adiponectin also developed significantly more severe fibrosis in the glomerular and tubulointerstitial compartments. In vitro experiments with cultured mesangial cells confirmed the anti-fibrotic effect of adiponectin as it reduced the phosphorylation of Smad2 and Smad3 after TGF-$\beta$ stimulation. Akita/APN mice also showed increased inflammatory cell infiltration in the kidney as identified by the F4/80 stain. Accordingly, adiponectin inhibited the activation of NF$\kappa$B activation in mesangial cells in response to high glucose stimulation, which could be partially mediated through reactive oxygen species as adiponectin suppressed the activation of NADPH oxidase induced by high glucose stimulation. Our data suggested that adiponectin deficiency was associated with aggravation of kidney injury in diabetic mice and was possibly a contributor to the development of DN.
4.2 Introduction

One of the most common complications in diabetic patients is kidney disease, and diabetic nephropathy (DN) is a major form of chronic kidney disease that may eventually lead to end stage renal failure [370, 371]. At present, the incidence of chronic kidney disease is on the rise, which has posed a heavy burden on health care globally [613]. Much effort has been put into studying the molecular mechanisms by which increased circulating glucose levels causes tissue injury, and a number of reviews have been published on this subject [376, 614]. Nonetheless, factors involved in the pathogenesis of DN and their roles have yet to be completely elucidated.

Recent experimental evidence suggests that adiponectin, an adipokine, has important implications in the development of diabetic complications [413, 615]. The 30kDa hormone is predominantly secreted by adipose tissue and is present abundantly in blood under normal physiological conditions. In the body, adiponectin molecule can interact with each other through the collagen-like tail domains to form trimers, hexamers and high molecular weight (HMW) multimers, and give rise to a bioactive type of globular fragment, gAD, by leukocyte elastase-mediated degradation [411, 414]. The various forms of adiponectin activate downstream signaling pathways by interacting with 2 major receptors, the adiponectin receptor 1 (adipoR1) and 2 (adipoR2), although T-cadherin has also been shown to interact with the adipokine and critically mediate many of its functions in cardiac tissue [422, 428]. Binding of adiponectin to its receptors impacts on several signaling pathways, including the AMPK, PPAR-α, MAPK, Akt and cAMP signal transduction pathways [423, 616, 617] Adiponectin functions to modulate metabolism and can augment the effect of insulin, as it positively regulates fatty acid beta oxidation and glucose uptake in skeletal muscle but negatively regulates gluconeogenesis in liver [412]. Blood adiponectin levels decrease in obese individuals and diabetic patients, and lower circulating adiponectin levels have been associated with a higher risk for developing type 2 diabetes mellitus [440, 441, 445].

Previous studies have demonstrated that adiponectin can improve insulin sensitivity and inhibit inflammation, and hence may be protective against diabetic injury. Replenishing adiponectin in obese mice attenuated insulin resistance and hyperglycemia [467]. Mutant
mice lacking adiponectin developed more severe vascular endothelial cell dysfunction in response to high-fat diet, in contrast to which adiponectin suppressed the expression of adhesion molecules on endothelial cells in response to TNFα stimulation [448, 452]. Adiponectin has also been found to influence kidney function. Using a line of adiponectin knock-out mice, Sharma and colleagues identified a critical role of adiponectin in maintaining the health of podocytes, which was dependent on its inhibitory effects on oxidative stress [514]. A pharmacological activator of AMPK, a major downstream signal mediator of adiponectin, also suppressed the generation of H₂O₂ and MCP-1 in the kidneys of mice fed a high-fat diet [518]. However the role of adiponectin in the development of DN has yet to be completely delineated. We hypothesize that adiponectin is beneficial to kidneys against hyperglycemic insults. To test this hypothesis, we crossed adiponectin gene knock-out (APN) mice with type 1 diabetic Ins2+/C96Y (Akita) mice to create a line of double mutant diabetic Akita/APN mice and examined the functional and structural changes in kidneys of these animals.
4.3 Materials and Methods

Animals - Wild-type (WT) and diabetic Ins1+/C96Y mutant mice of C57BL/6J strain were purchased from the Jackson’s Laboratory. Adiponectin-/- (APN) mice were generously donated by Dr. Subodh Verma. Double mutant Ins2+/C96Y adiponectin-/- (Akita/APN) were generated through crossing the Akita and APN line and maintained on a C57BL/6 background. All animals were housed at the Division of Comparative Medicine at University of Toronto, with standard chow diet and free access to food and water. All experiments were conducted under the guidelines of the University of Toronto Animal Care Committee.

Male mice were followed from 4 weeks of age until sacrifice and tissue harvest at 16 weeks of age, with weekly blood glucose and body weight recordings. Blood glucose was measured by Bayer Contour® glucometer (Bayer Inc., Toronto, ON, CA) at the end of 6 hour-fasting period starting at 7 AM with tail vein blood. 24 hour-urine samples were obtained at 16 weeks of age by placing animals individually in metabolic cages. At sacrifice, mice were anesthetized with inhaled isoflurane, and body weights and kidney weights were recorded. Blood samples were collected from the carotid artery and both kidneys were dissected out. Kidneys were cross-sectioned into 3 parts, with two polar sections snap-frozen in liquid nitrogen and stored at -80 °C until use. Middle sections of kidneys were fixed in buffered formalin in preparation for immunohistochemistry analysis.

Cells – Human renal mesangial cells were purchased from Lonza (Clonetics™, Lonza, Walkersville, MD, USA) and cultured RPMI-1640 medium (R1383, Sigma Aldrich, Saint Louis, MO, USA) supplemented with NaHCO3 (2g/L), HEPES (0.01M), glucose (5.6mM), fetal bovine serum (17%), insulin (6mg/L), transferrin (6mg/L), penicillin (100u/ml) and streptomycin (100ug/ml). Cells were maintained at 37°C, with 5% CO2 and 95% air, and cells of passage 4 -7 were used for experiments. Full-length recombinant adiponectin was used for in vitro experiments.

Blood biochemistry – Blood samples were collected from the carotid artery at sacrifice with Microvette® (Sarstedt Inc., Montreal, QC, CA) according to the manufacturers’
protocol. Samples were spun at 2000g at room temperature for 5 min. Plasma was isolated and stored at -80°C. Levels of blood biochemical parameters, including cholesterol, triglycerides, urea, creatinine, sodium, potassium and chloride, were measured at the Toronto Centre for Phenogenomics (Toronto, ON, CA).

**Urinary albumin excretion** – 24-hour urine samples were obtained by placing experimental animals individually in metabolic cages (Nalgene, Nalge Nunc International, Rochester, NY). Urine samples were centrifuged at 8000g for 5 min at room temperature and the supernatants were collected and stored at -80°C until use. Albumin levels were determined with Albuwell M kit (Exocell, Philadelphia, PA, USA) following the manufacturers’ guide.

**Histology and immunohistochemistry** – At tissue harvest, the middle 1/3 of the kidney was fixed in 10% neutral buffered formalin (Sigma Aldrich) for 24 hours and then transferred to 80% ethanol before embedding. Paraffin embedding, sectioning, staining and scanning were done at the Pathology Research Program (PRP) Laboratory at Toronto General Hospital (Toronto, ON, CA). 3μm periodic acid-Schiff (PAS) stained sections were used for scoring histopathological injury and measuring glomerular volume. Mean glomerular volumes ($\bar{V}_G$) were calculated from mean cross-sectional areas ($A_G$) derived by Aperio ImageScope software (Aperio Technologies Inc., Vista, CA, USA) using the equation $\bar{V} = \frac{\beta}{k} \times \bar{A}_G^\frac{3}{2}$, where the shape coefficient for sphere $\beta = 1.38$ and the size distribution coefficient $k = 1.1$. For immunohistochemical staining, 3% hydrogen peroxide was used to quench endogenous peroxidase activity before staining for αSMA, collagen IV and F4/80. Only glomerular regions were selected for ColIV quantification, and tubulointerstitial areas (excluding blood vessels) were selected for αSMA quantification. Other stains were quantified for the whole kidney section. Measurements for all staining were achieved by using the Positive Pixel Count algorithm of Aperio ImageScope software (Aperio Technologies, Inc.).

**Western blot** – All antibodies were purchased from Cell Signaling (Cell Signaling Technology, Inc., Danvers, MA, USA) unless otherwise specified: phosphor-mTOR (#2974), total-mTOR (#2972), phosphor-p70 S6K (S6K, #9205), total-p70 S6K (#9202),
phosphor-Smad2 (#3104), total-Smad2 (#5339), phosphor-Smad3 (#9520), total-Smad3 (#9523), anti-rabbit IgG (#7074). Mesangial cells were serum starved overnight before being incubated with high glucose (30mM D-glucose, 1 hour) or TGF-β (2ng/ml, 1 hour, R&D Systems Inc., Minneapolis, MN, USA) with or without adiponectin (100ng/ml, 30min pre-incubation, BioVision, Inc., Milpitas, CA, USA). Cells were collected and lysed with lysis buffer (Cell Signaling Technologies, Inc.). Proteins in cell lysates were separated by SDS-PAGE gels and blotted onto PVDF membrane, which were then detected by enhanced chemiluminescence system (ECL) kit (Luminata™ Forte Western HRP Substrate, Millipore Corp., Billerica, MA, USA). Scion Image software (Scion Corp., Frederick, MD, USA) was used for densitometry calculation.

**NFκB activity assay** – Human mesangial cells were sub-cultured in 6-well plates for 24 hours and co-transfected with pNFκB-Luc plasmid (Stratagene, Agilent Technologies, Inc., Santa Clara, CA, USA) and pRL-TK reporter vector (Promega Corp., Madison, WI, USA) using the Effectene Transfection Reagent kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturers’ guide. Cells were incubated for 24 hours then subjected to high glucose (30mM) treatment with or without adiponectin (100ng/ml) or apocynin (100μM, Calbiochem, Millipore Corp., Billerica, MA, USA) for 24 hours. Luciferase activity was determined with the Dual-Luciferase Reporter Assay System (Promega) and the light emission was read by FB12 Luminometer (Titertek-Berthold, Berthold Detection Systems GmbH, Pforzheim, Germany). Total protein measured with Bradford assay (Bio-Rad, Hercules, CA, USA) was used to normalize the activity results.

**Lucigenin enhanced chemiluminescence assay** – Human mesangial cells were grown to full confluence after being sub-cultured in 6-well plates. Then cells were incubated in serum-free medium containing normal glucose (5.6mM), high glucose (30mM) and high glucose with adiponectin (100ng/ml, pretreat 1 hour) for 18 hours. At the end of the incubation period, cells were collected and spun down to remove the medium. Mesangial cell pellets were resuspended with ice cold PBS and mixed with NADPH (final concentration = 100μM, Calbiochem) and lucigenin (final concentration = 5μM, Sigma Aldrich). The mixture was incubated at 37°C and light emission was monitored by FB12
Luminometer (Berthold Detection Systems). Peak emission rate was later normalized with total protein concentration in cell lysates as determined by Bradford assay.

**Dihydroethidium (DHE) stain:** Human mesangial cells were seeded onto glass cover slides and cultured to 90% confluence. Cells were then treated for 24 hours in medium containing normal (5.6mM) or high glucose (30mM) and/or adiponectin (100 ng/ml). After washing with PBS, cells were incubated with DHE (2 µM, Invitrogen) at 37°C for 1 hour in dark. At the end of incubation, cells were washed twice with PBS then once with distilled water. The cover slips were mounted onto glass slides using Dako Faramount Aqueous Mounting Medium (S3025, Dako Canada, Inc.). Fluorescent images were captured with a Zeiss confocal laser-scanning microscope (LSM510, Carl Zeiss Inc.). At least 11 fields (magnification: 400x) were selected for each experimental group and the fluorescence intensity for each field was scored semi-quantitatively on a scale of 0 to 4 (0 being dark and 4 being the strongest florescence) by a trained nephrologist blinded to the experimental conditions.

**Statistical analysis** – Results are shown as mean ± S.E., and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analyses. For multiple group comparison, one-way ANOVA with Bonferroni post-test was performed, and for two-group comparison, unpaired t-test was performed. Statistical significance was set at p value less than 0.05.
4.4 Results

4.4.1 Whole animal data: All 4 groups of experimental animals exhibited growth in body weight between 4 to 16 weeks of age (Figure 4.1A) and both the diabetic Akita and Akita/APN mice developed hyperglycemia as expected and experienced sustained high blood glucose levels beginning from 8 weeks of age (Figure 4.1B). Blood glucose levels were comparable in the 2 diabetic groups. At 16 weeks of age, both Akita and the double mutant animals had lower plasma cholesterol level but higher plasma triglyceride levels than the control animals (Table 4.1). Adiponectin knockout (APN) mice also had decreased plasma cholesterol levels compared to wild-type controls. Blood urea levels were elevated in the diabetic groups but decreased in the APN mice (Table 4.1).
Figure 4. 1 Body weight and blood glucose measurements in the experimental animals
Change in body weight (A) and blood glucose (B) levels were followed in the WT (●), APN (■), Akita (▲) and Akita/APN (▼) mice weekly from 4 to 16 weeks of age. At least 6 animals were used in each experimental group at any given age.
Table 4. Whole animal data

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT</th>
<th>APN</th>
<th>Akita</th>
<th>Akita/APN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose (mmol/M)</td>
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<td>10.3±0.62</td>
<td>30.7±0.67*</td>
<td>29.9±1.02*</td>
</tr>
<tr>
<td>Body weight (g)</td>
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<td>26.5±0.73*</td>
<td>26.5±0.44*</td>
<td>23.7±0.60*#</td>
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<td>Kidney weight (g)</td>
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<td>0.18±0.005</td>
<td>0.27±0.009*</td>
<td>0.30±0.008**#</td>
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<td>Cholesterol (mM)</td>
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<td>2.2±0.12*</td>
<td>2.5±0.12*</td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
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<td>1.1±0.10</td>
<td>1.7±0.21**#</td>
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<tr>
<td>Urea (mM)</td>
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<td>16.1±1.13*</td>
<td>21.6± 0.96*</td>
<td>21.4± 0.78*</td>
</tr>
<tr>
<td>Creatinine (µM)</td>
<td>17±1.5</td>
<td>16±1.1</td>
<td>22±1.0</td>
<td>21±0.8</td>
</tr>
<tr>
<td>Sodium (mM)</td>
<td>146±1.4</td>
<td>146±1.6</td>
<td>138±1.5*</td>
<td>141±0.627</td>
</tr>
<tr>
<td>Potassium (mM)</td>
<td>5.0±0.17</td>
<td>5.2±0.13</td>
<td>4.7±0.15</td>
<td>4.6±0.19</td>
</tr>
<tr>
<td>Chloride (mM)</td>
<td>116±0.8</td>
<td>114±0.6</td>
<td>105±1.6*</td>
<td>103±1.5*</td>
</tr>
</tbody>
</table>

Blood glucose was measured at 16 weeks of age before tissue harvest. Body weight and kidney weight (average of left and right kidney) were recorded at the time of sacrificed. Concentrations of cholesterol, triglycerides, urea, creatinine, sodium, potassium and chloride were determined in plasma samples. At least 19 animals in each group were used to derive blood glucose, body weight and kidney weight data and blood chemistry was measured for 8 mice in each group. Results are presented at mean ± S.E. * p < 0.05 vs. WT. # p < 0.05 vs. Akita.
4.4.2 Structural and functional changes in kidneys: At the end of the experimental period, body weights were reduced while kidney weights were increased in the diabetic mice (Figure 2A, 2B). Deletion of the adiponectin gene was associated with more exaggerated changes in body weight and kidney weight in mice, which indicated an exacerbation of wasting and kidney injury in Akita/APN mice comparing to the diabetic mice with intact adiponectin gene (Figure 2A-C). Interestingly, normoglycemic APN mutant mice also showed lower body weight than control animals at 16 weeks of age (Figure 2A). Although the absolute kidney weight was not changed in APN mice, the kidney-to-body weight ratio was increased in these animals in comparison to wild-type controls (Figure 2B, 2C). As expected, diabetic Akita mice had higher kidney-to-body weight ratio and the Akita/APN double mutant mice showed an even more significant increase in this measurement as compared to Akita mice (Figure 2C). The Akita mice developed impairment in kidney function as measured by urine albumin excretion rate (UAE, Figure 2D). The Akita/APN mice also had elevated UAE, but despite a trend of further increase the change in albuminuria level failed to reach statistical significance when compared to the Akita mice (Figure 2D). Deletion of the adiponectin gene did not alter the baseline UAE level.
Figure 4. 2 Body weight (A), kidney weight (B), kidney/body weight ratio (C) and urinary albumin excretion (UAE, D) were assessed in 16 week-old animals. Average kidney weights were present and used to calculate kidney/body weight ratio. Data are shown as mean ± S.E. n ≥ 19 for body weight, kidney weight, kidney/body weight ratio in each experimental group. For UAE, n = 6 for WT; n = 7 for APN and Akita; n = 9 for Akita/APN. * p < 0.05 vs. WT. # p < 0.05 vs. Akita.
**4.4.3 Assessment of kidney hypertrophy:** Because of the rise in kidney-to-body weight ratio in Akita/APN mice compared to Akita mice (Figure 2C), we sought to further define the effect of adiponectin on kidney hypertrophy. Microscopic structural changes in the kidney were examined in PAS stained kidney sections (Figure 3A), and semiquantitative scores were derived from PAS sections based on the degree of glomerular injury (Figure 3B). Mice with diabetes showed mesangial matrix expansion as expected, where loss of adiponectin further exacerbated the pathological changes. In agreement with the elevated kidney weight, mean glomerular volume was increased in the diabetic Akita and Akita/APN mice as compared to the wild-type mice (Figure 3C). Mean sizes of glomeruli were not significantly different between the diabetic groups despite a higher mean glomerular volume in the double knockout mice. To determine the specific effect of adiponectin on mesangial cell growth in hyperglycemic environment, cultured human renal mesangial cells were stimulated with 30mM glucose and tested for activation in the mTOR signaling pathway. Phosphorylations of mTOR and S6K were increased by high glucose treatment, which was suppressed by co-incubation with adiponectin (Figure 4).
Figure 4. 3 Structural changes in renal tissue
(A) Representative images for PAS staining of kidney sections in WT, APN, Akita and Akita/APN mice. Magnification: 200x. (B) Semiquantitative glomerular injury scores were derived from PAS sections by a trained renal pathologist blinded to the experimental groups. (C) Glomerular volumes were calculated from mean glomerular areas in each section. Results are presented as mean ±S.E. n ≥ 8 in each group. * p < 0.05 vs. WT.
Figure 4. Adiponectin was able to prevent high glucose induced activation of the mTOR signaling pathway in cultured human mesangial cells
Representative images for immunoblotting against phospho- and total-mTOR and S6K (A) and the respective densitometry measurements (B,C) were shown. Results are presented as mean ± S.E. n = 3 in each group. * p < 0.05 vs. normal glucose (5.6mM). # p < 0.05 vs. high glucose (30mM).
4.4.4 Detection of renal fibrosis: To assess the presence of fibrotic tissue within the glomeruli, the area positive for collagen IV immunohistochemical staining was quantified using the ImageScope software (Figure 4.5A). Compared to wild-type animals, Akita/APN double mutant mice but not other groups of animals showed significantly increased collagen IV-positive area in their glomeruli (Figure 4.5B). Glomerular collagen IV-positive region in the double mutant mice was also enlarged as compared to the Akita mice (Figure 4.5B). A similar trend on fibrosis was observed in the tubulointerstitial compartment, where degree of fibrotic change was measured by α-SMA immunohistochemical stain (Figure 4.6A). Mice without adiponectin accumulated significantly more α-SMA in kidneys after becoming diabetic, whereas diabetic mice with adiponectin were not significantly different from the control animals in α-SMA-positive area even though a trend of increase was recognized (Figure 4.6B). To investigate the molecular mechanisms potentially responsible for the role of adiponectin on fibrosis, mesangial cells were cultured in the medium containing TGF-β1, with or without adiponectin. Phosphorylation of Smad2 and Smad3 were strongly stimulated by TGF-β1 treatment (Figure 4.7). Treatment with adiponectin partially blocked this activation of Smad2 and Smad3 by TGF-β1.
Figure 4. 5 Immunohistochemical stain for type IV collagen (ColIV) on renal sections
(A) Representative images of glomerular ColIV staining in the experimental animals. Magnification: 200x. (B) Quantification of glomerular ColIV positive area. Results are presented as mean ± S.E. n = 10 in each group. * p < 0.05 vs. WT. # p < 0.05 vs. Akita.
Figure 4. 6 αSMA staining on kidney sections
(A) Representative images from the experimental groups. Antibody was purchased from Abcam. Magnification: 200x. (B) Corresponding quantifications of αSMA positive area in the tubulointerstitial compartment. Results are presented as mean ± S.E. n = 6 for WT and Akita group; n = 7 for APN and Akita/APN group. * p < 0.05 vs. WT.
Figure 4. Adiponectin partially inhibited phosphorylation of Smad2 and Smad3 following TGF-β1 stimulation in mesangial cells

Representative images of immunoblot (A) and the densitometry quantification for Smad2 (B) and Smad3 (C) were shown. Results are presented as mean ± S.E. n = 4 for Smad2 immunoblot; n = 3 for Smad3 immunoblot. * p < 0.05 vs. control group. # p < 0.05 vs. TGF-β1 treated group.
4.4.5 Pathological changes in inflammation and oxidative stress: Infiltrating macrophages in the kidney were detected by F4/80 immunohistochemical stain (Figure 4.8A). F4/80 positivity was increased significantly only in the Akita/APN mice compared to controls, while the amount of infiltrating macrophages was not statistically different in the Akita mice when compared with wild-type control animals (Figure 4.8B). NFκB is a central mediator of inflammation. Cultured mesangial cells were transfected with NFκB-sensitive luciferase reporter constructs and then subjected to treatments with high glucose and adiponectin. High glucose strongly activated NFκB in mesangial cells, and adiponectin reversed this effect (Figure 4.9). Reactive oxygen species have been implicated in mediating many injurious effects of diabetes. In support of this, co-treatment with the antioxidant apocynin also inhibited the high glucose-induced NFκB activation similarly as adiponectin (Figure 4.9). The direct effect of adiponectin on the generation of reactive oxygen species was studied by measurement of NADPH oxidase activity and DHE fluorescent stain. NADPH oxidase activity was significantly higher in mesangial cells incubated in high glucose medium, and treatment with adiponectin obliterated this high-glucose-induced activation (Figure 4.10). Similarly, the intensity of DHE stain was increased in mesangial cells treated with high glucose, which was suppressed by adiponectin co-incubation (Figure 4.11).
Figure 4. 8 F4/80 stain for macrophage in kidney
(A) Representative images of F4/80 stainings. Magnification: 200x. (B) Corresponding quantifications of F4/80 positive area on whole kidney section. Results are presented as mean ± S.E. n = 8 for WT and Akita/APN group; n = 9 for APN and Akita group. * p < 0.05 vs. WT.
Figure 4. High glucose-induced NFκB activation in mesangial cells was inhibited by adiponectin
Co-incubation with apocynin also suppressed the NFκB activity after high glucose treatment. Mesangial cells transfected with NFκB response vectors and culture in normal glucose (5.6mM) were used as controls. Results are shown as mean ± S.E. n = 3 in each group. * p < 0.05 vs. control condition. # p < 0.05 vs. high glucose treated group.
Figure 4. Adiponectin reversed the increase in NADPH oxidase activity induced by high glucose in mesangial cells
Results are presented as mean ± S.E. n = 4 for normal and high glucose treated groups; n = 5 for high glucose and adiponectin treated group. * p < 0.05 vs. normal glucose (5.6mM) treated group. # p < 0.05 vs. high glucose (30mM) treated group.
Figure 4. Adiponectin suppressed the increase in ROS generation caused by high glucose.
Representative images of DHE stain (A) in cultured mesangial cells were shown with corresponding semi-quantitative scores (B). Results are presented as mean ± S.E. n ≥ 11 in each experimental group. * p < 0.05 vs. normal glucose (5.6mM) treated group. # p < 0.05 vs. high glucose (30mM) treated group.
4.5 Discussion

The circulating levels of adiponectin fall in patients with diabetes and low adiponectin levels in blood have been correlated to risks of developing type 2 diabetes. Well-characterized as an anti-inflammatory and anti-atherosclerotic factor in the cardiovascular system, adiponectin provides benefits to other organ systems as well, including the kidney. Since the role of adiponectin in the development and progression of diabetic nephropathy has yet to be fully understood, we sought to study the degree of kidney injury in mice deficient in adiponectin on a type 1 diabetes mellitus background. We observed that several aspects of DN were worsened in mice without adiponectin.

The first major finding in the current study was that adiponectin attenuated kidney hypertrophy in response to persistent hyperglycemia. Adiponectin null mice were reported to develop insulin resistance and altered metabolism even when maintained on standard chow [618, 619], and increased kidney oxidative stress has been shown in adiponectin mutant animals [514]. The adiponectin knock-out mice used in our study had reduced body weight as compared to the wild-type animals. Although the absolute kidney weight was not different in the APN and the control mice, the kidney-to-body weight ratio showed a slight yet statistically significant increase in the APN knockout mice, suggesting low levels of kidney hypertrophy occurred in animals without adiponectin before any functional impairment emerged. The hypertrophic changes in kidneys of adiponectin null mice were aggravated by diabetes, as the kidney-to-body weight ratio was significantly increased in the double mutant mice when compared with the Akita mice. An elevated trend of glomerular volume was identified in the Akita/APN mice in comparison to Akita mice, but the difference did not reach statistical significance, indicating involvement of the tubulointerstitial compartment in the hypertrophic process induced by diabetes. Pathological proliferation and matrix protein synthesis in the glomerular mesangial cells are features of DN. The mTOR signal transduction pathway has been known to mediate cellular hypertrophy, and previous studies have discovered an interaction between adiponectin and this signalling pathway via AMPK in the vascular smooth muscle cells [620] and in mesangial cells treated with PDGF [621]. Our experimental data in cultured human mesangial cells further confirmed that adiponectin was able to suppress high glucose-induced mTOR and S6K activation, suggesting that
the mTOR pathway could potentially mediate the anti-hypertrophic effect of adiponectin in kidney against hyperglycemic stimulation.

Additional to tissue growth, fibrosis can also arise as a result of chronically elevated blood glucose levels. Ohashi and colleagues demonstrated that adiponectin knockout mice suffered worse kidney fibrosis after subtotal nephrectomy, as indicated by increased levels of type I and III collagens and the profibrotic cytokine TGF-β [516]. In Wistar rats subjected to streptozotocin toxicity, overexpression of adiponectin via adenovirus ameliorated the increase in mRNA levels of TGF-β, endothelin-1 and PAI-1, which were known contributors to renal fibrosis [517]. However in contrast, Yang and colleagues discovered that adiponectin deficiency in mice was associated with less accumulation of matrix proteins such as collagens, α-SMA and fibronectin after both unilateral ureteral obstruction and renal ischemia-reperfusion [521]. The investigators also showed that adiponectin could stimulate monocyte-to-fibroblast differentiation and thus promote the infiltration of bone marrow-derived fibroblast in injured kidney and lead to renal fibrosis. Therefore how adiponectin affect fibrotic changes in kidney may be stimulus- and cell type-specific. In the current study, we quantified the degree of glomerulosclerosis from collagen IV immunohistochemical staining and the degree of tubulointerstitial fibrosis from α-SMA staining. In both compartments, only the Akita/APN mice developed statistically more significant fibrosis compared to the wild-type control animals, while the increasing trends in the Akita mice did not reach significance in statistical tests. Interestingly the APN mice showed a similar increasing trend of collagen IV accumulation in their glomeruli compared to control animals, suggesting some glomerular injury may have been initiated even without the occurrence of diabetes. The direct effect of adiponectin against tissue fibrosis was verified in the mesangial cells, where the activation of Smad2 and Smad3 was attenuated by adiponectin treatment.

Another function of adiponectin that has been demonstrated by both experimental models and epidemiological studies is its role as an anti-inflammatory factor. In cultured human aortic endothelial cells, adiponectin inhibited the expression of the pro-inflammatory cytokine IL-8 and the activation of NFκB after TNF-α stimulation [622]. Adiponectin null mice showed increased TNF-α production in cardiac tissue following myocardial
infarction, which was mediated by cyclooxygenase (COX)-2 [457]. In a case-control study in Pima Indians, circulating levels of adiponectin were negatively correlated with various inflammatory markers, including CRP, IL-6 and phospholipase A2 [475]. We assessed the possible role of adiponectin on inflammation in DN by examining the number of infiltrating macrophages in kidney tissue, and found that the number of F4/80 positive cells was only significantly increased in the double mutant Akita/APN mice compared to wild-type controls whereas a trend of elevation existed for the Akita mice. Treatment with adiponectin also inhibited the high glucose-induced NFκB activation in cultured mesangial cells. Reactive oxygen species (ROS) was found to activate NFκB in cultured adult rat ventricular myocytes, which was prevented by adiponectin incubation [623]. By co-incubating the mesangial cells with high glucose medium and the antioxidant apocynin, we found a similar suppression on the increase in NFκB activity. In addition, adiponectin inhibited the activation of NADPH oxidase and increase in DHE-detected cellular ROS content caused by high glucose stimulation, further indicating that adiponectin may prevent NFκB activation by suppressing the generation of ROS.

In the current study, loss of adiponectin in the type 1 diabetic Akita mice was associated with exacerbated kidney hypertrophy, fibrosis and inflammation. In vitro experiments on cultured mesangial cells also showed that adiponectin negatively regulates mTOR, TGF-β and NFκB signaling as well as ROS generation in response to high glucose stimulation.

In conclusion, adiponectin is a beneficial factor in the kidney against diabetic injury, and regimens to augment adiponectin functions may be potential therapies for patients with DN.
Chapter 5

Discussion and Future Directions
Activation of the renin-angiotensin system is one of the most important contributors to kidney disease development and progression, and RAS blockade will continue to be a cornerstone therapy for kidney disease. Nonetheless, RAS is a very complicated system involving a number of enzymatic metabolic cascades that generate various bioactive angiotensin peptides. As the incidence of kidney diseases rises, it is necessary to have a better understanding of the specific roles of the RAS components in different disease settings, as well as factors that can influence RAS activity and modulate the pathogenesis of kidney disease. To address this, the first part of my project focused on the role of ACE2, a known negative regulator of the RAS, in an acute kidney injury (AKI) model. I then studied the potential interactions between adiponectin, an adipose-tissue secreted hormone, and Ang II, the main effector of RAS activation, in cultured renal tubular cells. The effect of adiponectin on kidney injury was also examined in an animal model of diabetic mellitus, the Akita mice. I have demonstrated that ACE2 plays a protective role in ischemic-reperfusion injury in kidney, that adiponectin counteracts Ang II-induced ROS generation and that loss of adiponectin accelerates nephropathy in mice with experimental type 1 diabetic mellitus.

**ACE2 as a treatment for AKI**

Despite the observation that impaired kidney function (indicated by decline in GFR) is reversible after the resolution of AKI, epidemiological studies comparing populations with or without previous AKI episodes have identified an increased risk to develop chronic kidney disease (CKD) in those who had AKI [624]. Therefore the management of AKI needs to be optimized to prevent the possible long-term deleterious effects. As introduced in Chapter 1, activation of the renin-angiotensin system has been linked to the development and progression of CKD, and evidence has emerged that it may influence the disease process of AKI as well. In a report by Efrati and colleagues, concurrent treatment with an ACE inhibitor captopril in rats with renal ischemia/reperfusion injury prevented the increase in Ang II, TGF-β, IL-6 and IL-10 levels within 48 hours of reperfusion [625]. Interestingly, captopril was also associated with increased STAT-8-isoprostane content and decreased NO levels, suggesting worse renal oxidative stress in
the ACEI treated rats. In Chapter 2, I showed that the mice with a deletion in the gene for ACE2 have increased neutrophil, macrophage and T-cell infiltration in the kidney after I/R injury. The expression of the pro-inflammatory cytokines, IL-1β, IL-6, TNFα, MIP-2 and MCP-1, were also elevated in the mutant mice compared to wild-type control animals following I/R. In addition to effects on inflammation, the number of apoptotic cells and intra-renal oxidative stress as measured by nitrotyrosine were both higher in the mutant mice. Parallel with exacerbated kidney injury, Ang II levels were higher in the ACE2 knock-out mice in comparison to control mice following I/R injury, suggesting that ACE2 may function to limit the accumulation of Ang II and thus reduce inflammation and oxidative stress in the kidney after I/R injury.

Given these findings, it will be interesting to see whether treatment with recombinant ACE2 after the initial renal ischemia will ameliorate the kidney damage later in the reperfusion period. Currently both murine and human recombinant ACE2 are available and have been shown to inhibit hypertension induced by Ang II infusion [243]. Previously it has also been shown that treatment with human recombinant ACE2 reduced oxidative stress and fibrosis in kidneys in response to Ang II-infusion and diabetes stimuli [241, 626]. These research findings support future studies testing the potential effects of ACE2 as a therapeutic agent for AKI.

In the current study, the effects of ACE2 were examined at the end of a 48-hour reperfusion period, while the recovery and long-term outcomes of I/R in kidney have not been addressed. As data on AKI accumulate, it is evident that AKI predisposes patients to CKD and ESRD [627]. In rats that survived AKI, proteinuria was established by 16 weeks post-operation. Elevated levels of TGF-β and decreased peri-tubular capillary density were also found in post-AKI rats when compared with sham rats 40 weeks after the initial I/R injury [628]. Increased intrarenal Ang II levels and the consequential increased oxidative stress were proposed to be at least partially responsible for this effect, as Ang II concentration was found to be higher by 5 weeks post-AKI along with more intense DHE staining in the renal tissue and the antioxidant apocynin was able to rescue kidney fibrosis in post-AKI rats [629]. In Chapter 2, I have shown that cell proliferation as measured by Ki67 immunostaining was similarly increased by 48 hours in the ACE2
null mice and wild-type control mice after I/R. However exacerbated cell apoptosis, as measured by TUNEL stain, caspase-3 stain and BAX and Bcl-2 immunoblots, suggests that deletion of the gene for ACE2 in mice surviving AKI may be associated with an overall insufficient restoration of kidney cell number, which may be linked to future tubulointerstitial fibrosis [627]. Additionally, I have demonstrated that more macrophages were present in the kidney tissue of ACE2 null mice after I/R, which is in agreement with higher renal MCP-1 content. Macrophages have been shown to exert multiple effects on kidneys damaged by acute ischemia-reperfusion. They may promote inflammation at early stage post-AKI, but a shift to the M2 phenotype may aid tissue reparation 48-72 hours after the initial injury [630]. Ko and colleagues reported that macrophage depletion by liposome clodronate from Day 3 post-I/R reduced the level of TGF-β and the deposition of fibrotic extracellular matrix protein in kidneys 4 and 8 weeks after I/R operation [631], suggesting that macrophages may be important players in mediating the fibrotic change in the kidney following AKI. In our laboratory we have also found that profound fibrosis in kidney tissue is developed 28 days after original bilateral renal ischemia (data not shown). Hence it will be of interest to examine whether the ACE2 null mice will suffer more severe kidney fibrosis following I/R in the long term, and whether alteration(s) in oxidative stress and macrophage phenotype are potentially responsible for these changes in the kidney.

The comparison between ACE2 null and wild-type mice has demonstrated that ACE2 is protective against kidney damage following I/R. However, as shown in Figure 2.11 and Figure 2.12, there was a reduction of ACE2 protein on the renal tubules in wild-type mice with I/R injury even though the mRNA expression of ACE2 appeared to be up-regulated in these animals. The mechanism(s) by which ACE2 is lost from acutely I/R-injured kidneys were not explored in the current project. In a recent study by Lambert and colleagues, ACE2 was subjected to post-transcriptional modifications by a microRNA miR-421 in cardiac myofibroblasts [632]. miR-421 was able to bind to the 3’UTR of the ACE2 transcript, which interefered with translation and down-regulated the protein. Increased Ang II may also negatively regulate the expression of ACE2 in the context of AKI: Ang II, as well as endothelin-1, was found to have suppressed the expression of ACE2 mRNA and ACE2 activity in rat cardiac myocytes and fibroblasts.
Recent experimental evidence also suggests that ACE2 could be lost from the renal tubule surface through shedding by proteolytic processing of a disintegrin and metalloproteinase 17 (ADAM17). In type 2 diabetic db/db mice, urinary ACE2 fragments were increased along with higher expression of ADAM17 in renal tissue, which was reversed by treatment with rosiglitazone [225]. The same research group also identified this potential interaction in type 1 diabetic Akita mice [634]. ADAM17 was shown to be co-localized with ACE2 in renal tubules. Elevated renal tissue ADAM17 expression and urinary ACE2 fragment concentration was again confirmed in Akita mice, which were normalized by insulin treatment. In primary cultured mouse proximal tubular cells, ACE2 fragments were detected in the medium under normal culture conditions, suggesting shedding of ACE2 might occur at baseline [635]. The shedding of ACE2 in tubular cells was enhanced by treatments with high glucose and Ang II, and blocked by ADAM17 inhibitors. For future experiments, it will be of potential importance to understand the mechanism(s) underlying the loss of ACE2 from renal tubules in AKI, especially the role of ADAM17 in this process.

As described in Chapter 1, ACE2 may influence the pathogenesis of kidney disease through both the degradation of Ang II and the generation of Ang-(1-7). Inconsistent results were obtained with regard to the use of Ang-(1-7) as a therapeutic agent in kidney disease. More specifically for AKI, the level of Ang-(1-7) decreased shortly after onset of I/R injury in rats, which was accompanied by a concurrent increase in the expression of the Mas receptor [261]. In the adriamycin-induced acute kidney injury model, deletion of the Mas gene in mice was not associated with worse outcomes, while treatment with AVE0991, a Mas receptor agonist, reduced damage in kidney tissue [636]. Therefore more studies are necessary to fully determine the exact roles of Ang-(1-7) and the Mas receptor in AKI. Direct infusion of Ang-(1-7) may help to clarify some of its effects in I/R kidney injury.
Signaling cascades linking adiponectin to NADPH oxidase activity

Ang II has pleiotropic effects in the kidney and one important way it induces injury is by promoting the generation of reactive oxygen species. In Chapter 3, I studied the effect of the adipose-secreted hormone adiponectin on the inductive effect of Ang II on NADPH oxidase, and found that adiponectin suppressed ROS generation by NADPH oxidase following Ang II stimulation.

NADPH oxidase is a major source of superoxide in the renal cortex as well as the outer medullary region [637]. As briefly introduced in Chapter 1, various research publications have confirmed the critical role of NADPH oxidase activation in mediating the deleterious effects of Ang II in the kidney. The exact mechanism(s) by which Ang II induces NADPH oxidase activation have been most fully explored in vascular smooth muscle cells (VSMC) [637, 638].

Briefly, Ang II stimulates the activity of NADPH oxidase in the short term through promoting the assembly of the NADPH oxidase complex and in the long term through up-regulating the expression of NADPH oxidase subunits. In the acute phase, Ang II increases the phosphorylation and membrane translocation of NADPH subunit p47phox via activation of PKC, which may be mediated upstream via PLD, PLC and/or PLA2. Another essential subunit required for NOX1 and NOX2 activation is Rac, which has been regulated by the kinase Src. Upon activation, Src in turn trans-activates the EGF receptor, which recruits PI3K and then leads to activation of Rac. Src has been shown to modulate the activity of p47phox as well, which is potentially dependent on its regulation on cortactin, a regulator of the cytoskeleton that may facilitate the transit of p47phox and the assembly of the NADPH oxidase complex. Hydrogen peroxide also induces Src activation, suggesting that the initial rapid generation of ROS may positively feed-back to the signaling cascade and promoting further increase in NADPH oxidase function. Also in VSMCs, sustained activation of NADPH oxidase may depend on the increased expression of its subunits, since experimental evidence showed that Ang II raised the levels of Nox1, p22phox and p47phox. In the kidney, p47phox-independent Nox4 based NADPH oxidase is highly expressed, where it has been proposed to mediate the Ang II-stimulated hypertrophic reaction and elaboration of extracellular matrix proteins in
mesangial cells [170]. Although the experimental results presented in Chapter 3 clearly support that adiponectin can inhibit Ang II-induced NADPH oxidase activation via adipoR1 and the AMPK and cAMP-Epac signaling pathways, the molecular events connecting these two pathways to NADPH oxidase have not been explored. More experiments are required to sort out the relative contribution of Nox4- and Nox2-based superoxide production following Ang II treatment in proximal tubular cells. It is of importance to investigate if activity status of p47phox and Rac, and further the assembly of the NADPH oxidase complex are influenced by adiponectin, as well as which upstream signaling mediators, such as PKC and Src, may be involved. Studies on the possible effect of adiponectin on Ang II-altered expression levels for NADPH oxidase subunits, for example Nox4, Nox2, p47phox, p22phox and Rac, will also be enlightening. Previous research confirmed that adiponectin can indeed regulate the expression of Nox4 in podocytes [514] and Nox2 in the brain [639]. It remains to be verified whether the effect of adiponectin on Ang II-induced NADPH oxidase activation is through suppression of expression of NADPH oxidase subunits.

Another interesting finding in Chapter 3 is the involvement of both AMPK and cAMP-Epac signaling. Interactions of these two important pathways in regulating cellular events have been reported. For instance, Al-Bataineh and colleagues discovered that AMPK and cAMP-PKA promote the apical expression of vacuolar H⁺-ATPase in renal tubular cells in S3 segment, which might be influenced by metabolic stress and/or hormonal signals, respectively [640]. In pancreatic β-cell-like INS-1 cells, AMPK and cAMP-PKA signaling mediates the membrane translocation of ATP-sensitive potassium channels after leptin stimulation, which could potentially regulate insulin secretion from β-cells [641]. Using pharmacological agonists and antagonists, I have shown that the inhibitory effect of adiponectin on Ang II-induced superoxide generation in proximal tubular cells was dependent on both AMPK and cAMP-Epac signal transduction pathways. Epac, also known as the cAMP-regulated guanine exchange factor, is a cAMP binding protein that can activate small GTPases Rap1 and Rap2 of the Ras superfamily. That Epac conveys signaling downstream of cAMP in addition to PKA has provided more specificity to cAMP signaling, and studies on Epac have demonstrated its role in modulating various cellular functions such as cell adhesion, cell differentiation, gene expression and
apoptosis [642]. As discussed in Chapter 3, cross-talk between the AMPK and cAMP-Epac pathways has been reported in hepatocytes and adipocytes [643, 644]. The data presented in Chapter 3 also showed that the AMPK antagonist compound C blocked the effect of the Epac activator 8-pCPT-2’O-Me-cAMP on NADPH oxidase activation following Ang II stimulation, suggesting that AMPK was possibly downstream of the cAMP-Epac pathway. Hence it will be interesting to learn if LKB-1 or CaMKK, 2 kinases known to phosphorylate and activate AMPK, are regulated by cAMP-Epac signaling.

**Adiponectin as a therapeutic agent for DN**

Several published reports have established that adiponectin is negatively associated with insulin resistance and low adiponectin levels have been linked to the risk of developing diabetes mellitus. However clinical observations resulted in inconsistent findings on the correlation between adiponectin levels and mortality rate in patients with chronic kidney disease, which may be affected by the severe wasting in patients with advance chronic kidney disease and the lack of detailed analyses on different forms of circulating adiponectin molecules (HMW oligomers vs. LMW oligomers).

More studies are needed to better understand the role of adiponectin in chronic kidney disease. I examined the effect of adiponectin gene deletion on the development of diabetic nephropathy. Diabetic mice with an Ins2 gene mutation were chosen as T1DM model animals to avoid possible toxicity of streptozotocin in kidney [645] and to reduce the potential confounding stimuli of dyslipidemia additional to hyperglycemia. As the experimental data presented in Chapter 4 suggested, adiponectin null mice experienced more severe diabetic kidney injury when evaluated by kidney hypertrophy and albuminuria, fibrosis, and inflammatory cell infiltration. These findings were supported by *in vitro* data on cultured mesangial cells, in which treatment with adiponectin inhibited the HG-induced activation of mTOR (hypertrophy), TGF-β (fibrosis), NFκB (inflammation) and NADPH oxidase activity (oxidative stress). However the signaling
events responsible for these effects of adiponectin in diabetic kidney injury were not studied and should become part of the direction for future experiments.

Studies by Gou and colleagues showed that the expression level of adipoR1 and phosphorylation of AMPK declined in the kidneys of rat with STZ-caused diabetes, while expression levels of adipoR2 were not altered in the kidneys of diabetic rats [646]. Downregulation of adipoR1 was also found in the kidneys of type 2 diabetic db/db mice [647]. Therefore it will be of interest to verify the relative contribution of adiponectin receptors in the diabetic Akita mice, which can be accomplished by using knock-out mice of adipoR1, adipoR2 and T-cad. The energy sensing AMPK is important in modulating the normal physiology of the kidney [648], and it has been shown to suppress kidney hypertrophy in STZ-induced diabetic rats and in high glucose-stimulated cultured glomerular epithelial cells through inhibiting mTOR activation [649]. In Chapter 4 I have demonstrated that adiponectin inhibited both mTOR and S6K phosphorylation in cultured mesangial cells, and the role of AMPK on these signal transduction molecules in the kidneys of diabetic Akita mice should be investigated in future studies.

Activation of the RAS is a well-established mechanism of pathogenesis in DN, as described in Chapter 1, and ACEI and ARB are still the mainstay of DN treatment. Adiponectin has been shown to directly counteract many of the deleterious effects of Ang II in the cardiovascular system: Ang II infusion into the adiponectin null mice resulted in exacerbated fibrosis and inflammation in heart, and globular adiponectin was able to inhibit activation of NFκB in macrophages after Ang II treatment [650]. Adiponectin also prevented the increase in oxidative stress and attenuated cellular apoptosis signalling by the adipoR1-AMPK pathway in vascular endothelial cells stimulated with Ang II [651]. Activation of β-catenin in the heart of Ang II-infused mice was also suppressed by the delivery of adiponectin-expressing adenovirus, which was implicated in Ang II-induced cardiac hypertrophy [652]. In Chapter 3 I have also demonstrated that adiponectin was capable of inhibiting the activation of NADPH oxidase caused by Ang II treatment in renal proximal tubular cells. Given these findings, potential changes in the level of Ang II and the expression of other RAS components in the kidney of diabetic mice with a deletion in adiponectin should be tested in future studies. In our laboratory, we also
discovered and verified the use of heme oxygenase-1 up-regulation as a marker of increased Ang II activity [653], and this can also be examined in the kidney of diabetic adiponectin-null mice.

Finally, based on the accumulated experimental data in the kidney and the cardiovascular system, it is hypothesized that raising the level and activity of adiponectin could protect the kidney from injury secondary to either AngII or high glucose. In STZ-induced diabetic rats, Nakamaki and colleagues reported adenovirus-mediated adiponectin overexpression reduced the expression of TGF-β, ET-1, PAI-1 and iNOS while increased expression of nephrin and eNOS in kidney [517]. Progression of proteinuria in early nephropathy in diabetic rats was also delayed by adiponectin overexpression. However as discussed in Chapter 1, the existence of different circulating adiponectin oligomers in the body adds some complexity to the simple notion of targeting adiponectin levels directly as a therapeutic regimen. Small molecules that can mimic the effect of adiponectin in vivo, for example adipoRon, and AMPK agonists, such as AICAR, will prove to be interesting reagents to test in models of diabetic nephropathy in future studies.
References


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316. !!! INVALID CITATION !!!


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